Czech Annual Cancer Research Meeting

November 18–20 2024 | Olomouc, CZ

19th Czech Annual Cancer Research Meeting
former Diagnostic, Predictive and Experimental Oncology Days

19th Molecular Pathology Days

3nd Conference of the National Institute for Cancer Research

ABSTRACT BOOK

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Conference Coordinator & Administrator

Peter Vanek, M. Sc.
+420 775 050 355
vanek.peter@icloud.com

Graphic design & typesetting

Denisa Pavelková

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Organizers

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Author: Authors are listed for individual abstracts.
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CZECH RECOVERY PLAN
MINISTRY OF EDUCATION, YOUTH AND SPORTS

MONDAY NOVEMBER 18, 2024

12:30 - 12:45 **GRAND OPENING**

Genetic and Genomic Cancer Biomarkers
12:45 – 14:45
Chairs: Petra Kleiblova, Jiri Drabek

12:45 - 13:15 Cancer risk factors: The role of tobacco harm reduction
David Khayat

13:15 - 13:30 Parallel DNA/RNA NGS using an identical target enrichment panel in the analysis of hereditary cancer predisposition
Petra Kleiblova

13:30 - 13:45 Electrochemical biochip technologies for clinical sample analysis in molecular oncology
Martin Bartosik

13:45 - 14:00 Spatiotemporal organization of biomolecules in cancer cells by phase separation
Martin Sztacho

14:00 - 14:15 Mapping genetic alterations in colorectal cancer: The promise of liquid biopsy
Sona Krivonoskova

14:15 - 14:30 Everyday practice of molecular pathology in the National Institute of Oncology
Erika Toth

14:30 - 14:45 Presence of *TP53*-mediated clonal hematopoiesis in *BRCA1/2* variant carriers
Jana Kotaskova

14:45 - 15:15 **COFFEE BREAK**

Anticancer Drugs and Therapies I
15:15 – 16:45
Chairs: Michal Hocek, Petr Dzubak

15:15 - 15:30 Enzymatic synthesis of potential modified RNA therapeutics with engineered DNA polymerases
Michal Hocek

15:30 - 15:45 Polymeric conjugates bearing mitoxantrone derivatives with immunomodulatory properties for treatment of androgen-independent prostate carcinoma and other mitoxantrone-sensitive tumors
Daniil Starenko

15:45 - 16:00 Preclinical assessment of enhanced blood retention and tumor uptake PSMA-targeting 225Ac-labeled radioconjugates
Zbynek Novy

16:00 - 16:15 Discovery of tubulin-stabilisation properties of novel A-ring-fused isoxazoles of dihydrotestosterone leading to mitotic block and apoptosis in cancer cells
Miroslav Perina

16:15 - 16:30 Structure-assisted humanization of 5D3 antibody targeting prostate-specific membrane antigen (PSMA)
Cyril Barinka

16:30 - 16:45 Improving the selectivity of cytotoxic active triterpenoids containing Michael acceptor
Milan Urban

TUESDAY NOVEMBER 19, 2024

Cancer Immunology and Immunotherapy I
9:00 – 10:30

Chairs: Lucie Kucerova, Karel Smetana

9:00 - 9:15	European Cancer Plan and Mission on Cancer - Challenges and opportunities **Marek Svoboda**
9:15 - 9:45	T cell immunotherapies: Some molecular insights from the basic science perspective **Jonathan Duke-Cohan**
9:45 - 10:00	Distribution and prognostic significance of T cells in colorectal cancer between normal mucosa, primary tumor and liver metastasis **Andriy Trailin**
10:00 - 10:15	Lymphocyte-produced cytokines drive tumor dissemination in the liver in a non-cell autonomous manner **Jan Kosla**
10:15 - 10:30	Therapeutic potential of antibody–drug conjugates in multiple-relapsed/recurrent germ cell tumors **Lucia Kucerova**
10:30 - 11:00	**COFFEE BREAK**

Molecular Basis of Cancers: Tumor Heterogeneity
11:00 – 12:30

Chairs: Martin Mistrik, Pavel Bouchal

11:00 - 11:30	Dissecting tumor and immune heterogeneity in breast cancer metastasis **Juliane Winkler**
11:30 - 11:45	Breast cancer subclassification based on proteomics/proteogenomics for targeted therapy **Pavel Bouchal**
11:45 - 12:00	Polygenic risk score (PRS) and its potential for breast cancer risk stratification in the Czech Republic **Marketa Janatova**
12:00 - 12:15	Multiplex data utilizing a single-step staining and imaging workflow for the investigation of multiple sample types **Alex Darmoise**
12:15 - 12:30	Genomic instability, microenvironment and telomere homeostasis in solid malignancies **Pavel Vodicka**
12:30 - 13:30	**LUNCH**

TUESDAY NOVEMBER 19, 2024

Early Cancer Detection and Prevention
13:30 – 15:30

Chairs: Marian Hajduch, Aleksi Sedo

13:30 - 13:55	Ecological study of lung cancer incidence and mortality in the Czech Republic **Marian Hajduch**
13:55 - 14:15	Biomarkers of lung cancer and COPD identified using mass-spectrometry based proteomics analysis of exhaled breath condensate **Jana Vaclavkova**
14:15 - 14:35	Proteomics of ascitic extracellular vesicles reveals tumor microenvironment in ovarian cancer **Vendula Pospichalova**
14:35 - 14:55	Suitability of the tear proteome for cancer biomarker studies **Tomas Ozdian**
14:55 - 15:15	Real-time monitoring of light-triggered protein-DNA interaction with a surface plasmon resonance biosensor **Giusy Finocchiaro**
15:15 - 15:30	Unraveling the proteome by untargeted mass spectrometry proteomics at scale **Maik Pruess**
15:30 - 16:00	**COFFEE BREAK**

Anticancer Drugs and Therapies II
16:00 – 17:45

Chairs: Tomas Etrych, Milan Urban

16:00 - 16:30	Drug-free macromolecular therapeutics **Jindrich Kopecek**
16:30 - 16:45	Stimuli-responsive polymer therapeutics and theranostics employing photodynamic therapy **Tomáš Etrych**
16:45 - 17:00	Reversing the tumor chemoresistance by using polymer-based nanotherapeutics with P-gp overcoming capacity **Milada Sirova**
17:00 - 17:15	HPMA polymer-porphyrin conjugates with potential application in photodynamic therapy **Alzbeta Turnovska**
17:15 - 17:30	*In situ* synthetic approach of HPMA diblocks for tumor treatment **Michaela Hrochova**
17:30 - 17:45	Gemcitabine nanotherapeutics based on HPMA copolymers: the effect of drug release kinetics on the cancer treatment efficacy **Katerina Behalova**
19:00 - 23:00	**SOCIAL EVENT**

PROGRAM / Evropa Hall Big

WEDNESDAY NOVEMBER 20, 2024

Molecular Basis of Cancer and Molecular Targets
9:00 – 11:00

Chairs: Marek Mraz, Josef Srovnal

9:00 - 9:30	Germline genetics of multiple myeloma **Asta Forsti**
9:30 - 10:00	Cardiac troponins and natriuretic peptides for monitoring cardiovascular safety of cancer treatment **Petr Jarolim**
10:00 - 10:15	A novel model to study proliferation signaling in chronic lymphocytic leukemia identifies unique drugs with anti-proliferative effect **Marek Mraz**
10:15 - 10:30	T-maps: Evaluation of new cell surface markers in pediatric T-cell acute lymphoblastic leukemia to monitor minimal residual disease **Tomas Kalina**
10:30 - 10:45	Epigenetic control of stem cell decisions via ISWI ATPase Smarca5 **Tomas Stopka**
10:45 - 11:00	Synergistic role of aldehyde dehydrogenase inhibition in combination with hypomethylating agents in relapse/refractory acute myeloid leukemia **Srdjan Grusanovic**
11:00 - 11:30	**COFFEE BREAK**

PROGRAM / Evropa Hall Small

Molecular Basis of Cancers and Biomarkers
9:00 – 10:30

Chairs: Jan Bouchal, Stjepan Uldrijan

9:00 - 9:15	Identification and validation of novel prognostic factors and therapeutic targets in osteosarcoma **Petr Benes**
9:15 - 9:30	Regeneration initiation expression signatures found in single cell and spatial transcriptomics tumor data **Radek Sindelka**
9:30 - 9:45	Expression of germline *Jak2* R1063H represents increased risk of thrombosis and impairs normal hematopoietic development in mice **Lucie Lanikova**
9:45 - 10:00	CDK4 and CDK6 expression pattern in B-cell non-Hodgkin lymphomas as a predictive marker of response to CDK4/6 inhibitors **Denisa Vesela**
10:00 - 10:15	New insights into the ERK MAPK pathway regulation in malignant melanoma **Stjepan Uldrijan**
10:15 - 10:30	eIF4F controls AMPK activity in malignant melanoma **Natalia Vadovicova**

WEDNESDAY NOVEMBER 20, 2024

Personalised Medicine: From Genes to Therapeutic Approaches
11:30 – 13:15

Chairs: Ondrej Slaby, Marek Kovar

11:30 - 12:00	Investigating tumor heterogeneity using *in vivo* CRISPR gene editing technologies in head and neck cancer **Sampath Loganathan**
12:00 - 12:30	Opportunities to deliver the vision of precision oncology through whole genome sequencing **Richard Houlston**
12:30 - 12:45	Employing dual nature of iron to combat cancer via mitochondrial targeting of deferasirox **Jaroslav Truksa**
12:45 - 13:00	Pro-apoptotic proteins Bax and Bak modulate in cell-specific manner mitochondrial respiration via regulation of TEFM expression **Ladislav Andera**
13:00 - 13:15	Tricking cancer cells into an apoptotic response to the false alarm triggered by MDM2 inhibitors **Zdenek Andrysik**
13:15 - 14:05	**LUNCH**

14:05 - 15:05	**POSTER PRESENTATIONS – EVROPA HALL SMALL**

Cancer Immunology and Immunotherapy II
15:15 – 17:30

Chairs: Karel Smetana, Lucca Vanucci

15:15 - 15:45	Immunosuppression in tumor microenvironment mediated by myeloid-derived suppressor cells (MDSC) **Viktor Umansky**
15:45 - 16:00	Targeting the IL-6 receptor alpha chain to manipulate the cancer microenvironment **Karel Smetana, Jr.**
16:00 - 16:15	CD25-biased IL-2 agonists synergize with immune checkpoint blockade in cancer immunotherapy despite robust Treg cell expansion but timing is crucial **Marek Kovar**
16:15 - 16:30	Exploring fibroblast activation protein (FAP) expression in brain metastases and novel alpha-ketoamide FAP inhibitors for theranostic applications **Petr Busek**
16:30 - 16:45	Experimental targeting of tumor microenvironment by nano-constructs. Our experience. **Luca Vannucci**
16:45 - 17:00	Cytoskeletal crosslinkers as a target for inhibiting hepatocellular carcinoma growth and metastasis **Martin Gregor**
17:00 - 17:15	Modulation of Lck in immune and tumors cells and spheroids. The importance of specificity **Juan De Sanctis**
17:15 - 17:30	**CLOSING CEREMONY**

PARALLEL SESSIONS / Evropa Hall Small

MONDAY NOVEMBER 18, 2024

10:00 - 12:00	NICR Education workshop (satellite workshop)
13:00 - 15:00	SALVAGE project meeting
17:15 - 18:00	Project meeting of the NICR Research Program 2 – Research and development of anticancer pharmaceuticals and therapeutic methods

TUESDAY NOVEMBER 19, 2024

11:00 - 12:30	Project meeting of the NICR Research Program 4 – Early detection and prevention of tumours Project meeting of the NICR Research Program 3 – Biomarkers of cancers and cancer diagnostics
16:45 - 18:00	Project meeting of the NICR Research Program 1 – Molecular basis of cancer and molecular targets Project meeting of the NICR Research Program 5 – Translational oncology: Verification clinical studies of the proof-of-concept type

PARALLEL SESSIONS / Ventana Lounge

MONDAY NOVEMBER 18, 2024

14:00 - 16:00	NICR administrative meeting
18:00 - 21:00	Board meeting of the National Institute for Cancer Research

WEDNESDAY NOVEMBER 20, 2024

18:00 - 21:00	NICR International Science and Advisory Board Meeting

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MONDAY NOVEMBER 18, 2024

Chairs: Petra Kleiblova, Jiri Drabek

Cancer Risk Factors: The Role of Tobacco Harm Reduction

David Khayat

Health, Nutrition and Wellness Experts, Paris, France

Smoking is a major risk factors for lung cancer and therefore, for any smoker, quitting is the best approach. However, many smokers do not quit, even in the face of serious disease, creating a need for a different approach that may reduce the risk and impact of smoking-related diseases. Toward that end, doctors and public health authorities have begun to examine the role that harm reduction can play in reducing the negative impact of smoking on health.

While the long-term data supporting the reduction of disease associated with smoking is not yet available, we can begin to understand the potential benefits of switching based on the totality of evidence including a reduction in exposure to harmful and potentially harmful constituents (HPHCs) that are present in cigarette smoke and early signals of improvements tied to the pathomechanisms involved in the development of smoking related diseases. For cancer, epidemiology shows us that the lower the exposure to carcinogens, the lower the risk of cancer. Therefore, it can be reasonably expected that reductions in exposure to HPHCs would positively impact the development of longer-term diseases, such as cancers.

A harm reduction approach to smoking takes into consideration the individual risk reduction of switching to non-combustible alternatives to cigarettes along with the potential of an alternative product to move people away from cigarettes. As nicotine is the primary driver for smoking, but not a direct cause of smoking-related diseases, there is a need to understand the role that nicotine has in a harm reduction approach to smoking, evaluating both the risks and the benefits of nicotine as a drug.

To reduce the risk of smoking-related disease, including cancer, the best option is to stop smoking. But for those who don't we need to have a holistic view on emerging data and trends to understand the risk reduction potential of alternatives. Global approaches to tobacco harm reduction can offer insights into how non-combustible alternatives that still deliver nicotine influence smoking prevalence and how these new products may benefit public health.

Parallel DNA/RNA NGS using an identical target enrichment panel in the analysis of hereditary cancer predisposition

Petra Kleiblová[1,2], Marta Černá[1], Petra Zemánková[1], Kateřina Matějková[1], Petr Nehasil[1], Klára Horáčková[1], Markéta Janatová[1], Jana Soukupová[1], Barbora Šťastná1, Zdeněk Kleibl[1]

[1]Institute of Medical Biochemistry and Laboratory Diagnostics, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic.

[2]Institute of Biology and Medical Genetics, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic

Germline DNA testing using next-generation sequencing (NGS) technology has become the analytical standard for the diagnostics of hereditary diseases, including cancer. Its increasing use places high demands on correct sample identification, independent confirmation of prioritized variants, and their functional and clinical interpretation. To streamline these processes, we introduced parallel DNA and RNA capture-based NGS using identical capture panel CZECANCA, which is routinely used for DNA analysis of hereditary cancer predisposition. Here, we present the analytical workflow for RNA sample processing and its analytical and diagnostic performance. Parallel DNA/RNA analysis allowed credible sample identification by calculating the kinship coefficient. The RNA capture-based approach enriched transcriptional targets for the majority of clinically relevant cancer predisposition genes to a degree that allowed analysis of the effect of identified DNA variants on mRNA processing. By comparing panel and whole-exome RNA enrichment, we demonstrated that the tissue-specific gene expression pattern is independent of the capture panel. Moreover, technical replicates confirmed a high reproducibility of tested RNA analysis. We concluded that parallel DNA/RNA NGS using the identical gene panel is a robust and cost-effective diagnostic strategy. In our setting, it allows routine analysis of 48 DNA/RNA pairs using NextSeq 500/550 Mid Output Kit v2.5 (150 cycles) in a single run with sufficient coverage to analyze 226 cancer predisposition and candidate genes. This approach can replace laborious Sanger confirmatory sequencing, increased testing turnaround, reduce analysis costs, and improve variants impact interpretation by analyzing their effect on mRNA processing.

This work was supported by Ministry of Health of the Czech Republic grant projects (NU23-03-00150 and RVO-VFN 64165); Charles University research projects (SVV 260516 and Cooperatio); and the Ministry of Education Youth and Sports of the Czech Republic grant (Programme EXCELES, ID Project No. LX22NPO5102 - Funded by the European Union – Next Generation EU).

Decode the Secrets of Genome

Get-to-know

Electrochemical biochip technologies for clinical sample analysis in molecular oncology

Martin Bartosik[1] Ludmila Moranova[1], Nasim Izadi[1], Ravery Sebuyoya[1], Johana Strmiskova[1,2], Milan Anton[3], Roman Hrstka[1]

[1]Masaryk Memorial Cancer Institute, Brno, Czech Republic. [2]Masaryk University, Brno, Czech Republic. 3University Hospital Brno, Brno, Czech Republic

Electrochemical (EC) detection of nucleic acids presents a promising approach for identifying these biomolecules as important cancer biomarkers. Primary benefits include affordability, rapidity, simplicity, minimal sample requirements, and potential for miniaturization, making it ideal for personalized, decentralized medicine at point-of-care or in limited resource settings. By integrating the EC detection with with novel, PCR-free isothermal amplification techniques (IAT) like loop-mediated amplification (LAMP), rolling circle amplification (RCA), or recombinase polymerase amplification (RPA), high sensitivity and selectivity can be achieved.

Application of these technologies in analyzing clinical samples from cancer patients, targeting a variety of DNA/RNA biomarkers, is demonstrated. Examples include the development of bioassays for detecting HPV oncoviruses in cervical cancer, long non-coding RNAs in prostate cancer, and DNA point mutations in BRAF or KRAS oncogenes in colorectal cancer. Consequently, EC methods coupled with IATs could serve as a valuable alternative in contemporary molecular cancer diagnostics. This work was supported by grant projects AZV NU21-08-00078, AZV NU21-08-00057, National Institute for Cancer Research (Programme EXCELES, ID Project No. LX22NPO5102) - Funded by the European Union - Next Generation EU, Large research infrastructure BBMRI.cz (Project No. LM2023033) and MH CZ - DRO (MMCI, 00209805).

Spatiotemporal organization of biomolecules in cancer cells by phase separation

Agnieszka Chytła[1], Elena Herencia Lagunar[1], Rabia Gonül[1], Barbora Šalovská[2,3], Jakub Červenka[4,5], Ludovica Antiga[6], Peter Hoboth[6], Pavel Hozák[6], Martin Sztacho[1]

[1]Laboratory of Cancer Cell Architecture, Institute of Biochemistry and Experimental Oncology, First Faculty of Medicine, Charles University, Prague, Czech Republic. [2]Department of Genome Integrity, Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic. [3]Yale Cancer Biology Institute, Yale University School of Medicine, West Haven, USA. [4]Laboratory of proteomics, Institute of Biochemistry and Experimental Oncology, First Faculty of Medicine, Charles University, Prague, Czech Republic. [5]Laboratory of Applied Proteome Analyses, Research Center PIGMOD, Institute of Animal Physiology and Genetics of the Czech Academy of Sciences, Libechov, Czech Republic. [6]Department of Biology of the Cell Nucleus, Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic

Our research investigates the spatiotemporal organization of biomolecular material within cells, emphasizing its critical role in the efficacy and kinetics of cellular processes, particularly in the context of cancer transformation. We are investigating the molecular mechanisms that drive pathophysiological changes in cellular architecture during oncogenesis, with a particular interest in the principles of biomolecular condensation by phase separation driven by phosphoinositides and RNA molecules. We use a combination of *in vitro* and *in vivo* methods, integrating biochemistry, molecular cell biology, microscopy, proteomics and bioinformatics. These approaches are applied to a range of cancer cell culture models, from glioblastoma to human papillomavirus-infected cell lines, and our findings are corroborated with patient-derived material in translational projects. An important aspect of our study is the role of RNA and phosphatidylinositol 4,5-bisphosphate (PIP2) in the formation of nuclear compartments such as nuclear speckles and nucleoli. PIP2 found in these compartments is involved in RNA polymerase I/II transcription and exhibits RNA-dependent nuclear localization. We investigated the cooperative interaction between PIP2 and RNA in the establishment of nuclear architecture and determined the RNA-dependent PIP2-associated (RDPA) nuclear proteome in human cells using mass spectrometry. Our results show that intrinsically disordered regions (IDRs) with polybasic PIP2-binding K/R motifs are important features of RDPA proteins. We found that the RDPA protein BRD4 associates with PIP2 in an RNA-dependent manner via electrostatic interactions. Elevated PIP2 levels were found to increase the number of BRD4 nuclear foci, suggesting that PIP2 spatiotemporally orchestrates nuclear processes by associating with RNA and RDPA proteins, thereby influencing their phase separation ability. These findings highlight the critical role of PIP2 in establishing a functional nuclear architecture competent for gene expression, advancing our molecular understanding of cancer and offering new avenues for early diagnosis and treatment strategies.

This project is supported by the project National Institute for Cancer Research (Programme EXCELES, ID Project No. LX22NPO5102) by the European Union – Next Generation EU. COST Action 19105-Pan-European Network in Lipidomics and EpiLipidomics

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Mapping Genetic Alterations in Colorectal Cancer: The Promise of Liquid Biopsy

Eliška Jandová[1], Soňa Křivonosková[2], Anna Oplištilová[2], Radka Lohynská[2], Ludmila Boublíková[1,2]

[1]CLIP – Laboratory of Molecular Genetics, Clinic of Pediatric Hematology and Oncology, 2nd Faculty of Medicine, Charles University and University Hospital Motol, Prague, Czech Republic. [2]Clinic of Oncology, 1st Faculty of Medicine, Charles University and Thomayer University Hospital, Prague, Czech Republic

Introduction

Despite advances in understanding and treatment, colorectal cancer (CRC) remains the second leading cause of cancer-related death worldwide, and the global burden of this disease is expected to increase significantly. Early detection and treatment are crucial; however, many cases are diagnosed at advanced stages where treatment options become limited and less effective.

Mapping the molecular background of CRC is essential to guide effective treatment decisions. During the disease course and progression, the molecular background changes due to cancer cells' evolution and clonal selection. Analysis of circulating tumor DNA (ctDNA), in addition to the primary tumor sample, offers valuable insights into the actual cancer molecular profiles through a minimally invasive procedure.

This study delves into the molecular characteristics of metastatic colorectal cancer and their changes over time to elucidate the disease's development, resistance mechanisms, and treatment responses.

Goals

The primary objectives are to identify pathogenic molecular alterations in primary tumors, metastases, and ctDNA from different time points, compare their profiles and relation to patient´s and disease characteristics, assess ctDNA's diagnostic utility as a predictor of treatment response and marker for disease monitoring, and evaluate the evolution of molecular background of metastatic CRC during the course of the disease.

Methods

This research employs next-generation sequencing (NGS) to examine sequence variants in primary tumors, metastases, and ctDNA from patients with metastatic CRC (a test cohort of 10 patients, and an investigation cohort of over 100 patients). Formalin-fixed paraffin-embedded (FFPE) samples from primary tumors and metastases (if available), as well as blood samples were collected at defined stages of the disease and treatment. Germline DNA was also extracted from blood leukocytes to serve as a control for distinguishing somatic mutations from germline polymorphisms.

Results

In the test cohort of patients with known RAS mutations in primary tumors, 7 of 10 pts including all patients with high tumor burden had this mutation present also in ctDNA samples.

In the main study, numerous pathogenic variants have been identified across primary tumors, metastases, and ctDNA, most of them in genes with well-established roles in CRC. In 18 patients with a completed analysis of all samples, the median number of altered genes in primary tumor samples was 6 (2 - 24), the most commonly mutated genes being *TP53* (72%), APC (61%), and KRAS (44%).

In 28% of patients were these mutations found also in ctDNA samples, representing 14% of all mutations found in primary tumors. In the same percentage of patients (28%), novel molecular variants were identified in ctDNA that have not been present in the original primary tumor. Only 4 of the 18 patients had also metastatic samples available and analyzed. All of them had a proportion of mutations shared in all samples (17 - 50%), the remaining mutations were distinct for the primary tumor and metastatic lesions, respectively. The enrollment and evaluation of patients´ samples are ongoing.

Conclusion

This study highlights the dynamic and heterogeneous nature of tumor evolution in CRC and underscores the importance of understanding its molecular background to improve diagnosis, treatment, and disease monitoring. The potential of ctDNA as a non-invasive biomarker for real-time monitoring of disease progression and treatment response holds great promise for personalized medicine. However, the sensitivity of ctDNA detection remains a challenge, particularly for low-frequency mutations, necessitating further methodological optimizations. Overall, the findings support the integration of comprehensive molecular profiling, including ctDNA analysis, into clinical practice to enhance personalized treatment strategies and improve patient outcomes in metastatic CRC. By advancing our understanding of the genetic and molecular landscape of CRC, we can develop more effective strategies for early detection, stratification, and optimal targeted therapies to improve patient outcomes.

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All authors declare they have no conflicts of interest.

Everyday practice of molecular pathology in the National Institute of Oncology

Erika Toth

National Institute of Oncology, Budapest, Hungary

Recent developments in molecular genetic methods (e.g. next-generation sequencing -NGS-panels) largely accelerated the process of finding the most appropriate targeted therapeutic intervention for cancer patients based on molecularly targetable genetic alterations. Comprehensive genomic profiling was introduced into our routine clinical practice in 2019.

In this presentation, I will demonstrate the practice of molecular pathological testing of solid tumors in our institute. In the majority of cases we use NGS.

Libraries are prepared using the Ion Chef™ System with Ion 540™ Chips (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions with a DNA input of approximately 8.5 ng. Sequencing is performed using an Ion S5™ Plus Sequencer and Genexus Integrated Sequencer (Thermo Fisher Scientific, Waltham, MA, USA). We use Ion Reporter™ Software (v. 5.18) (Thermo Fisher Scientific, Waltham, MA, USA) for data analysis. We also use Oncomine Comprehensive Assay v3, Oncomine Focus Assay and Oncomine Comprehensive Assay Plus.

The most frequent tumor types are non small cell lung cancer, pancreato-biliary, breast, prostate and gynecological cancers. The analysis is performed on formalin-fixed paraffine-embedded samples. I will shortly summarize the most problematic aspects of these types of samples and routine workflow.

NGS-based CGP was successfully introduced in our Institution and significant number of patients benefited from comprehensive genetic tests, the number of cases tested has tripled in four years

Presence of *TP53*-mediated clonal hematopoiesis in *BRCA1/2* variant carriers

Šárka Pavlová[1,2,3], Michaela Bilčíková[1], Eva Zapletalová[1], Ivona Blaháková[1,2], Robert Helma[1,2], Johana Mayerová[1], Jitka Malčíková[1,2,]3, Jakub Trizuljak[1,3], Lucie Ehrlichová[1], Karla Plevova[1,2,3], Anna Panovská[1], Boris Tichý[1,2,3], Šárka Pospíšilová[1,2,3], Markéta Bednaříková[1], Michael Doubek[1,2,3], Jana Kotašková[1]

[1]Department of Internal Medicine, Hematology and Oncology, University Hospital Brno and Faculty of Medicine, Masaryk University, Brno, Czech Republic.
[2]Central European Institute of Technology, Masaryk University, Brno, Czech Republic.
[3]Institute of Medical Genetics and Genomics, University Hospital Brno and Faculty of Medicine, Masaryk University, Brno, Czech Republic

Introduction: Myeloproliferative disorders (MPDs) resulting from cytotoxic therapy are rare but serious conditions. Recent studies have shown that clonal hematopoiesis underlies the development of MPDs. Clonal hematopoiesis is defined as the presence of a subclone with a somatic mutation with variant allele frequency (VAF) greater than or equal to 2 %, a threshold based on the limit of detection (LOD) of conventional NGS (next-generation sequencing) panels. Retrospective analyses of cancer patients have demonstrated that the clones responsible for the MPD development can be detected prior to the onset of therapy, with mutations in the tumor suppressor gene *TP53* being particularly significant.

Aims: This study aimed to evaluate the frequency of subclones carrying *TP53* gene variants in patients with pathogenic or likely-pathogenic germline variants in *BRCA1/2* genes. We included patients tested in our laboratory for hereditary cancer syndrome between 2018 and 2022, allowing for the longitudinal monitoring of their disease progression.

Material and methods: We analyzed a cohort of 80 patients with confirmed germline pathogenic or likely-pathogenic variants in *BRCA1/2* who consented to the use of their biological material for research purposes. Using a previously established ultra-deep NGS-based approach, we examined the entire coding region of the *TP53* gene (exons 2 – 11) for variants with VAF as low as 0.1 %. DNA was extracted from peripheral white blood cells. For 90 % of the patients, the sample was collected prior to the initiation of cancer therapy.

Results: *TP53* variant-carrying subclones were identified in eight out of 80 patients (10%), with multiple variants detected in two patients. The allelic frequency of the detected variants ranged from 0.12 to 1.6 % VAF. During follow-up periods of 38 months, MPD developed in one patient treated for triple-negative breast cancer, with clonal expansion of the *TP53* variant initially identified at the time of *BRCA1/2* testing.

Conclusion: Our study confirms the presence of leukocyte clones with *TP53* variants in the peripheral blood of *BRCA1/2* variant carriers even before the initiation of antitumor therapy. Although none of the detected clones exceeded the 2% VAF threshold that defines clonal hematopoiesis, these minor clones can still lead to severe MPDs. Further studies are needed to determine whether *TP53*-mediated clonal hematopoiesis is more prevalent in *BRCA1/2* mutation carriers compared to the general population.

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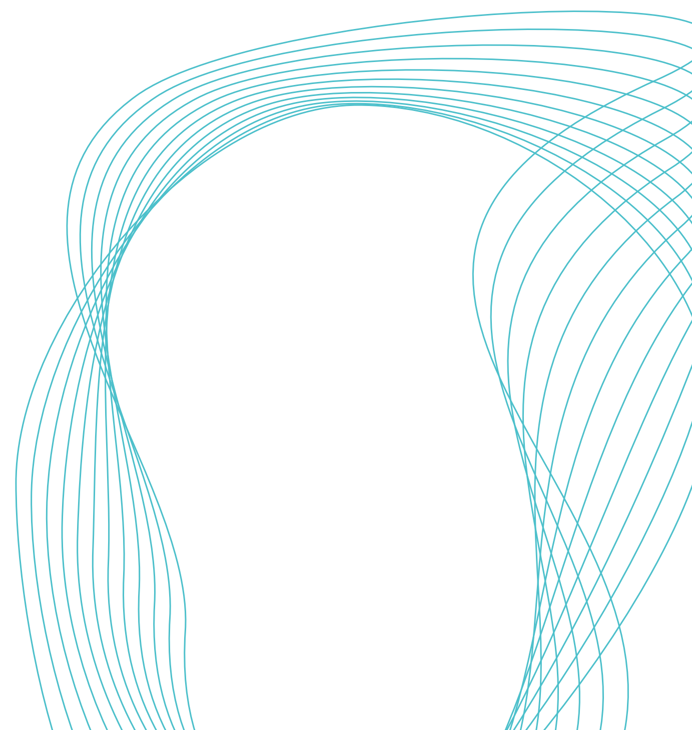

MONDAY NOVEMBER 18, 2024

Chairs: Michal Hocek, Petr Dzubak

Enzymatic synthesis of potential modified RNA therapeutics with engineered DNA polymerases

*Michal Hocek,** [a,b]
Mária Brunderová, [a] *Matouš Krömer,*[a] *Samanta Rožánková,* [a,b]

[a] *Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Flemingovo namesti 2, 16000 Praha 6, Czech Republic; hocek@uochb.cas.cz*
[b] *Dept. of Organic Chemistry, Faculty of Science, Charles University, 12843 Prague 2, Czech Republic*

A new method for enzymatic synthesis of base-modified RNA was developed using engineered thermophilic DNA polymerases [1] in primer extension of an RNA primer to afford RNA strand containing one or several different base-modified nucleotides (Scheme 1). [2] The method can be used for the synthesis of hypermodified RNA containing all four modified nucleotides using a set of four base-modified NTPs. We also developed site-specific or segmented introduction of one or two modifications at defined positions in diverse RNA molecules, including mRNA. The approach is also useful for the synthesis of modified XNAs using 2'-modified NTPs. This methodology can be used for expedient synthesis of diverse types of base-modified RNA and XNA for development of potential nucleic acids therapeutics.

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Polymeric conjugates bearing mitoxantrone derivatives with immunomodulatory properties for treatment of androgen-independent prostate carcinoma and other mitoxantrone-sensitive tumors

Daniil Starenko[1], *Petr Chytil*[2], *Jana Kousalová*[2], *Tomáš Etrych*[2], *Marek Kovář*[1]

[1]*Institute of Microbiology of the Czech Academy of Sciences, Prague, Czech Republic.*
[2]*Institute of Macromolecular Chemistry of the Czech Academy of Sciences, Prague, Czech Republic*

Mitoxantrone (MTX) is one of the chemotherapeutics approved for the treatment of androgen-independent prostate cancer (AIPC). Nowadays, it is mainly used as palliative treatment for AIPC as well as one of the first line treatments for triple-negative breast carcinoma (TNBC) and acute myeloid leukemia (AML). MTX differs from most other cancerostatics due to the feature that it is one of the most potent inductors of immunogenic cell death (ICD). ICD is a special type of regulated cell death which enables cells of the immune system (mainly dendritic cells) to detect dying tumor cells. The process occurs thanks to expression of "eat me" signals by dying tumor cell (e.g. membrane-bound calreticulin, HSP70/90 or extracellular ATP), facilitating phagocytosis of dead tumor cells and subsequent presentation of tumor-associated and tumor-specific antigens to T cells. Here, we evaluated *in vitro* and *in vivo* antitumor activity of polymeric conjugates based on N-(2-hydroxypropyl) metharcylamide bearing MTX modified with 5-methyl-4-oxohexanoic acid to enable its attachment to the polymeric backbone via pH-sensitive hydrazone bond. Cytostatic and cytotoxic properties of prepared conjugates were evaluated *in vitro* to determine IC50 and apoptosis induction in AIPC, TNBC and AMC mouse and human cell lines. Toxicity and antitumor activity were tested *in vivo* in mice bearing progressively growing syngeneic or xenogeneic (human) tumors. Finally, we evaluated immunogenic properties of conjugates in comparison to free MTX and confirmed that conjugates are capable of successful ICD induction both *in vitro* and *in vivo*. This opens a potential possibility to assess their therapeutical effects in combination with immunotherapeutics such as immune checkpoint inhibitors or IL-2/anti-IL-2 monoclonal antibody complexes.

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Preclinical assessment of enhanced blood retention and tumor uptake PSMA-targeting 225Ac-labeled radioconjugates

Zbynek Novy[1], Katarina Hajduova[1], Milos Petrik[1], Falco Reissig[2], Daniela Kurfurstova[1], Marian Hajduch[1], Constantin Mamat[2]

[1]Palacky University Olomouc, Olomouc, Czech Republic.
[2]Helmholtz Zentrum Dresden-Rossendorf, Dresden, Germany

Introduction: The prostate-specific membrane antigen (PSMA) is overexpressed in prostate cancer at significantly higher levels compared to healthy tissue. Therefore, PSMA has emerged as very suitable target for molecular imaging as well as targeted radionuclide therapy of metastatic castration-resistant prostate cancer (mCRPC). In this study, we have investigated the *in vivo* behavior of two novel macropa-based PSMA inhibitors, namely [225Ac]Ac-mcp-D-PSMA and [225Ac]Ac-mcp-M-alb-PSMA modified with albumin binding moiety. The main motivation behind this project was to improve tumor uptake and thus therapeutic efficacy of those novel 225Ac-PSMA inhibitors.

Methods: We have performed *in vivo* studies involving long-term toxicity study in healthy mice with subsequent immunohistochemical examinations of kidneys and salivary glands. We have also done therapeutic efficacy study in LNCaP-tumor bearing animals employing three different doses (5/15/45 kBq/mouse). Kidneys, livers and tumors were examined using immunohistochemical staining methods to detect PSMA expression, DNA damage (γH2AX), proliferation status (Ki67) and necrosis (H&E).

Results: The toxicity study have not revealed any significant toxic effect in studied parameters. Insignificant DNA damage was observed in the kidney tissue compared to the untreated controls. The therapy study showed no significant effect of two lower doses (5 and 15 kBq/animal) onto tumor volume or survival. The dose of 45 kBq/mouse had significant impact to both mentioned parameters, whereas [225Ac]Ac-mcp-M-alb-PSMA performed better than other two 225Ac-PSMA inhibitors.

Conclusion: *In vivo* experiments in healthy mice showed very low toxicity of tested PSMA inhibitors. Histological examination of the organs in therapy study confirmed substantial DNA damage in the tumor tissue of mice injected with both studied novel 225Ac-compounds, on the other hand the same parameter revealed only low DNA harm in the kidneys. The therapeutic efficacy of novel compounds was comparable to gold standard 225Ac-PSMA-617, in case of [225Ac]Ac-mcp-M-alb-PSMA seemed to be even higher.

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Discovery of tubulin-stabilisation properties of novel A-ring-fused isoxazoles of dihydrotestosterone leading to mitotic block and apoptosis in cancer cells

Miroslav Peřina[1], Márton Kiss2, Jakub Bělíček[1], Éva Frank[2], Radek Jorda[1]

[1]Department of Experimental Biology, Faculty of Science, Palacký University Olomouc, Šlechtitelů 27, 78371 Olomouc, Czech Republic, Olomouc, Czech Republic.
[2]Department of Molecular and Analytical Chemistry, Interdisciplinary Excellence Centre, University of Szeged, Dóm Tér 7-8, H-6720 Szeged, Hungary, Szeged, Hungary

Tubulin-targeting drugs, such as taxanes, are integral to the treatment of advanced cancers, particularly in patients progressing on milder chemotherapeutic regimens. However, their clinical efficacy is often limited by significant off-target toxicity and adverse side effects. In this study, we report the synthesis and characterization of novel A-ring fused isoxazoles of dihydrotestosterone (DHT), achieved through iodine-mediated oxidative cyclization of α,β-unsaturated oximes derived from DHT-enones. These derivatives were structurally confirmed and evaluated for antiproliferative activity. Several derivatives demonstrated unexpectedly high antiproliferative potency, with GI50 values around 5 μM, selectively inhibiting the growth of rapidly proliferating cancer cells while sparing non-cancerous cells. Mechanistic studies revealed that the most potent compounds induced mitotic arrest and disrupted cytoskeletal integrity in low micromolar concentrations. The lead compound, 2j, notably increased the rate of tubulin polymerization *in vitro* and stabilized polymerized tubulin in the cells, leading to strong G2/M block, which was reversible upon short treatment, but led to apoptosis upon longer treatment. Molecular docking studies indicated that novel compounds bind preferably to the taxane site on tubulin, forming conserved interactions. This binding was further proved by micro-scale thermophoresis, which demonstrated high-affinity binding with dissociation constants in the nanomolar range. Importantly, the candidate compounds maintained or even increased their activity against docetaxel-resistant prostate cancer cells (DU145), highlighting their potential to overcome resistance mechanisms associated with current taxane therapies. Our findings suggest that A-ring isoxazoles of DHT are promising taxane-site binding agents on a steroid scaffold, exhibiting taxane-like biological activity. These results support further development and optimization of these compounds

for potential therapeutic use in cancer treatment, particularly in cases where resistance to existing therapies poses a significant challenge.

Structure-assisted humanization of 5D3 antibody targeting prostate-specific membrane antigen (PSMA)

Cyril Bařinka[1], Barbora Havlínová[1], Andrea Smith[1], Arne Skerra[2], Zora Nováková[1]

[1]Institute of Biotechnology of the Czech Academy of Sciences, Vestec, Czech Republic.
[2]Technische Universität München, Freising, Germany

Prostate-specific membrane antigen (PSMA) is a clinically used biomarker for the imaging and therapy of prostate cancer (PCa). Currently, PSMA-targeted imaging agents and therapeutics are primarily based on small-molecule ligands yet macromolecular modalities, including antibodies and nanoparticles, are being actively researched and developed. We have recently developed and engineered a PSMA-specific antibody, 5D3, which exhibits sub-nanomolar affinity and high specificity for human PSMA *in vitro* and *in vivo*. To advance 5D3 into preclinical testing, we solved crystal structures of free 5D3 as well as its complex with PSMA. The structural data were then used for structure-assisted humanization of 5D3 (h5D3). The lead humanized molecule was further engineered to mitigate its sequence liabilities and structural variability. Biophysical and cell-based assays confirmed the suitability of h5D3 for ongoing *in vivo* preclinical testing.

Improving the selectivity of cytotoxic active triterpenoids containing Michael acceptor

Milan Urban[1,2], Ivo Frydrych[1], Jan Bachořík[2,1], Viktor Zamazal[1,2], Natálie Maršálková[2,1], Kristína Kúdelová[2,1], Soňa Gurská[1], Barbora Lišková[1], Petr Džubák[1], Marián Hajdúch[1]

[1]IMTM, Faculty of Medicine and Dentistry, Palacký University Olomouc, Czech Republic.
[2]Dept. of Organic Chemistry, Faculty of Science, Palacký University in Olomouc, Olomouc, Czech Republic

Pentacyclic triterpenoids are natural compounds with significant biological activities.1 Among them, one of the most interesting compounds is 30-oxobetulinic acid.2 This compound is highly cytotoxic in multiple cancer cell lines, however, it is also cytotoxic in non-cancerous fibroblasts. This lack of selectivity is likely associated with the presence of an electron-deficient double bond (Michael acceptor) which interacts with random nucleophiles in living cells (such as cysteine, threonine or lysine residua in proteins). This non-specific binding results in general toxicity. In this work, several sets of derivatives of 30-oxobetulinic acid were prepared in order to weaken or completely remove the Michael acceptor. As a result, new compounds with much better selectivity against cancer cells than the parent molecule were obtained. The activity, selectivity and the influence of the structure on the biological behaviour will be discussed.

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TUESDAY NOVEMBER 19, 2024

Chairs: Lucie Kucerova, Karel Smetana

European Cancer Plan and Mission on Cancer - Challenges and opportunities

Marek Svoboda

T cell immunotherapies: Some molecular insights from the basic science perspective

Jonathan Duke-Cohan

Dana-Farber Cancer Institute, Boston, USA. Harvard Medical School, Boston, USA

The mammalian αβ T cell exhibits exquisite specificity discriminating “foreign” peptides displayed by self-MHC from self-peptides, a property that is now being recruited in immunotherapies for recognition of neoepitopes presented by tumours, as well as for amplification ex vivo and reintroduction of apparently tumour-specific T cells into patients. Nevertheless, the results in general of these techniques are not as successful as might be expected. In fact, to gain even a modest effect of tumour-specific T cells, 109- 1011 cells are usually transplanted. Part of the reason for the limited efficacy is a lack of understanding of the molecular interaction properties of the αβT cell receptor (αβTCR) with peptide-MHC (HLA) that confer specificity in a proper physiological context. Here we will review the properties and caveats recently revealed by our research and others clearly identifying the TCR as a mechanotransducing receptor – a functionality not discriminated by current modal techniques for assessing TCR specificity.

Distribution and prognostic significance of T cells in colorectal cancer between normal mucosa, primary tumor and liver metastasis

Andriy Trailin[1], Esraa Ali[1], Wenjing Ye[1], Sergii Pavlov[1], Lenka Červenková[2], Ondřej Vyčítal[3], Filip Ambrozkiewicz[1], Ondřej Daum[4,5], Václav Liška[3], Kari Hemminki[6,1]

[1]Laboratory of Translational Cancer Genomics, Biomedical Center, Faculty of Medicine in Pilsen, Charles University, Pilsen, Czech Republic. [2]Laboratory of Cancer Treatment and Tissue Regeneration, Biomedical Center, Faculty of Medicine in Pilsen, Charles University, Pilsen, Czech Republic. [3]Department of Surgery and Biomedical Center, Faculty of Medicine in Pilsen, Charles University, Pilsen, Czech Republic. [4]Sikl’s Institute of Pathology, Faculty of Medicine and Teaching Hospital in Pilsen, Charles University, Pilsen, Czech Republic. [5]Bioptická Laboratoř s.r.o., Pilsen, Czech Republic. [6]Department of Cancer Epidemiology, German Cancer Research Center, Heidelberg, Germany

Introduction. We aimed to assess the role of local adaptive immunity in the metastatic process by comparing densities of T cells between normal colorectal mucosa (NM), primary colorectal cancer (pCRC) and liver metastases of CRC (LM) in patients with synchronous and metachronous disease. Another goal of the study was to investigate the prognostic value of T cells between NM, pCRC and its paired synchrononous and metachronous LM for disease-free survival (DFS) after liver surgery.

Patients and methods. We enrolled CRC patients, who underwent resection of pCRC and LM in Pilsen University Hospital between 1999 and 2022. 55 patients presented at the time of diagnosis with LM (stage IV, synchronous) and 44 patients (stage II/III) developed LM later (metachronous). After immunohistochemical staining of FFPE sections and whole slide scanning densities of CD3+ and CD8+ T cells per mm2 were quantified in NM, pCRC and LM using QuPath image analysis software. In pCRC and LM cell densities were measured in tumor center (TC), inner invasive margin (IM), outer invasive margin (OM) and peritumor zone (PT). IM and OM were defined as 500 µm on each side of the tumor border towards TC or PT.

Results. Median densities of CD3+ and CD8+ T cells in NM were 1014 and 319 in synchronous group, and 1042 and 390, respectively, in metachronous group. For all examined cells types in both groups we found smaller densities in TC of pCRC and LM compared to NM. In pCRC of synchronous group compared to LM densities of all examined cells were smaller in IM, OM and PT. In pCRC of metachronous group compared to LM densities of T cells were greater in TC, densities of CD3+ were smaller in OM and PT region. Compared to metachronous group patients with synchronous metastases had smaller densities of CD3+ T cells in IM and CD8+ T cells in TC and IM of pCRC.

High densities of $CD8^+$ T cells in TC, OM and PT of synchronous LM were associated with longer DFS, besides, the longest DFS was observed when high densities of CD8+ T cells in OM of both pCRC and LM. Greater densities of CD3+ T cells in IM and PT and CD8+ T cells in IM, OM and PT in synchronous LM over pCRC were associated with longer DFS.

Conclusion. Development of pCRC is accompanied by decreased densities of T cells in TC compared to NM irrespective of presence of LM. Lower densities of T cells in IM,

OM and PT of pCRC compared to LM is a hallmark of synchronous group, whereas in metachronous group TC of pCRC harbors larger numbers of T cells compared to LM. Low numbers of CD3+ and CD8+ T cells in IM and CD8+ T cells in TC of primary CRC may contribute to development of synchronous metastases.

This study demonstrated a clear difference in prognostic significance of T cells between synchronous and metachronous disease: high densities of CD8+ T cells in synchronous LM were associated with longer DFS, besides, the longest DFS was observed when high densities of CD8+ T cells in both pCRC and LM. Greater densities of CD3+ T cells and CD8+ T cells in selected regions of synchronous LM over pCRC were associated with longer DFS. Prognostic associations of T cells in peritumor zone and outer tumor margin may require refinement of current guidelines for assessment of tumor-infiltrating lymphocytes in CRC.

Lymphocyte-produced cytokines drive tumor dissemination in the liver in a non-cell autonomous manner

Jan Kosla

Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic. - Division of Chronic Inflammation and Cancer, German Cancer Research Center (DKFZ), Heidelberg, Germany

Tumor dissemination is the spread of tumor cells from the primary site to distant organs and accounts for up to 90% of all cancer-related deaths. It is a highly complex but inefficient process involving multiple steps. The liver is one of the most common sites of tumor dissemination due to both physical constraints and specific mechanisms. Nevertheless, treatment options for disseminated tumors remain poor and there is still a great need to better understand the underlying mechanisms driving this process of dissemination. In this context, chronic inflammation has been shown to be critical for carcinogenesis, from initiation and progression to dissemination. Here, we identified that lymphocyte-produced cytokines are expressed in both lymphoma and colorectal carcinoma cells disseminated in the liver and that primary colorectal carcinoma cells upregulate these cytokines during the process of dissemination - metastasis. We identified a liver-specific cell type that promotes tumor dissemination in response to lymphocyte-produced cytokines in both *in vitro* and *in vivo* models. To demonstrate this, we used two cancer entities - lymphoma and colorectal carcinoma cells – that naturally lack and express receptors for the lymphocyte-produced cytokines, respectively. Furthermore, we determined a signaling pathway induced in the identified liver-specific cell type that promotes tumor cell adhesion and transmigration. Abrogation of this pathway prevents liver dissemination of both cancer entities. Altogether, our results suggest a potential interventional target for tumor dissemination.

Therapeutic Potential of Antibody – Drug Conjugates in Multiple-Relapsed/ Recurrent Germ Cell Tumors

Lucia Kucerova[1,2], Natalia Udvorkova[3], Adriana Fekiacova[4], Michal Mego[1]

[1]Translational Research Unit, the [2]nd Oncology Clinic of the Medical Faculty, Comenius University, and the National Cancer Institute, Bratislava, Slovakia.
[2]Cancer Research Institute, Biomedical Research Centre, Slovak Academy of Sciences, Bratislava, Slovakia.
[3]Medical Faculty, Comenius University, Bratislava, Slovakia.
[4]Faculty of Natural Sciences, Comenius University, Bratislava,

Background: Platinum-based chemotherapy is highly efficient in treating germ cell tumors (GCT) being a standard-of-care in this patient group. However, relapsing patients often present with refractory disease, resulting in poor prognosis due to lack of novel treatment options besides chemotherapy and surgery. The aim of our research was to identify novel modalities for the treatment of GCT by screening for targets of clinically-approved antibody-drug conjugates (ADC) sacituzumab govitecan and mirvetuximab soravtasine in GCT. Additionally, we evaluated the cytotoxic efficacy of ADC in model cisplatin-resistant GCT to identify most promising candidates for clinical application.

Methods: Protein and mRNA levels of putative targets were measured by flow cytometry, immunohistochemical staining, and qRT-PCR. Cell viability of cisplatin-resistant GCT was assayed by luminescent viability assays, impedance and image-based cytotoxicity assays. Efficacy of the ADC treatment was evaluated in preclinical study on the experimental metastasis model of GCT.

Results: In a cell line-dependent manner, we detected expression of targets Trop2 and FolRα in multiple model GCT cell lines. Broad panel covered all histology types of GCT including seminoma, embryonal carcinoma, yolk sac tumor, choriocarcinoma and teratocarcinoma. We demonstrated that treatment with ADC induced potent cytotoxic effect in the subtypes with high expression of Trop2 and FolRα, respectively. Furthermore, we identified biomarkers of response in the GCT and confirmed the presence of selected targets on patient samples by immunohistochemistry staining. More importantly, potent therapeutic effect was observed in preclinical study on metastatic model of human choriocarcinoma.

Conclusions: In summary, this study offers a novel ADC to target multiple-relapsed/refractory GCT. Our work underlines the potency of the

antibody–drug conjugate treatment in chemoresistant hard-to-treat solid tumors including GCT. Moreover, we suggest that mrrGCTs represent another clinical entity where these novel agents will substantially improve the clinical outcome in a specific patient group with dismal prognosis due to refractoriness of their disease to currently used chemotherapeutic regimen.

Keywords: Antibody-drug-conjugates; Germ cell tumors; Resistance; Therapy.

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TUESDAY NOVEMBER 19, 2024

Chairs: Martin Mistrik, Pavel Bouchal

Dissecting tumor and immune heterogeneity in breast cancer metastasis

Juliane Winkler

Center of Cancer Research, MedUni Vienna, Vienna, Austria

Metastasis is the major cause of cancer-related deaths because conventional therapies often fail to eradicate metastatic disease. While the phenomenon of tumor heterogeneity has been studied in greater detail in the recent decade, its impact on metastasis remains largely unknown. Moreover, metastasis requires systemic remodeling of distant organ microenvironments that impacts immune cell phenotypes, population structure, and intercellular communication networks. However, our understanding of immune phenotypic dynamics in the metastatic niche remains incomplete. Here, we use single cell omics technologies to study the impacts of tumor heterogeneity on metastatic progression and metastatic niche development in breast cancer. Heterogeneous human primary tumor and metastatic cells showed profound transcriptional differences and displayed a dynamic continuum of cell states that are associated with metastatic potential. Additionally, we longitudinally assayed lung immune transcriptional profiles in the genetically engineered polyomavirus middle T antigen (PyMT) and the syngeneic 4T1 metastatic breast cancer mouse models from the onset of primary tumorigenesis and early stages of spontaneous metastatic dissemination to metastatic outgrowth at single-cell resolution. This temporal-resolved immune cell atlas revealed surprising dynamic changes in all immune cell types during metastatic progression. Specifically, we uncovered a TLR-NFκB inflammatory program that correlates with metastatic progression. This inflammatory gene program is conserved across multiple myeloid cell types in both models indicating shared inflammatory functions of different immune cell types that may orchestrate early stages of metastasis. Our PyMT and 4T1 longitudinal scRNA-seq cell atlases of spontaneous breast cancer metastasis provide new insights into the complex mechanisms of metastatic niche formation and the dynamic pro-metastatic reprogramming of immune cells which represent candidate pathways for anti-metastatic immunotherapy development.

Breast cancer subclassification based on proteomics/ proteogenomics for targeted therapy

Petr Lapčík[1], Jan Šimoník[1], Pavla Bouchalová[1], Kateřina Jurásková[2], David Potěšil[3], Lucia Janáčová[1], Boris Tichý[4], Vojtěch Bystrý[2], Rudolf Nenutil[5], Roman Hrstka[6], Pavel Bouchal[1]

[1]Masaryk University, Faculty of Science, Department of Biochemistry, Brno, Czech Republic.
[2]Masaryk University, Central European Institute for Technology, Core facility Bioinformatics, Brno, Czech Republic.
[3]Masaryk University, Central European Institute for Technology, Core facility Proteomics, Brno, Czech Republic.
[4]Masaryk University, Central European Institute for Technology, Core facility Genomics, Brno, Czech Republic.
[5]Masaryk Memorial Cancer Institute, Department of Oncological Pathology, Brno, Czech Republic.
[6]Masaryk Memorial Cancer Institute, Research Centre for Applied Molecular Oncology, Brno, Czech Republic

Accurate classification of breast tumors is vital for patient management decisions and enables more precise cancer treatment. We have shown that a quantitative proteotyping approach based on next-generation, data independent acquisition mass spectrometry (DIA-MS) is able to establish key proteins for breast tumor classification. The study was based on 96 tissue samples representing five conventional breast cancer subtypes [1]. Proteotype patterns largely recapitulated these subtypes; however, they also revealed varying heterogeneity within the conventional subtypes, with triple negative breast cancer (TNBC) being the most heterogeneous. To further address TNBC heterogeneity, we analyzed another set of 105 TNBC tissues via whole exome sequencing, RNA-Sequencing and DIA-MS proteomics using diaPASEF. A hybrid proteomics library contained 244,464 precursors, 168,006 peptides and 11,564 protein groups (FDR=1%), representing the deepest coverage of TNBC proteome to date, according to our knowledge [2]. Hierarchical clustering of TNBC patients was performed, based on 1223 proteins the mostly correlating with transcripts, and identified TNBC clusters typical of enriched pathways relevant for androgen receptor, immune response, DNA repair, steroid synthesis, and for basal and mesenchymal phenotype. These data well complement previous mRNA based classifications [3] moreover identifying core proteins associated with patient survival. Overall, our work highlights how cancer multiomics can lead to more accurate patient stratification, identifying pathways and proteins relevant for TNBC subclassification which could potentially serve as targets of biological therapy.

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Polygenic risk score (PRS) and its potential for breast cancer risk stratification in the Czech Republic

Milena Hovhannisyan, Petra Zemankova, Marketa Janatova

Institute of Medical Biochemistry and Laboratory Diagnostics, First Faculty of Medicine of Charles University and General University Hospital in Prague, Praha 2, Czech Republic

Background

The Polygenic Risk Score (PRS) allows to quantify an aggregated polygenic effect of many low-penetrance alleles to the risk of various multifactorial diseases including cancer. For breast cancer, two sets comprising 77 or 313 low penetrance loci (PRS77 and PRS313) have been designed. We aimed to evaluate the performance of both sets in breast cancer patients in the homogeneous (Czech) population allowing to compare their performance in breast cancer risk prediction.

Methods

In a retrospective case-control study we genotyped variants from both PRS77 and PRS313 sets in 1329 breast cancer patients and 1324 non-cancer control individuals, all women without germline pathogenic variants in breast cancer predisposition genes. Odds ratios (ORs) were calculated according to the categorical PRS in individual deciles. Weighted Cox regression analysis was used to estimate the hazard ratio (HR) per standard deviation increase in PRS, with age at diagnosis as a time-dependent variable. Set performance was assessed on the individual level.

Results

The distributions of standardized PRS in patients and controls were significantly different (p-value<2.2×10-16) using both PRS77 and PRS313 sets. The predictive performance of PRS313 was better than the performance of PRS77 for both categorical and continuous PRS analysis for risk estimation. For patients in the top 2.5% of PRS313, the risk of breast cancer was calculated as OR=3.05 (95% CI=1.66–5.89; p=1.76×10-4) and the risk of the disease was estimated as HRper SD=1.64 (95% CI=1.49–1.81, p<2.0×10-16) resulting in an absolute cumulative risk of 21.03% at age 80 years for individuals in the 95th percentile of PRS313. In addition, 248 (9.3%) individuals were discordantly classified into PRS deciles using different variant sets.

Conclusion

Both PRS77 and PRS313 are able to stratify individuals according to their breast cancer risk also in the Czech population. However, PRS313 shows better discriminatory ability. The results of our validation study support the potential clinical utility of using PRS313 in individualized breast cancer risk prediction and its ability to refine personalized clinical management including timing of screening and preventive measures in our population.

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Multiplex data utilizing a single-step staining and imaging workflow for the investigation of multiple sample types

Alex Darmoise

RareCyte

Tissue consists of heterogenous cell types with diverse functions and states, where spatial organization can inform – and impact - patient health status. Understanding the tumor microenvironment has proven particularly important for oncology and immune oncology studies, with fast-turnaround insights into immune response to tumors being critical for biomarker discovery, validation and the ultimate development of cancer therapeutics and diagnostic tests. Resolving tissue complexity across a statistically relevant number of patient biopsies at a cellular level has historically been challenged by image resolution, the number of targets that can be simultaneously assessed and sample throughput. These barriers have recently been broken with generation of high plex, whole-slide immunofluorescence (IF) imaging data in a single cycle using the Orion™ platform to deliver multiplex biomarker quantitation in just hours. Described here are some specific examples of how the established Orion one-step stain-and-scan approach has been used to rapidly generate highquality, subcellular quantitative IF data for multiple biomarkers across whole

tissue sections. Further, we describe how this, combined with traditional H&E on the same platform, can be used to provide accretive insights across the same cells and tissue microenvironments from the same biopsy sample.

Genomic instability, microenvironment and telomere homeostasis in solid malignancies

Pavel Vodicka[1,2,3], Kristyna Tomasova[1,3], Michal Kroupa[1,3], Anna Siskova[1,2], Anusha Uttarilli[1], Petr Hanak[1], Sona Vodenkova[4,3], Rajiv Kumar[1], Natalie Danesova[1,3], Kari Hemminki[3], Ludmila Vodickova[1,2,3]

[1]Institute of Experimental Medicine, Czech Academy of Sciences, Prague, Czech Republic.
[2]Institute of Biology and Medical Genetics, First Faculty of Medicine, Charles University, Prague, Czech Republic.
[3]Biomedical Center, Faculty of Medicine in Pilsen, Charles University, Pilsen, Czech Republic.
[4]Institute of Experimental Medicine, Czech Academy of Sciences, Prague, Djibouti

Impaired DNA repair mechanisms and disrupted telomere length homeostasis represent key steps in cancer initiation, progression, and prognosis. Understanding the mechanisms and dynamics of tumor genomic diversification, where DNA damage response and telomere homeostasis are important players, is critical to understanding carcinogenesis and overcoming drug resistance. Telomere shortening has a dual role in tumorigenesis. It promotes cancer initiation by inducing chromosomal instability, while telomere length (TL) maintenance characterized by telomerase expression is required for cancer cell proliferation and tumor growth. First, we made a Genome-wide association study comparing TL in peripheral blood lymphocytes in a cohort of 5,371 individuals consisting of patients with colorectal adenomas, colorectal cancer (CRC), and healthy non-cancerous controls with the aim to identify polymorphisms associated with TL differences and consequently, to reveal individuals in higher risk of developing cancer. We also studied TL as a biomarker for patient therapy response, and/or survival and in other cohort we also investigated mtDNA-CN in CRC tissues and adjacent mucosa in relation to the TL. The mitochondrial dysfunction seems to be linked with DNA repair capacity as well and compensates for damage by increasing the mitochondrial DNA copy number (mtDNA-CN). Moreover, gene expressions within mtDNA repair pathways correlated with mtDNA damage. The association of mtDNA damage and gene expressions has been found among 48 genes representing all putative repair pathways in mitochondria. In comparison with adjacent mucosa, CRC tumors exhibited a lower extent of mtDNA damage (P=0.047), but there was higher expression of the majority of mtDNA repair genes. Out of the 48 mtDNA repair genes, 14 with the strongest correlation between the expression and mtDNA damage (LIG3, MUTYH, RAD50, BRCA2, BRCA1, LIG1, PARG, NEIL3, RFC3, POLD3, POLD4, MRE11, NEIL1, UNG) are validated on a larger cohort of patients.

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TUESDAY NOVEMBER 19, 2024

Chairs: Marian Hajduch, Aleksi Sedo

Ecological study of lung cancer incidence and mortality in the Czech Republic

Marian Hajduch

Laboratory of Experimental Medicine, Institute of Molecular a Translational Medicine, Faculty of Medicine and Dentistry Palacky University, Olomouc, Czech Republic

Biomarkers for lung cancer and COPD diagnosis identified using mass-spectrometry based proteomics analysis of exhaled breath condensate

Jana Václavková[1], Petr Džubák[1,2], Jana Vrbková[1], Pavla Kouřilová[1], Dušan Holub[1], Juraj Kultan[3], Petr Jakubec[3], Ondřej Fisher[3], František Kopřiva[2], Vendula Látalová[2], Marián Hajdúch[1]

[1]Laboratory of Experimental Medicine, Institute of Molecular a Translational Medicine, Faculty of Medicine and Dentistry Palacky University, Olomouc, Czech Republic.
[2]Department of Pediatrics, University Hospital Olomouc and Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic.
[3]Department of Respiratory Medicine, University Hospital Olomouc and Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic

The malignant tumours a of tracheae, bronchi and lungs is a leading cause of death among the cancer deaths in the Czech Republic. It is usually diagnosed too late, only 15 – 20 % patients are diagnosed in stage I and II when the cancer can be cured entirely. Thus, the population pilot programme for lung cancer early detection was established in the Czech Republic in 2022. This project is a part of the European screening programme SOLACE (Strengthening the screening of lung cancer in Europe) and its goal is to implement a optimize the effective screening of lung carcinoma within EU member states. The ongoing national cancer programme is focused on men and women aged 55 to 74 years with smoking history (current or former smokers who have smoked 20 or more pack-years). The individuals are sent to be screened using low-dose CT (LDCT).

Collection of exhaled breath condensate is a cheap and non-invasive method to obtain samples from human respiratory tract. Finding new non-invasive methods for early detection of lung diseases (such as lung cancer, asthma, COPD, cystic fibrosis, various lung injuries) would be highly beneficial. Exhaled breath condensate (EBC) represents a rich source of biomarkers which can provide valuable information about respiratory and systemic diseases. Proteomic analysis of EBC is a prospective method to detect early changes in the status of the respiratory system and possibly other organs. It could also replace or complement some invasive sampling methods in future and provide non-invasive lung diseases screening technique.

We are focused on mass spectrometry based proteomic analysis of the EBC. We have optimized a method including gel-free sample preparation, Orbitrap based HPLC-MS analysis and powerful search tool to obtain high number of protein identifications. We have analysed many samples provided by Department of Respiratory Medicine as well as by the Czech early detection programme for lung cancer.

In our recent work, we focused on non-small cell lung cancer (NSCLC) and COPD diagnostics. Combining univariate and multivariate statistical approaches and sensitivity analysis, we have suggested potential biomarkers that could distinguish NSCLC patients from COPD and healthy individuals. Our models for NSCLC and COPD biomarker prediction worked well, seem promising, and will be further studied and validated. The validation using SureQuant approach for targeted quantitation was implemented. The validation phase is currently ongoing. The confirmed biomarkers will be used to develop an MS-independent kit for preventive screening of NSCLC, COPD, and possibly other lung cancer types.

Proteomics of ascitic extracellular vesicles reveals tumor microenvironment in ovarian cancer

Anna Vyhlídalová Kotrbová[1], Kristína Gömöryová[1], Antónia Mikulová[1], Hana Plešingerová[1], Stanislava Sladeček[1], Marek Kravec[1], Šárka Hrachovinová[1], David Potěšil[2], Markéta Bednaříková[3], Jitka Hausnerová[3], Michal Felsinger[3], Zbyněk Zdráhal[2], Vít Weinberger[3], Vítězslav Bryja[1], Vendula Pospíchalová[1]

[1]Faculty of Science, Masaryk University, Brno, Czech Republic.
[2]CEITEC, Masaryk University, Brno, Czech Republic
[3]University Hospital Brno, Brno, Czech Republic

High-grade serous carcinoma (HGSC) of the ovary, fallopian tube, and peritoneum is the most prevalent and lethal form of ovarian cancer. A significant number of HGSC patients develop ascites, an accumulation of excess fluid in the peritoneal cavity, which serves as a complex tumor microenvironment (TME) comprising various cells, proteins, and extracellular vesicles

(EVs). In this study, we employed orthogonal methods to isolate EVs from the ascites of HGSC patients and conducted a detailed analysis using mass spectrometry. This approach enabled us to identify a set of core ascitic EV-associated proteins and to further delineate a subset unique to HGSC ascites. By integrating single-cell RNA sequencing data, we traced the origins of these HGSC-specific EVs to various cell types within the ascitic fluid.

Contrary to expectations, our findings revealed that the majority of EVs did not originate from tumor cells but rather from non-malignant cell types such as macrophages and fibroblasts. This surprising discovery was corroborated through flow cytometry analysis of ascitic cells, coupled with a comprehensive evaluation of the EV protein composition in matched samples. Our results demonstrated that analyzing cell type-specific EV markers in HGSC provides more substantial prognostic insights compared to merely analyzing ascitic cells.

In conclusion, our research provides compelling evidence that proteomic analysis of EVs can effectively define the cellular composition of the HGSC TME. This advancement holds significant promise for enhancing our understanding of the role of EVs in tumor progression and inhibition, thereby paving the way for improved diagnostic and therapeutic strategies for HGSC.

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Suitability of the tear proteome for cancer biomarker studies

Tomáš Oždian, Denisa Kroupová, Martina Kintlová, Petr Džubák, Marián Hajdúch

Laboratory of Experimental Medicine, Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University and University Hospital Olomouc, Olomouc, Czech Republic

There is a growing interest in discovering biomarkers from body fluids, particularly those obtainable non-invasively. While this approach is feasible for many tissues and diseases, the brain presents a unique challenge due to its location within the skull and protection by the blood-brain barrier. Cerebrospinal fluid, the only body fluid providing direct information about the brain, requires invasive sampling. Another theoretically possible, but not yet practical, body fluid is tear fluid. This fluid washes the eye, which is connected to the brain via the optic nerve.

The tear proteome has been explored in connection with various diseases, including Parkinson‘s disease, breast and colon cancer, confirming its diagnostic potential. For this purpose, we conducted a preliminary experiment consisting of two groups: patients with retrobulbar neuritis and healthy volunteers. The retrobulbar neuritis cohort provided about 600 proteins per sample in the initial experiment. Later, the protocol used for the healthy volunteer cohort was deeply optimized and transferred to state-of-the-art Orbitrap instruments equipped with ion mobility separation, led to the identification of approximately 3000 proteins.

Tissue expression analysis of these results revealed 20 proteins specific to brain tissue. This discovery, together with the published crosstalk between tears and cerebrospinal fluid, suggests the presence of neuronal proteins in the tear proteome and confirms the suitability of tears as an interesting source of biomarkers for brain malignancies.

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Real-time monitoring of light-triggered protein-DNA interaction with a surface plasmon resonance biosensor

Giusy Finocchiaro[1], Tomáš Špringer[1], Kateřina Kralová[1], Markéta Bocková[1], Karel Chadt[1], Jan Bukáček[1], Gustavo Fuertes[2], Aditya Suresh Chaudhari[2], Bohdan Schneider[2], Jiří Homola[1]

[1]Institute of Photonics and Electronics, Prague, Czech Republic.
[2]Institute of Biotechnology, Vestec, Czech Republic

Light-responsive transcription factors are crucial for synthetic biology and optogenetic applications, holding the potential for targeted therapeutic strategies in cancer treatment [1]. EL222 is a light-responsive transcription factor from the bacterium Erythrobacter litoralis, which consists of an effector DNA-binding domain and a light-oxygen-voltage (LOV) domain. Upon blue light illumination, the LOV domain triggers a conformational change in EL222, rendering the effector domain accessible for binding to the target double-stranded DNA (dsDNA). Methods such as the electrophoretic mobility shift assay (EMSA) and the transient grating technique (TG) have been applied to study the interaction between EL222 and dsDNA [2], enabling the identification of a consensus dsDNA sequence [3]. However, the EL222-dsDNA dissociation process and the dynamics of the interaction remain

largely unknown.

In this work, we developed a novel label-free surface plasmon resonance (SPR) biosensor and monitored the real-time association and dissociation processes between EL222 and dsDNA. We advanced the SPR biosensor with a transparent microfluidic cartridge and backside illumination of the sensor surface, enabling EL222 *in situ* illumination. To investigate the interaction between EL222 and dsDNA, we immobilized one of the two components on the sensor surface, while injecting the other along the sensor surface. We hypothesized a mechanism for EL222-dsDNA complex formation and determined the association and dissociation rate constants. Our results revealed that the illuminated EL222 bound several times faster to dsDNA than the non-illuminated protein. We confirmed that EL222 specifically interacted with its consensus dsDNA sequence, without binding to random sequences. We also observed that the interaction between EL222 and dsDNA was affected by the buffer ionic composition, the illumination intensity and the light wavelength.

This work provides pivotal insights into the light-triggered interaction between EL222 and dsDNA, paving the way for the application of the SPR biosensor technology to the in-depth investigation of other photobiological processes.

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Unraveling the proteome by untargeted mass spectrometry proteomics at scale

Maik Pruess

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TUESDAY NOVEMBER 19, 2024

Chairs: Tomas Etrych, Milan Urban

Drug-Free Macromolecular Therapeutics

Jindřich Kopeček

Center for Controlled Chemical Delivery, University of Utah, Salt Lake City, Utah, USA. Departments of Molecular Pharmaceutics and of Biomedical Engineering, Salt Lake City, Utah, USA

Inactivity of immune effector cells in patients pose a challenge for anti-CD20 antibody-based immunotherapy of hematological malignancies. This signifies the need for alternative strategies that circumvent the resistance without involving immune effector function. Drug-free macromolecular therapeutics (DFMT) is a new paradigm in nanomedicine for the treatment of hematological B cell malignancies. Apoptosis is induced by crosslinking of receptors no low molecular weight drug is needed. DFMT is comprised of two nanoconjugates: a) the bispecific engager: an antibody Fab' fragment or antibody conjugated with 25 base pair morpholino oligonucleotide MORF1; and b) the crosslinking effector: human serum albumin (HSA) grafted with multiple copies of complementary oligonucleotide MORF2. Crosslinking of surface CD20 acts as a surface switch that triggers calcium influx, mitochondrial depolarization and caspase activation to induce apoptosis. Hyper-crosslinking of CD20-bound type II antibodies generates the effects of type I anti-CD20 antibodies and creates a new therapeutic that combines mechanisms of both antibody types, producing greater antitumor activity. Heteroreceptor crosslinking, involving CD20 and CD38 induces synergistic levels of apoptosis in CD20(+)/CD38(+) Raji cells. A murine model demonstrated dual-target DFMT significantly increased animal survival compared to single-target DFMT treated mice.

The efficacy of HSA-based DFMT in the treatment of 56 samples isolated from patients diagnosed with Chronic Lymphocytic Leukemia (CLL) was evaluated. Fab' fragments from Obitunuzumab and Isatuximab were employed in the synthesis of anti-CD20 (Fab'OBN-MORF1) and anti-CD38 (Fab'ISA-MORF1) bispecific engagers. The efficacy of DFMT was significantly influenced by the expression levels of CD20 and CD38 receptors. Dual-targeting DFMT strategies (CD20 + CD38) were more effective than single-target approaches. Pretreatment of patient cells with gemcitabine or ricolinostat markedly increased cell surface CD20 and CD38 expression, respectively. Apoptosis was effectively initiated in 62.5% of CD20-targeted samples and in 42.9% of CD38-targeted samples. These findings demonstrate DFMT's potential in personalized CLL therapy.

Recently, we expanded the DFMT concept to Multi-Antigen T Cell Hybridizers (MATCH). In this innovative technology hybridization of MORFs mediates T cell recruitment and activation against malignant B cells. Simultaneously targeting CD3 on an effector T cell surface with a B cell cancer antigen induces T cell cytotoxicity. Using four B cell antigens (CD20, CD38, BCMA, and SLAMF7), T cell activation against leukemia, lymphoma and myeloma cells was possible. *In vivo* efficacy was demonstrated on a mouse model of human NHL with a CD20-directed MATCH therapy. Cancer cell expression profiles can be matched with a corresponding Fab'-MORF1 combination therapy to activate T cells against multiple cancer-specific antigens. The interchangeability of MATCH introduces a technology that could overcome antigen-loss related relapse and provide a true patient-specific immunotherapy option for treating hematological malignancies.

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Stimuli-Responsive Polymer Therapeutics and Theranostics Employing Photodynamic Therapy

Marina Tavares[1], Kevin Kotalík[1], Alžběta Turnovská[1], Vladimír Šubr[1], Jun Fang[2], Tomáš Etrych[1]

[1]Institute of Macromolecular Chemistry, Praha, Czech Republic. [2]Sojo University, Kumamoto, Japan

Nano-sized polymer-based systems are widely studied as suitable candidates for the advanced delivery of bioactive molecules such as drugs and diagnostics. Indeed, efficient theranostic strategies concurrently bring and use both therapeutic and diagnostic features. Such Theranostics could serve as a cutting-edge tool to combat advanced cancers. Photodynamic therapy (PDT) has received considerable attention for the treatment of solid tumors (1). There is considerable evidence for the use of PDT in the treatment of numerous solid tumors, such as breast, prostate, ovarian, bladder, skin, and head and neck cancers (2). Herein, the development of long-circulating stimuli-responsive water-soluble or amphiphilic micelle-forming polymer-based systems employing various photoactive molecules or their precursors, e.g. pyropheophorbide-a (dPyF) or 5-aminolevulinic acid, covalently attached by the stimuli-sensitive bonds, were developed. The highly improved tumor-targeted delivery of dPyF was observed by fluorescence imaging. The pH-responsiveness and controlled activation of dPyF fluorescence and singlet oxygen production were determined using

the *in vivo* experiments focusing on both therapy and diagnostic of the solid tumors in mice. The nanomedicines showed superior anti-tumor PDT efficacy and huge tumor-imaging potential while reducing their accumulation and potential side effects in the liver and spleen. The developed theranostics exhibited clear selective tumor accumulation at high levels in the mouse sarcoma S180 tumor model with almost no dPyF found in the healthy tissues after 48 h. Once in the tumor, illumination at λexc = 420 nm reached the therapeutic effect due to the singlet oxygen generation. Indeed, an almost complete inhibition of tumor growth was observed up to 18 days after the treatment. The clear benefit of the specific dPyF release and activation in the acidic tumor environment for the targeted delivery and tissue distribution dynamics was proved. Conjugates carrying dPyF attached by pH-sensitive hydrazone bonds showed their excellent antitumor PDT effect and its applicability as advanced theranostics at very low doses of PyF.

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Reversing the tumor chemoresistance by using polymer-based nanotherapeutics with P-gp overcoming capacity

Milada Šírová[1], Martin Kaňa[1,2], Alena Braunová[3], Markéta Frejková[3], Jan Bouček[2], Tomáš Etrych[3], Blanka Říhová[1], Marek Kovář[1]

[1]*Institute of Microbiology CAS, Prague, Czech Republic.*
[2]*University Hospital Motol, Prague, Czech Republic.*
[3]*Institute of Macromolecular Chemistry CAS, Prague, Czech Republic*

Chemotherapy is still the mainstay of cancer therapy. However, cancer cells often fail to respond to chemotherapeutic regimens because of either inherent or acquired resistance. Several factors contribute to the development of this unfavorable phenomenon, including epigenetics, the presence of cancer stem cells, the tumor microenvironment, and changes that affect the cell's signaling pathways. It has been found that critical mechanism involved in (multi)drug resistance (MDR) is increased drug clearance by the transporter-facilitated efflux of the drugs from cancer cells. It can be mediated by overexpression of membrane transporters, such as adenosine triphosphate binding cassette (ABC) pumps, from which P-gp (ABCB1, MDR1) is the most prominent. A significant array of specific inhibitors of individual ABC family transporters exists. However, their effect is burdened with a number of undesirable toxicities and interactions, because ABC transporters also have extensive physiological expression in healthy cells and organs. Thus, there is still a need to find ways how to bypass the resistance phenomenon. Some recently developed nanodrug delivery systems may represent a significant approach in cancer therapy.

We used an amphiphilic diblock polymer nanotherapeutics containing a hydrophilic block based on the N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer and a hydrophobic poly(propylene oxide) block (PPO). The amphiphilic character of the diblock polymer ensures self-assembly into micelles with hydrodynamic radius Rh ~15 nm in aqueous solutions. The diblock copolymer and its conjugates with doxorubicin (Dox) significantly increased the intracellular concentrations of Dox and markedly sensitized multidrug resistant tumor cells to chemotherapy. The underlying mechanisms included inhibition of P-gp-mediated drug efflux, alteration of mitochondrial membrane potential, depletion of intracellular ATP, and increased ROS production. Moreover, the DB-Dox conjugates inhibited tumor growth *in vivo* more effectively when compared to corresponding HPMA-based drug delivery system. Copolymers with additional PPO loaded in the micelle core demonstrated superior efficacy in terms of P-gp inhibition, ATP depletion, and chemosensitizing effect *in vitro*, as well as antitumor activity *in vivo*. In primary human tumor cells derived from patients with head and neck tumors, the diblock copolymers effectively decreased ATP levels both *in vitro* and *in vivo* using patient-derived xenograft (PDX) model. Variation in the polymer structure, resulting in controlled hydrolysis of the polymer structure consisting of HPMA and PPO blocks, and its implications for the biological effect will also be discussed. The data underscore the potential of this polymer drug delivery system to enhance the effectiveness of standard chemotherapy and show translational potential.

HPMA polymer-porphyrin conjugates with potential application in photodynamic therapy

Alžběta Turnovská[1], Marina Rodrigues Tavares[1], Shanghui Gao[2], Jan Hynek[3], Jun Fang[2], Kamil Lang[4], Tomáš Etrych[1]

[1]Institute of Macromolecular Chemistry, CAS, Prague, Czech Republic.
[2]Sojo University, Kumamoto, Japan. [3]Institute of MacroInstitute of Inorganic Chemistry, Prague, Czech Republic.
[4]Institute of Inorganic Chemistry, Prague, Czech Republic

Photodynamic therapy uses a light-sensitive photosensitizer (PS), such as tetraphenyl-porphyrin (TPP), in combination with illumination at appropriate wavelength for the treatment of cancer. The light-activated PS then reacts with oxygen present in the tumour tissue, form reactive oxygen species, such as singlet oxygen and induce tumour cell death. (1) The main drawbacks, restricting the PS´s use in PDT, involves limited solubility in physiological conditions and hydrophobicity, as well as their lack of tumour selectivity. To overcome these obstacles, water-soluble, and non-toxic polymer nanocarriers, such as N-(2-hydroxypropyl) methacrylamide (HPMA) copolymers, can be employed to carry PS and improve its physico-chemical properties. Moreover, due to the Enhanced Permeability and Retention (EPR) effect, the nanocarriers are passively accumulated in the tumour tissue amplifying the therapeutic outcome. (2) This study presents the synthesis, and subsequent biological and physico-chemical evaluation of polymer-TPP nanoconjugates. TPP derivatives were bound in the nanoconjugates by hydrazone bonds with either aliphatic or aromatic spacer, or by stable amide bond. The structure-release rate dependency was studied in conditions mimicking the neutral blood conditions and acidic tumour environment. *In vitro* evaluation of cytotoxicity and intracellular uptake, as well as preliminary *in vivo* experiments were carried out. Fluorescence and CMC were measured to understand micelle formation and overall behaviour in physiological conditions.

We were able to prepare systems with up to 6.2 wt% of TPP bound to polymer precursors. TPP release rate can be controlled by selection of spacer in between the sensitizer and polymer backbone. Remarkable increase of *in vitro* cytotoxicity was observed after illumination with λ=420 nm. In aqueous solutions, decreased fluorescence was observed due to micelle formation and quenching. CMC values of the three systems were correlated with structure and rigidity of the spacer. HPMA-based micellar nanocarriers with bound TPP exhibited enhanced solubility and stability in physiological conditions. Systems with hydrazone bonds allow the release of TPP and full activation of its properties during the treatment. Thanks to the passive accumulation in the tumour and illumination of only the affected area, these systems proved to be a promising candidate for minimally invasive photodynamic therapy.

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In situ synthetic approach of *HPMA* diblocks for tumor treatment

Michaela Hrochová[1], Lenka Kotrchova[1], Libor Kostka[1], Rafal Konefal[1], Markéta Frejková[1], Jun Fang[2], Tomáš Etrych[1]

[1]Institute of Macromolecular Chemistry, CAS, Prague, Czech Republic.
[2]Faculty of Pharmaceutical Sciences, Sojo University, Kumamoto, Japan

Recently, nanomedicines showed very good treatment efficacy of various tumors in preclinical research but there is still very limited amount of nanomedicine which entered or passed clinical trials. Here, we evaluated novel synthetic approach that allows us to *in situ* prepare two-arm drug carrier with important features. Due to the increased hydrodynamic radius of the polymer system, both increased accumulation in tumor tissue and long-term circulation in the bloodstream can be achieved [1]. The diblock system could then represents a depo carrying the anticancer drug and enabling long circulation of the carrier molecules in the blood stream. Importantly, developed HPMA diblocks could be easily degraded after the fulfilling its mission of drug delivery. After the hydrolytic degradation the polymers could be easily eliminated by the kidneys via glomerular filtration.

In our work we have designed four types of bifunctional chain transfer agents (CTA) that enable *in situ* RAFT polymerization of N-(2-hydroxyropyl)methacrylamide (HPMA) into diblock copolymers [2]. Polymers with molar mass up to 100 kg/mol and 8 nm in hydrodynamic diameter can be easily synthesized. The use of different CTAs allows the synthesis of diblocks with different circulation times in the bloodstream, where the half-life of the system can be set in range of 5 hours to 21 days. In the future, these systems could be used as depos for the various cytostatics suitable for the treatment

of solid tumors or hematological malignancies. This hypothesis was verified by synthesizing polymer conjugate carrying pirarubicin bound via a pH-sensitive hydrazone bond. *In vitro* and *in vivo* biological studies confirmed the high efficacy of treatment of the rapidly growing S180 sarcoma tumor. Comparison of the diblock with the linear system showed that the longer circulation time of diblock contributes to more effective treatment and also to the possibility to administer lower dose of the drug.

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Gemcitabine nanotherapeutics based on HPMA copolymers: the effect of drug release kinetics on the cancer treatment efficacy

Katerina Behalova[1], *Natalie Klusova*[1], *Martin Ivancik*[1], *Milada Sirova*[1], *Robert Pola*[2], *Tomas Etrych*[2], *Marek Kovar*[1]

[1]*Laboratory of Tumor Immunology, Institute of Microbiology of the Czech Academy of Sciences, Prague, Czech Republic.*
[2]*Department of Biomedicinal Polymers, Institute of Macromolecular Chemistry of the Czech Academy of Sciences, Prague, Czech Republic*

Gemcitabine, a potent chemotherapeutic agent, has been used in clinical practice for decades. Its main clinical applications include the treatment of breast, lung, pancreatic, and ovarian cancers. It is used both as monotherapy and in combination with other cytostatics. Although it is a commonly used drug, its main limitation is its short half-life in circulation, as it is very rapidly metabolised by the enzyme cytidine deaminase upon administration.

We have therefore devised a way to protect this chemotherapeutic from degradation, significantly extend its half-life in the circulation and increase its efficacy. Four polymer conjugates based on HPMA copolymers were synthesized, differing in the linkage between the drug and the polymeric carrier that dictates the release kinetics of gemcitabine. The conjugates contain biodegradable spacers - the tetrapeptide GFLG (P-Gem1), B-alanine (P-Gem2), valeric acid (P-Gem3) and aminocaproic acid (P-Gem4). By these systems, gemcitabine is transported as a prodrug, protected from metabolisation by cytidine deaminase, released in a controlled manner with kinetics corresponding to its linker, and its half-life in circulation is significantly prolonged.

We studied the antitumor activity of these polymer conjugates in models corresponding to the main clinical applications of gemcitabine both *in vitro* and *in vivo*. The *in vitro* data obtained correlate with the kinetic release of the individual conjugates. Additionaly, we have developed a method to study the kinetics of the persistence of P-Gem conjugates in the organism, directly studying the release kinetics *in vivo*. We confirmed that the conjugate with the slowest release kinetics (P-Gem4) can prolong the circulation of gemcitabine in the organism and for up to 96 hours, whereas free gemcitabine persists in the bloodstream for only 2 hours.

These results are reflected in the therapeutic efficacy of the individual conjugates in *in vivo* tumor models 4T1 (breast cancer), LL2 (lung cancer) and Panc02 (pancreatic cancer), where slow-release conjugates (P-Gem3 and 4) are strongly favored. They effectively slow tumor growth, prolong survival and in some cases provide complete tumor remission (in 4T1 model up to 50 % of the treated mice were completely cured from the cancer). Therefore, we believe that we have found a potentially promising tool for the treatment of various solid malignancies, especially difficult-to-treat panreatic carcinomas.

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WEDNESDAY NOVEMBER 20, 2024

Chairs: Marek Mraz, Josef Srovnal

Germline Genetics of Multiple Myeloma

Asta Försti

Hopp Children's Cancer Center (KiTZ), Heidelberg, Germany. German Cancer Research Center (DKFZ), Heidelberg, Germany

Multiple myeloma (MM) is a plasma cell disease and its somatic genetic events are well-established. They include IGH translocations and hyperdiploidy as primary genetic events followed by regional chromosomal deletions or gains, mutational events and secondary translocations. Germline genetic alterations started to gain interest first in the genome-wide association study (GWAS) era. In our international collaboration of nearly 10,000 cases and 245,000 controls we have identified most of the so far reported low-penetrance susceptibility variants for MM and functionally characterized many of them. We have also identified the CCND1 c.870G>A variant as the first constitutive genetic factor associated with the risk of a specific chromosomal translocation, t(11;14)(q13;q32) in MM. More recently we and others have collected germline samples from MM families for identification of high-risk genes for MM using whole exome or genome sequencing. Among 21 families with 46 affected individuals we identified potentially pathogenic variants in a tumor suppressor gene DAB2IP and an oncogene ABL2. Two genes, KMT2A and USP28, are related to previously reported MM predisposition genes related to DNA methylation and apoptosis, respectively. One germline variant in a somatic driver in MM, SAMHD1, was also found in one family. We also identified an interesting copy-number variant, the region covering the genes FGFBP1, FGFBP2 and PROM1. In the same families, we also identified non-coding variants especially in the promoters and 5'UTRs of genes implicated in important biological pathways in MM development, including MAPK and ErbB signaling pathways. Our study has enhanced the knowledge of germline variant contribution in the development of MM.

Cardiac troponins and natriuretic peptides for monitoring cardiovascular safety of cancer treatment

Petr Jarolim

Dana-Farber Cancer Institute, Boston, USA. Brigham and Women's Hospital, Boston, USA. Harvard Medical School, Boston, USA

Cardiovascular biomarkers such as cardiac troponins I and T and natriuretic peptides are well established biomarkers for diagnosing acute coronary syndrome and for monitoring of patients with heart failure. Their use in cardio-oncology is steadily increasing. We will review these biomarkers and their clinical use.

An important contributing factor to the increased use of troponin testing is the emergence of high sensitivity cardiac troponin assays that enable detection of not only the gradual, cumulative cardiac damage during cancer chemotherapy but also early diagnosis of potentially severe, or even fatal fulminant autoimmune myocarditis during treatment with immune checkpoint inhibitors (ICIs). Essentially all clinical trials at our institutions that utilize ICIs stipulate regular testing of patients using high sensitivity troponin assays prior to the next drug application with prespecified sex-specific cutoff values and additional evaluation if these cutoffs are exceeded. These cutoffs differ from the typical diagnostic cutoffs and algorithms used for early detection of acute coronary syndrome.

Natriuretic peptide testing, including both the detection of the B-type natriuretic peptide (BNP) as well as its surrogate marker, the N-terminal pro-B-type natriuretic peptide (NT-proBNP) has been a mainstay of diagnosis and monitoring of patients with acute and chronic heart failure. In a subset of patients with cancer, the treatment is associated with left ventricular dysfunction detected by this type of testing. Moreover, both natriuretic peptides and cardiac troponins are associated with adverse outcomes in patients with cancer and in childhood cancer survivors.

We will discuss classification of cardiac troponin assays according to their sensitivity, differences between cardiac troponin I and T performance, potential novel assays aiming at distinguishing acute and chronic cardiac injury, and the work performed in our laboratory on various versions of cardiac troponin tests including the ultrasensitive single molecule assays. Particular attention will be paid to the use of the high sensitivity assays for monitoring cardiac safety in patients with cancer on various treatment modalities. We will then explain principal differences between the BNP and NT-proBNP assays both in terms of their use for monitoring of certain cancer patients as well as their role in predicting patient survival.

A novel model to study proliferation signaling in chronic lymphocytic leukemia identifies unique drugs with anti-proliferative effect

Eva Hoferkova[1,2], Daniel Filip1[,2], Laura Ondrisova[1,2], Vaclav Seda[1,2], Jan Verner[2], Kvetoslava Matulova[3], Hana Skuhrova Francova[2], Miroslav Boudny[1,2], Josef Vecera[1], Peter Kacz[1,2], Krystof Hlavac[1,2], Petra Pavelkova[1], Lenka Kostalova1,2, Androniki Michaelou[1], Sarka Pospisilova[2], Jana Dorazilova[4], Michael Doubek[2], Lucy Vojtova[4], Ales Hampl[5], Leos Kren[3], Jiri Mayer[2], Marek Mraz[1,2]

[1]*CEITEC Masaryk University, Brno, Czech Republic.*
[2]*Dept. of Internal Medicine, Hematology and Oncology, University Hospital Brno and Faculty of Medicine, Masaryk University, Brno, Czech Republic.*
[3]*Dept. of Pathology, University Hospital Brno and Faculty of Medicine, Masaryk University, Brno, Czech Republic.*
[4]*Central European Institute of Technology, Brno University of Technology, Brno, Czech Republic.*
[5]*Dept. of Histology and Embryology, Faculty of Medicine, Masaryk University, Brno, Czech Republic*

Objectives Several *in vitro* model systems have been developed to mimic chronic lymhocytic leukemia (CLL) proliferation in lymph nodes typically utilizing CD40L-expressing stromal cells and addition of various recombinant factors. However, in such models, CLL proliferation is relatively low and variable. Here we aim to prepare a novel co-culture model based on mimicking T cell signals *in vitro* and in patient-derived xenografts (PDX).

Methods. We prepared six cell lines by engineering HS5 stromal cells with stable expression of human CD40L, IL4, IL21, and their combinations, namely HS5-CD40L, HS5-IL4, HS5-IL21, HS5-CD40L-IL4, HS5-CD40LLOW-IL4, and HS5-CD40L-IL4-IL21 cells. HS5 cells were co-cultured with CLL cells for proliferation and gene expression studies, drug testing and co-transplanted on 3D scaffolds into NSG mice.

Results. We co-cultured primary CLL cells (n=16) with a panel of six HS5 lines, revealing a CLL cell proliferation of 7 % cells (at day 7) in HS5-CD40L-IL4 co-cultures (P=0,019), and 44 % in HS5-CD40L-IL4-IL21 co-cultures (P<0,001). The proliferation in HS5-CD40L-IL4-IL21 co-cultures was more prominent when compared to the use of recombinant CD40L, IL4, and IL21 (P<0,05), and IGHV status did not affect the proliferation rate. To test the applicability of the models for drug screening purposes, we performed its downscaling to microliter volumes. Custom poly(dimethylsiloxane)-based inserts of 5 ul volume were seeded with ~250 HS5-CD40L-IL4 cells and ~2500 CLL cells. CLL cells in microwells (n=5) had a proliferation rate and viability identical to standard co-cultures (3 ml wells). Next, we performed transcriptional profiling of sorted primary CLL cells cultured on plastic or co-cultured with HS5-WT or HS5-CD40L-IL4/IL21 cells. We identified 3833 differentially expressed mRNAs (Padj<0,05) between CLL cells co-cultured with HS5-WT vs. HS5-CD40L-IL4 cells, and 2057 differentially expressed mRNAs in HS5-CD40L-IL4 vs. HS5-CD40L-IL4-IL21 co-cultures. This included activation of MYC, NFκB and E2F signatures induced by HS5-CD40L-IL4/IL21 co-cultures (as defined from CLL lymph nodes [Herishanu et al, 2011]) and other pathways. The other induced pathways reveal novel CLL vulnerabilities in context of CLL-T cell-induced proliferation. We tested 10 inhibitors based on these data rrevealing for the first time that RAF inhibitors and FOXO1 inhibitors block CLL proliferation, and this has been validated in xenograft animal models. Finally, we demonstrate that CLL cell engraftment in NSG mice can be supported by engineered HS5 cells, thus bypassing the need to use primary T cells in PDX (Bagnara et al, 2011). We co-transplanted 41 NSG mice with CLL cells and HS5-CD40L-IL4/IL21 using a subcutaneous scaffold and intraperitoneal injection, resulting in engraftment of CLL cells in >50% of cases. However, only about ~20% PDXs had evidence of a direct clonal relationship to original CLL, suggesting that often either minor CLL subclones or normal EBV+ B cells are engrafted.

Conclusion. We engineered a co-culture model that induces robust CLL cell proliferation via a supportive cell line expressing CD40L, IL4, and IL21. These co-cultures can be downscaled to microliter volumes for drug screening purposes or upscaled to a PDX model. The novel model allowed us to identify pan-RAF and FOxO1 inhibitors as having a striking anti-leukemic activity.

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T-maps: Evaluation of new cell surface markers in pediatric T-cell acute lymphoblastic leukemia to monitor minimal residual disease

Adéla Vávrová, Daniela Kužílková, Tomáš Kalina

CLIP Cytometry, Second Faculty of Medicine,Charles University, Prague, Czech Republic

Childhood T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive disease characterized by the presence of malignant T lymphoblasts in the bone marrow. At diagnosis, the prognosis is comparable to other types of ALL, but patients who relapse present with a very poor

prognosis. A key prognostic feature is the determination of the minimal residual disease (MRD) level on day 15 of treatment. Detection of MRN by flow cytometry is based on a distinct immunophenotype of malignant cells, but due to partial overlap with non-malignant T cells, its accurate detection remains challenging.

We set out to identify aberrantly expressed molecules on pediatric T-ALL cells in order to refine MRN detection by flow cytometry.

Using fluorescently labeled antibodies, we mapped the expression of surface markers (n=307) in diagnostic samples of pediatric T-ALL (n=34) by conventional and spectral cytometry and compared them with expression in T cells from healthy donors (n=12) and in developmental stages of T cells isolated from pediatric thymic patients (n=3). The BD PE Quantitation kit was used to convert fluorescence to the number of antibody molecules bound to the cell.

In the analysis of individual samples from pediatric T-ALL patients, we identified 14-39 aberrantly expressed markers (expression at least 2-fold increased or 2-fold decreased) compared with healthy T cells, and ten CD markers (CD229, CD44, CD226, CD52, CD48, CD6, CD71, CD38, CD82, and CD26) were aberrantly expressed in at least 75% of the samples examined. We further compared the expression of these traits between T-ALL samples and physiological T cell populations present in the pediatric thymus. Expression of seven CD features reflected their physiological development. Expression of three additional CD features in the pediatric T-ALL samples did not correlate with expression on healthy thymocytes, and thus their increased (CD38) or decreased (CD26 and CD52) expression is specific for tumor-altered pediatric T-ALL cells.

In total, we identified 10 potential CD markers that are suitable for MRN monitoring in at least 75% of pediatric T-ALL patients.

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Epigenetic control of stem cell decisions via ISWI ATPase Smarca5

Tomas Stopka

BIOCEV 1.LF, Prague, Czech Republic

Superfamily SWI/SNF2 (mating type switch/ sucrose non-fermenting) utilize energy to perturb histone-DNA contacts in order to change the position of histone octamers on DNA. The SWI/SNF2-mediated remodeling results in either the nucleosome-depleted DNA regions or densely packed arrays of nucleosomes. It is well known that chromatin remodeling influence any transaction on DNA such as transcription, replication or repair.

Chromatin structure changes throughout developmental checkpoints and loss or inappropriate activity of chromatin remodeling leads to distinct human pathologic states. SWI/SNF2 superfamily consists of the SWI/SNF2, ISWI, CHD and INO80 subfamilies. Based on extensive work on hematopoietic differentiation we previously identified ISWI ATPase Smarca5 as major regulator of chromatin fluidity in stem cells.

We generated a set of mouse knockout and transgenic models and found that Smarca5 is required in hematopoietic stem cells at commitment level. Upon Smarca5 deficiency, p53 program becomes activated and this leads to programmed cell death. However, at lower level, Smarca5 is critically important for development of lymphoid progenitor cells. Our work also suggests, that Smarca5 in acute myeloid leukemia is a prerequisite of leukemic proliferation and chromatid cohesion and thus could represent a therapeutic target.

Our work currently focus on Smarca5 protein partners and protein degradation in a tissue specific manner.

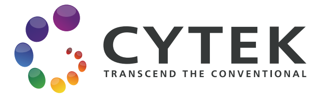
Synergistic Role of Aldehyde Dehydrogenase Inhibition in combination with Hypomethylating Agents in Relapse/ Refractory Acute Myeloid Leukemia

Mehak Shaikh[1,2], Muhube Yazir[3], Celine Baier[4], Srdjan Grusanovic[1], Andrada Constantineascu3, Miroslava Adamcova[1,5], Chloe Fourmound[3], Mileidys Perez-Alea[3], Meritxell Alberich-Jorda[1,5]

[1]Department of Hemato-oncology, Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic.
[2]Faculty of Science, Charles University, Prague, Czech Republic.
[3]Adavnced BioDesign, Lyon, France.
[4]Advanced BioDesign, Lyon, France.
[5]Childhood Leukemia Investigation Prague, Department of Pediatric Hematology and Oncology, 2nd Faculty of Medicine, Charles University in Prague, University Hospital Motol, Prague, Czech Republic

Acute myeloid leukemia (AML) is a heterogeneous malignancy characterized by a differentiation arrest and accumulation of blasts. Upon initial treatment, most patients exhibit remission, however, 50% of all patients who achieve remission relapse over time. We and others have reported, that there is an increase in the levels of the enzyme Aldehyde Dehydrogenase 1A (ALDH1A) in the group of refractory and relapse AML (R/R AML) patients, and that high ALDH1A activities are associated with drug resistance. ALDH1A plays a role in oxidizing the toxic aldehydes to carboxylic

acids, thus protecting cells from oxidative damage and proteo-toxic stress. DIMATE is an inhibitor of ALDH1A which could be employed to inhibit chemo-resistance in AML. Therefore, we first determined the effect of DIMATE in human AML cell lines, and murine MLL-AF9 leukemia both *in vitro* and *in vivo*. We studied that DIMATE does not affect the healthy cells and only targets the AML cells. We determined the molecular mechanism of DIMATE in ALDH1A inhibition. And lastly, we carried out *in vitro* and *in vivo* studies to observe the effect of DIMATE in combination with hypomethylating agents Decitabine, and 5-Azacytidine on primary AML cell lines, murine MLL-AF9 leukemia, and on R/R AML patient samples. Our findings suggest that ALDH1A inhibition using DIMATE in combination with hypomethylating agents plays a beneficial role in inhibiting the progression of AML both *in vitro* and *in vivo*, and thus can improve the quality of life of patients with R/R AML, and also of older patients who cannot uptake a higher dose of chemotherapy.



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Chairs: Jan Bouchal, Stjepan Uldrijan

Identification and validation of novel prognostic factors and therapeutic targets in osteosarcoma

Lucia Knopfová[1,2], Kamila Říhová[1,2], Jana Pokludová[1,2], Andrea Rousová[1], Jan Verner[3], Jiří Navrátil[4], Zuzana Kahounová[5], Iva Staniczková Zambo[6], Peter Múdry[7], Dagmar Adámková[8], Karel Souček[5,2,1], Petr Beneš[1,2]

[1]*Department of Experimental Biology, Faculty of Science, Masaryk University, Brno, Czech Republic.*
[2]*International Clinical Research Centre, St. Anne´s University Hospital, Brno, Czech Republic.*
[3]*CEITEC - Central European Institute of Technology, Masaryk University, Brno, Czech Republic.*
[4]*Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic.*
[5]*Institute of Biophysics of the Czech Academy of Sciences, Brno, Czech Republic.*
[6]*1st Department of Pathology, St. Anne's University Hospital and Faculty of Medicine, Masaryk University, Brno, Czech Republic.*
[7]*Pediatric Oncology Department, University Hospital Brno and Faculty of Medicine, Masaryk University, Brno, Czech Republic.*
[8]*Masaryk Memorial Cancer Institute and Faculty of Medicine Masaryk University, Brno, Czech Republic*

Osteosarcoma (OSA) is the most common primary malignant bone tumor. Its therapy is based on surgery and drugs that have been used since the 70s with various regimens that include namely methotrexate, doxorubicin and cisplatin. Recurrent disease, however, still occurs in 30 % to 40 % of patients. OSA is highly aggressive cancer and about 15-20 % of patients have secondary involvement at the time of diagnosis, mostly in lung parenchyma and distant skeletal sites. The 5-year overall survival for patients with metastatic spread is less than 30 %, frighteningly low and largely unchanged in the last decades. The identification of valid OSA prognostic markers is therefore needed to select patients at high risk of development of metastatic disease and to identify possible therapeutic targets. Although numerous markers/targets have been proposed so far, they have not yet improved the outcome of OSA patients. In our laboratory, we combine analysis of transcriptomic data from non-metastatic/metastatic cancer cell lines with analysis of available transcriptomic datasets in OSA patients to reveal novel metastatic regulators, prognostic markers and possible therapeutic targets. Using this strategy, we identify c-Myb, creatine kinase B (CKB), tropomyosin 2 (TPM2) and apoptosis-associated speck-like protein containing a CARD domain (PYCARD) as regulators of tumor growth, metastatic dissemination and chemosensitivity using cell lines and OSA mouse models. c-Myb proteins has been validated in retrospective cohort of OSA patients as a negative prognostic factor, similar analysis of other proteins revealed significant heterogeneity in patient tumor samples and their clinical validation is ongoing.

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Regeneration initiation expression signatures found in single cell and spatial transcriptomics tumor data

Radek Sindelka, Ravindra Naraine, Mikael Kubista

Institute of Biotechnology, CAS, Vestec, Czech Republic

Regeneration is an fascinating process that occurs in broad spectrum of animals including fishes, amphibians, and reptiles. In general, mammals show partial regenerative capabilities during embryonic stages. We have revealed a novel cell state during the initial hours of regeneration and we called it "Regeneration Initiation Cells" (RICs). Recently, we found similar RICs like expression signatures in various tumor single cell and spatial transcriptomic data. Here, we will present potential role of RICs like cells during tumor growth. We believe that our results could serve as a backbone for a comparative analyses of regeneration and tumor progression and in the future as an efficient diagnostic marker.

Expression of germline *Jak2* R1063H represents increased risk of thrombosis and impairs normal hematopoietic development in mice

Veronika Zimolova[1], Monika Buroczova[1], Jan Gursky[2], Lubos Janotka[2], Alena Pecinova[3], David Kundrat[4], Vladimir Korinek[1], Vladimir Divoky[2], Lucie Lanikova[1]

[1]*Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic.*
[2]*Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic.*
[3]*Institute of Physiology of the Czech Academy of Sciences, Prague, Czech Republic.*
[4]*Institute of Hematology and*

Blood Transfusion, Prague, Czech Republic

BACKGROUND: While the role of the acquired JAK2 V617F mutation in the development of myeloproliferative neoplasms (MPN) has been well characterized, the role of weakly activating JAK2 germline variants in the pathogenesis of MPN and/or acute myeloid leukemia (AML) remains unclear. We have previously described and functionally characterized the JAK2 R1063H germline variant, cooperating with the JAK2 E846D in a case of hereditary MPN with erythrocytosis and megakaryocytic atypia (PMID: 27389715) or with the acquired JAK2 V617F causing increased JAK2 signaling and higher disease severity in patients with essential thrombocythemia. In 28% of double mutant patients (4/14) there was at least one thrombotic event during the course of the disease (PMID: 30377194).

AIMS: The aim of this study was to investigate germline Jak2 R1063H variant's role in MPN as a potential risk factor for disease development and progression.

METHODS: The mouse model bearing Jak2 R1063H mutation was created using microinjection of synthesized R1063H ssODNs, sgRNA mRNA and Cas9 protein into C57BL6/N-derived zygotes. In order to intensify a possible effect of the mutation, we bred and analyzed the knock-in Jak2 R1063H homozygous mice.

RESULTS: In the cohort of Jak2 R1063H mice, we observed a relatively high frequency of sudden death in mice with median overall survival of 215 days (5/15) in comparison with the control group (0/15). The level of D-dimers, as a marker of thrombosis, was found statistically increased in the Jak2 R1063H group. Bioenergetic alterations in Jak2 R1063H platelets (PLT) suggested elevated basal mitochondrial respiration and demonstrated strong enrichment of fatty acid metabolism genes, including CD36. The Jak2 R1063H mice display increased PLT counts when compared to wild-type (wt) mice during life-span. The observed trend towards increased PLT was fully transplantable suggesting cell autonomous defect of Jak2 R1063H bone marrow. Despite increased PLT counts, the plasma thrombopoietin (TPO) level was normal whereas erythropoietin (EPO) levels were increased without marked erythrocytosis. The co-immunoprecipitation experiments with human EPO and TPO receptors (EPOR/TPOR) and JAK2 variants suggest increased TPOR and decreased EPOR binding with JAK2 R1063H kinase, possibly explaining the hematological phenotype. Also experiments with humanized mouse model of EPOR (PMID: 11158582) crossed with Jak2 R1063H mice supported these findings, where anemia due to hypoactivation of wt human EPOR signaling was more severe in Jak2 R1063H mice and mild thrombocytosis was still present in the double mutants. The Jak2 R1063H model displays increased number of long-term hematopoietic stem cells (HSC) (3 months) and shift to increased short-term HSC and multipotent progenitors with aging (12 months). The myeloid progenitor cells (LK) were significantly increased in Jak2 R1063H mice due to disproportionately increased megakaryocytic/erythroid progenitors over other myeloid progenitors at 3 months of age. The LK compartment is compromised in old Jak2 R1063H mice due to substantial myeloid bias by disproportionately increased CMP cells and markedly decreased MEP cells, explaining the more pronounced anemia of aging in these mice. The RNA-seq profiling of HSC and LK populations showed a progressive augmentation of a subset of Egr1 transcriptional signature in the Jak2 R1063H progenitors. Egr1 was significantly upregulated in the Jak2 R1063H 12 months HSCs vs. 3 months mutant HSCs and also in the Jak2 R1063H 12 months HSCs vs. wt 12 months HSCs. Egr1 expression coregulated with some of its homeostatic and inflammatory targets, among them, Thbs1, the gene encoding thrombospondin 1, a CD36 ligand, may contribute to the observed phenotype. While in mutant mice Thbs1 was significantly upregulated in both the aged HSCs as well as in aged LK cells, inflammatory INF-γ and TNF-α signaling signatures were upregulated only in young animals' Jak2 R1063H LK cells. Next, we assessed a possible cooperative role of Jak2 R1063H mutation in leukemic development. We injected MLL-AF9 splenocytes into the non-irradiated wt and Jak2 R1063H recipients and monitored development of leukemia, which was faster in Jak2 R1063H recipients than in wt recipients. In addition, Jak2 R1063H HSCs transduced with MLL-AF9 oncogene showed enhanced colony forming ability upon MLL-AF9 transduction and increased re-plating capacity. These data collectively suggested that Jak2 R1063H mutation may cooperate with a driver oncogene in promoting leukemic development in both cell extrinsic and intrinsic manner.

SUMMARY: Our study characterizes the Jak2 R1063H germline mutation as a risk factor for thrombosis, MPN development, progression and leukemic transformation. These findings have important clinical implications for the management of patients with MPNs carrying the JAK2 R1063H mutation.

CDK4 and CDK6 expression pattern in B-cell non-Hodgkin lymphomas as a predictive marker of response to CDK4/6 inhibitors

Denisa Veselá[1], Kristýna Kupcová[2], Pavel Klener[3,4], Ondřej Havránek[2,3], Vladimír Kryštof[1], Miroslav Strnad[5], Radek Jorda[1]

[1]*Department of Experimental Biology, Faculty of Science, Palacky University Olomouc, Olomouc, Czech Republic.*
[2]*BIOCEV, First Faculty of Medicine, Charles University, Vestec, Czech Republic.*
[3]*Department of Haematology, First Faculty of Medicine, Charles University and General University Hospital, Praha, Czech Republic.*
[4]*Institute of Pathological Physiology, First Faculty of Medicine, Charles University, Praha, Czech Republic.*
[5]*Laboratory of Growth Regulators, Palacky University Olomouc and Institute of Experimental Botany, The Czech Academy of Sciences, Olomouc, Czech Republic*

Targeting CDK4 and CDK6 kinases has radically changed the clinical perception of the treatment of advanced hormone receptor-positive breast cancer with four FDA-approved CDK4/6 inhibitors (CDK4/6i), palbociclib, ribociclib and abemaciclib. However, several tumour types possess only modest or no therapeutic benefit and biomarkers that could predict their sensitivity remain uncertain.

An investigation into the antiproliferative effects of palbociclib, ribociclib and abemaciclib on a panel of 20 lymphoma cell lines representing different subtypes revealed variable responses. The protein expression levels of CDKs differed significantly, with CDK4 predominantly expressed in mantle cell lymphomas contrary to the expression of CDK6. Most ABC subtypes expressed similar levels of both proteins, while GCB subtypes showed higher CDK6 expression. Burkitt lymphoma cell lines predominantly expressed higher CDK6 levels. The observed differences in cell line sensitivity to ribociclib correlated with the intrinsic expression levels of CDK4 and CDK6. Cells with low levels of CDK6 exhibited exquisitely high sensitivity, whereas those expressing both CDK4 and CDK6 showed little or no response to ribociclib. Doxycyclin-inducible CDK4 overexpression significantly increased the sensitivity of lymphoma cells to ribociclib, whereas CDK6 overexpression did not. Low CDK6 expression may predict sensitivity to ribociclib and could serve as a useful biomarker to stratify patient response to CDK4/6i therapy.

For lymphomas with poor outcomes from CDK4/6i treatment, we proposed a novel strategy involving combined therapy with PI3K inhibitors, such as idelalisib and alpelisib, or with ERβ modulators, tamoxifen and acolbifene. These approaches provide promising results for improving future lymphoma therapy.

New insights into the ERK MAPK pathway regulation in malignant melanoma

Stjepan Uldrijan[1,2], Natália Vadovičová[1], Barbora Valčíková[1]

[1]*Masaryk University, Faculty of Medicine, Brno, Czech Republic.*
[2]*St. Anne's University Hospital, International Clinical Research Center, Brno, Czech Republic*

Oncogenic BRAF and NRAS mutations, which activate the RAS/RAF/MEK/ERK mitogen-activated protein kinase (MAPK) pathway, are established drivers of malignant melanoma. Due to the strong activation potential of these mutations, the intensity of ERK MAPK signaling must be modulated by various negative feedback mechanisms to achieve optimal tumor growth. The quantitative analysis of ERK signaling downregulation by these feedback mechanisms and the spare signaling capacity of the MAPK pathway has not been thoroughly explored.

In our recent study, we identified several novel inhibitors targeting phosphatase-mediated negative feedback within the MAPK pathway. We utilized these inhibitors to examine changes in ERK activity in BRAF- and NRAS-mutant human melanoma cells following feedback disruption. Our findings reveal that the steady-state ERK MAPK signaling flux in melanoma cells harboring oncogenic BRAF and NRAS mutations constitutes only a small fraction of the pathway's total signaling capacity. Small-molecule compounds that disrupt phosphatase-mediated negative feedback can significantly perturb the control of MAPK signaling in melanoma cells.

In conclusion, our quantitative analysis of the MAPK signaling optimum in melanoma cells uncovered a substantial spare signaling capacity, which can be harnessed using drugs that disrupt selected negative feedback mechanisms.

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eIF4F controls AMPK activity in malignant melanoma

Natália Vadovičová[1], Barbora Valčíková[1], Kateřina Koždoňová[1], David Potěšil[2], Zbyněk Zdráhal[2], Stjepan Uldrijan[1,3]

[1]*Department of Biology, Faculty of Medicine, Masaryk University, Brno, Czech Republic.*
[2]*Central European Institute of Technology, Masaryk University, Brno, Czech Republic.*
[3]*International Clinical Research Center, St. Anne's University Hospital, Brno, Czech Republic*

Introduction

Malignant melanoma is an aggressive form of cancer with poor prognosis, often caused

by mutations in the MAPK ERK signaling cascade. Clinically relevant small-molecule drugs targeting BRAF and MEK kinases can prolong survival, but resistance rapidly emerges. Recently, the eukaryotic translation initiation complex (eIF4F) has been reported as the nexus of resistance to BRAF/MEK inhibitors. Furthermore, simultaneous inhibition of BRAF and eIF4F synergized in killing cancer cells. Therefore, we aimed to precisely characterize the crosstalk between the ERK and eIF4F pathways in melanoma.

Methods

We used MS-based proteomic approach and small-molecule MEK/eIF4F inhibitors to identify common targets of the ERK and eIF4F pathways in NRAS- and BRAFV600E-mutant melanoma cells. Then we validated the targets using RNA interference and western blotting.

Results

The proteomic analysis revealed a significant overlap of ERK and eIF4F targets in both melanoma subtypes. Interestingly, apart from the cell cycle and DNA repair regulators, we found regulators of the primary cellular energy sensor, AMP-dependent protein kinase (AMPK).

MO25, part of an AMPK-activating complex (LKB1-STRAD-MO25), and PP2Aα, an AMPK-inhibiting phosphatase, were found to be downregulated. Upon eIF4F inhibition, we observed ERK pathway activation and surprisingly, AMPK activation despite the downregulation of LKB1, the canonical activator of AMPK. This was also confirmed in LKB1-deficient BRAFV600E-mutant melanoma cells. These results suggest the existence of a novel, LKB1-independent mechanism of AMPK activation in melanoma cells.

Furthermore, PP2Aα downregulation seems to play an essential role, as RNAi-mediated knockdown of PP2Aα and a small-molecule PP2A inhibitor both potently promoted AMPK activity.

Discussion

Previous studies reported negative feedback regulation between the ERK pathway and LKB1 in BRAFV600E-mutant melanoma cells. Active ERK and its target RSK both phosphorylate LKB1, preventing it from activating AMPK, which would suggest mutually exclusive activity of AMPK and ERK.

In our recent study, we reported direct AMPK-mediated hyperactivation of the oncogene-driven ERK pathway in response to metabolic stress. While high metabolic stress affected ERK pathway differently in BRAFV600E- and NRAS-mutant melanoma cells, it caused cell cycle arrest in both melanoma subtypes.

Conclusion

Our findings reveal new insights into the molecular mechanisms of melanoma resistance and indicate the cooperation of the ERK and eIF4F pathways in controlling essential cellular processes.

eIF4F inhibition promoted non-canonical, LKB1-independent AMPK activation with simultaneous ERK activation. We describe a novel mechanism of AMPK activity control in BRAFV600E-mutant melanoma cells.

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WEDNESDAY NOVEMBER 20, 2024

Chairs: Ondrej Slaby, Marek Kovar

Investigating tumor heterogeneity using in vivo CRISPR gene editing technologies in head and neck cancer

Sampath Loganathan

McGill University/RI-MUHC, Montreal, Canada

Advancements in genetic sequencing technology uncovered hundreds of mutations in most adult solid tumors including head and neck squamous cell carcinoma (HNSCC) during the past decade. Most of these mutations form just randomly as the tumor grows and could be mere bystanders. However, a handful of these mutations trigger tumor development or metastasis and are hence called driver mutations. In addition, some of these driver gene mutations co-occur in different combinations in patients resulting in difference in treatment response, disease progression etc. creating an incredible challenge of tumor heterogeneity. Deciphering these complex combinations require advanced gene-editing technologies in animal models of head and neck cancer. Our lab utilizes CRISPR gene editing technology combined with an embryonic injection methodology to switch-off or switch-on 100s of genes simultaneously in the skin and oral cavity of mice and study novel driver mutations that trigger tumor development. Using transcriptomics and *in vivo* drop out screen in immune competent mouse, we further assess the vulnerabilities in Head and Neck Cancer in order to develop therapeutic strategies.

Opportunities to Deliver the Vision of Precision Oncology through Whole Genome Sequencing

Richard Houlston

Division of Genetics and Epidemiology, The Institute of Cancer Research, London, United Kingdom

Precision oncology aims to tailor therapy to the unique biology of the patient's cancer, thereby optimising treatment efficacy and minimising toxicity. Underpinning precision oncology is the concept of somatic driver mutations as the foundation of cancer biology. An expansion in the number of therapeutically actionable genes has exposed the limitations of single-analyte genomic assays. The incremental cost of adding additional cancer genes to high-throughput sequencing-based panels has made the development of drugs targeting increasingly smaller subsets of molecularly defined patients financially and logistically feasible. The development of inhibitors effective in cancers driven by rare genomic mutations has required the concurrent development of novel clinical trial designs such as basket trials, in which eligibility is based on mutational status instead of organ site, cancer stage, and histology. With the advent of basket studies, many oncologists now consider that tumour genomic profiling should be offered to all patients with cancer who are not candidates for curative-intent local or systemic therapy.

Currently, multiple standalone tests or a panel are typically used to capture a set of genomic, transcriptomic, or epigenomic features in a tumour to inform patient treatment9. However, falling costs are making whole genome sequencing (WGS) a potentially attractive proposition as a single all-encompassing test to identify cancer drivers and other genomic features, which may not be captured by standard testing but are clinically actionable10. This approach is being explored in the UK by the 100,000 Genomes Project (100kGP), which is seeking to deliver the vision of precision oncology through WGS to National Health Service (NHS) patients as part of routine care.

I will discuss an analysis of WGS data on 10,478 patients spanning 35 cancer types recruited to the 100kGP. Across all cancer types, 330 candidate driver genes were identified, including 74 which are novel to any cancer. Their actionability both in terms of currently approved therapeutic agents and through computational chemogenomic analysis to predict candidacy for future clinical trials will be discussed.

Employing Dual Nature of Iron to Combat Cancer via Mitochondrial Targeting of Deferasirox

Sukanya Jadhav[1], Cristian Sandoval-Acuna[1], Yaiza Pacior[1], Petra Potomova[1], Kristyna Klanicova[1], Jana Smigova[2], Radislav Sedlacek[3], Jan Stursa[1], Lukas Werner[1], Jaroslav Truksa[1]

[1]Institute of Biotechnology of the Czech Academy of Sciences, BIOCEV research centre, Vestec, Czech Republic.
[2]Faculty of Sciences, Charles University, BIOCEV research centre, Vestec, Czech Republic.
[3]Czech Centre for Phenogenomics, Institute of Molecular Genetics of the Czech Academy of Sciences, Vestec, Czech Republic

Iron has a dual nature, it is a vital micronutrient needed for the proper functioning of cellular metabolism, respiration and DNA replication, yet, excess free iron leads to the generation of toxic reactive oxygen species, eventually triggering a special type of cell death termed "ferroptosis". Deferasirox (DFX) is a cell-permeable iron chelator

approved for the treatment of chronic iron overload. We have designed and synthesized its mitochondrially targeted derivative, called mitoDFX, by coupling it with a triphenylphosphonium group. The compound significantly affects mitochondrial proteome and induces selective iron deprivation in cancer cells. mitoDFX exhibits marked cytostatic, cytotoxic, and migrastatic properties at nM concentrations *in vitro* while not affecting non-malignant cells. The underlying molecular mechanisms include impairment of [Fe-S] cluster/heme biogenesis leading to destabilization and loss of activity of [Fe-S] cluster/heme containing enzymes. This results in dysfunctional mitochondria with markedly reduced respiration, disassembled respiratory supercomplexes and increased mtROS production, all of which contribute to the induction of mitophagy. Importantly, mitoDFX leads to depletion of reduced glutathione levels and significant lipid peroxidation, pointing towards an oxidative “ferroptosis-like” cell death, which is further supported by markedly enhanced cell death in GPX4 KO cells and synergistic effect of glutathione synthesis inhibitor BSO with mitoDFX. Mitochondrial targeting of DFX, therefore, represents a way to deprive cancer cells of biologically active iron, which is incompatible with their proliferation, growth and invasion, while at the same time it exhausts their antioxidant defense mechanisms, leading to lipid peroxidation and cell death. Our findings highlight the novel concept of targeting mitochondrial iron metabolism as an anti-cancer approach and demonstrate that mitochondrially targeted deferasirox not only chelates the iron inside the mitochondria, but renders it redox-active, making mitoDFX an extremely effective anti-cancer drug.

Pro-apoptotic proteins Bax and Bak modulate in cell-specific manner mitochondrial respiration via regulation of TEFM expression

Dana Sovilj[1], Cristina Daniela Kelemen[1], Sarka Dvorakova[1], Renata Zobalova[1], Helena Raabova[2], Jan Kriska[3], Zuzana Hermanova[3], Tomas Knotek[3], Miroslava Anderova[3], Pavel Klener[4], Vlada Filimonenko[2], Jiri Neuzil[1,5], Ladislav Andera[1,2]

[1]IBT CAS, Vestec, Czech Republic.
2IMG CAS, Prague, Czech Republic.
[3]IEM CAS, Prague, Czech Republic.
[4]1.FM CU, Prague, Czech Republic.
5Griffith University, Southport, Australia

Proteins from the Bcl-2 family play an essential role in the regulation of intrinsic/mitochondrial apoptotic signaling, but they also display cell death-unrelated, less comprehended roles in cellular/mitochondrial metabolism. Thus in this communication, we aimed to contribute to a better understanding of apoptosis-unrelated activities of the Bax and Bak, pro-apoptotic members of the Bcl-2 family. Using CRISPR-Cas9 gene editing, we prepared Bax/Bak-deficient human cancer cells of epithelial, neural and hematopoietic origin and discovered that while respiration and cell proliferation of the glioblastoma-derived U87 Bax/Bak-deficient cells was greatly enhanced, respiration as well as *in vitro* proliferation of Bax/Bak-deficient B lymphoma HBL-2 cells was attenuated. Interestingly, Bax/Bak-deficient U87 cells also more rapidly formed tumours in mice, and showed modulation of metabolism with a considerably increased NAD+/NADH ratio. Follow-up analyses uncovered increased/decreased expression of mitochondria-encoded subunits of respiratory complexes and stabilization/destabilization of the mitochondrial transcription elongation factor TEFM in Bax/Bak-deficient U87 and HBL-2 cells, respectively. ShRNA-mediated downregulation of TEFM expression attenuated mitochondrial respiration in Bax/Bak-deficient U87 as well as in parental HBL-2 cells. Our findings suggest that (post)translational regulation of TEFM levels in Bax/Bak-deficient cells modulates the expression of subunits of mitochondrial respiratory complexes that, in turn, contribute to respiration and the accompanying changes in metabolism and proliferation in these cells.

Tricking cancer cells into an apoptotic response to the false alarm triggered by MDM2 inhibitors

Zdenek Andrysik

Department of Biology, Faculty of Medicine, Masaryk University, Brno, Czech Republic

Transcription factor p53 is encoded by the most frequently mutated tumor suppressor gene in human cancers. In response to a variety of stress stimuli, p53 induces transcription of hundreds of genes involved in numerous tumor-suppressive cellular programs including cell cycle arrest, apoptosis, and senescence. Gene network governed by p53 is highly conserved due to two key features of this transcription factor: First, p53 tetramers interact with response elements on the DNA by two half-sites, each 10bp long. Consensus sequences of over 98% of other transcription factors are shorter, limiting binding specificity. Second, p53 acts as a pioneering transcription factor which can interact with nucleosomal DNA, restricting p53’s dependence on the chromatin status. Finally, since p53 is expressed in virtually all tissue types, it can act as a universal coordinating hub for tumor suppression.

In about half of the cancer cases, a wild-type form of the protein is preserved, providing an opportunity for targeted activation in disease

treatment. Throughout the last two decades numerous compounds were developed to obstruct an interaction between p53 and a key negative regulator MDM2. However, induction of p53 by blocking MDM2 triggers the therapeutically desirable apoptosis only in few cancer models. Clinical trials in which small molecule MDM2 inhibitors were used in monotherapies fell short of expectations and revealed hematological toxicity as a characteristic adverse effect for the drug class. Currently, the leading strategy for both improving efficacy and mitigating adverse effects of MDM2 inhibitors is represented by combination treatments. Induction of the p53 network may be boosted by drugs modulating chromatin landscape, p53 transcriptome, translatome, and proteome. However, majority of targeted therapeutics synergizing with MDM2 inhibitors aim at specific cellular programs including apoptosis, cell cycle arrest, DNA repair, metabolic stress response, immune response, ferroptosis, and cancer cell vulnerabilities like growth signals addiction. Since almost all those pathways are also activated by p53 through a number of directly transactivated genes, the principle of functional similarity among the targets of synergizing drugs may be used as a guide for candidate pathway selection in combination treatments.

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WEDNESDAY NOVEMBER 20, 2024

Chairs: Karel Smetana, Lucca Vanucci

Immunosuppression in tumor microenvironment mediated by myeloid-derived suppressor cells (MDSC)

Viktor Umansky

German Cancer Research Center (DKFZ), Heidelberg, Germany. Ruprecht-Karl University of Heidelberg, Heidelberg, Germany

Myeloid-derived suppressor cells (MDSC) represent a heterogeneous myeloid cell population that is accumulated and activated in tumor microenvironment under chronic inflammatory conditions. They substantially contribute to immunosuppression in cancer, representing thereby a valuable therapeutic target. It has been recently demonstrated that such MDSC enrichment could be mediated not only by a long-term production of soluble inflammatory factors but also by extracellular vesicles (EV) secreted by tumor cells. Importantly, EV contain a broad range of proteins, mRNA, microRNA and lipids and are considered as mediators of intercellular communication. We analyzed the effect of EV cargo, in particular, S100A8/A9 and HMGB1 on the conversion of normal myeloid cells into MDSC.

There are different approaches to inhibit MDSC functions in cancer. One possibility of MDSC targeting is based on the inhibition of the transcription factor STAT3, orchestrating MDSC accumulation and acquisition of immunosuppressive properties. The STAT3 inhibitor Napabucasin abrogated the capacity of murine MDSC to suppress T cell proliferation. It induced apoptosis in murine MDSC and significantly increased expression of molecules associated with antigen processing and presentation on these cells. Melanoma bearing mice treated with Napabucasin showed prolonged survival accompanied by a strong accumulation of tumor-infiltrating antigen-presenting cells and activation of CD8 and CD4 T cells. In melanoma patients, circulating M-MDSC strongly expressed activated STAT3 that was associated with a worse progression free survival (PFS), indicating the role of STAT3 as a promising therapeutic target in these patients and as a predictive marker for their clinical outcome.

Targeting the IL-6 receptor alpha chain to manipulate the cancer microenvironment

Karel Smetana, Jr.[1], Lukáš Lacina[1,2], Milan Raška[3], Petr Malý[4]

[1]Charles University, 1st Faculty of Medicine, Institute of Anatomy and BIOCEV, Prague, Czech Republic. [2]Charles University, 1st Faculty of Medicine, Prague, Czech Republic. [3]Palacky University, Faculty of Medicine and Dentistry, Department of Immunology, Olomouc, Czech Republic. [4]Institute of Biotechnology , Czech Academy of Sciences, Vestec, Czech Republic

Human tumors are complex ecosystems consisting of cancerous and non-cancerous cells, including cancer-associated fibroblasts (CAFs) and infiltrating immune cells. The use of immune checkpoint inhibitors in tumor treatment has led to an interest in possibly manipulating CAFs for therapy. Across different types of tumors, the secretion of IL-6 is a common characteristic of iCAFs. In this context, IL-6 is highly biologically active and affects the differentiation, proliferation, and migration of cancer cells. Therefore, targeting the IL-6 receptor appears to be a potentially effective approach for treating tumors and also severe inflammatory disorders.

We utilized biotechnology and protein engineering to develop a series of small proteins called NEF blockers with a strong affinity for the α subunit of the IL-6 receptor (IL6-Rα). We demonstrated their predicted effect by inhibition of phosphorylation of the downstream protein STAT3. To examine the biological properties of the NEF blockers, we conducted tests using *in vitro* platforms such as iCelligence and IncuCyte. Through these methods, we studied the effects of the NEF blockers on the proliferation and migration of cancer cells. To verify their blocking function on the mouse version of the IL-6Rα we assessed their anti-inflammatory potential using a dextran-sulfate-induced acute model of mouse colitis.

NEF blockers are proven to be non-toxic. NEF blockers exhibited a negative effect on the proliferation and migration of cancer cells including melanoma and cells from ductal adenocarcinoma of the pancreas. Their application substantially suppressed intestinal inflammation such as the number of infiltrating cells, histological changes in mucosal architecture and secretion of inflammatory cytokines in mice.

NEF blockers are powerful inhibitors of IL6-Rα. This outcome motivates us to continue the research with the potential for the practical application of NEF blockers.

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CD25-biased IL-2 agonists synergize with immune checkpoint blockade in cancer immunotherapy despite robust Treg cell expansion but timing is crucial

Marek Kovář[1], Irfan Baki Kilic[1], Petra Weberová[1], Derek VanDyke[2], Milada Šírová[1], Kateřina Kubešová[1], Charina Fabilane[3,2], Vladyslav Mazhara[1], Kathy Liu[3], Kateřina Běhalová[1], Bohumil Ptáček[1], Blanka Říhová[1], Jamie Berta Spangler[2,3,4]

[1]Institute of Microbiology of the Czech Academy of Sciences, Prague, Czech Republic.
[2]Johns Hopkins University, Baltimore, USA.
[3]Johns Hopkins University School of Medicine, Baltimore, USA.
[4]Johns Hopkins University Bloomberg School of Public Health, Baltimore, USA

Numerous studies have described the improvement of IL-2 therapy through the design of IL-2 muteins with biased cytokine activity (either eliminating CD25 binding or increasing CD122 binding), PEGylated IL-2 and its variants, or IL-2/CD25 fusion proteins. Another interesting approach, described by Boyman et al.1, is based on complexes of IL-2 and αIL-2 mAb (IL-2co). IL-2co possesses dramatically increased *in vivo* biological activity, mostly mediated via a significant extension of IL-2 half-life. Moreover, they exert selective stimulatory activity for different IL-2 responsive cell subsets depending on the clone of anti-IL-2 mAb used. Thus, IL-2 complexed with S4B6 mAb (IL-2/S4B6 henceforth) was shown to predominantly stimulate CD122high populations via dimeric IL-2Rβγc, while IL-2 complexed with JES6-1A12 mAb (IL-2/JES6 henceforth) highly selectively stimulates CD25high populations via trimeric IL-2Rαβγc. However, IL-2/S4B6 also stimulates CD25high populations to some extent, albeit in a CD25-independent manner. Mechanistically, it has been shown that IL-2/S4B6 directly binds to IL-2Rβγc while completely avoiding interaction with CD25 due to the overlapping binding site for CD25 and the S4B6 mAb in the IL-2 molecule 2. On the other hand, induction of IL-2R downstream signaling by IL-2/JES6 requires interaction with CD25 in the first step, followed by dissociation of IL-2 from JES6-1A12 mAb, IL-2 binding to CD25, and subsequent transfer to IL-2Rβγc. Later on, chimeric proteins called immunocytokines (ICs), where IL-2 is linked through a flexible oligopeptide linker to the light chain of αIL-2 mAb, were introduced 3. ICs mimic IL-2co both structurally and functionally but possess an advantage over IL-2co since the possibility of having an excess of either IL-2 or αIL-2 mAb in the mixture (i.e. IL-2co) and dissociation to free cytokine leading to off-target effects is eliminated.

Since IL-2/S4B6 showed potent stimulatory activity for NK, memory, as well as activated CD8+ T cells, and demonstrated antitumor activity in several mouse tumor models, it has been established for more than a decade that these CD122-biased IL-2co are suitable for cancer immunotherapy, while CD25-biased IL-2/JES6, extensively expanding Treg cells, are suitable for the treatment of autoimmunity or to facilitate allograft acceptance 4-6. However, several studies have been published recently showing that targeting the high-affinity IL-2R may be beneficial in cancer immunotherapy and that CD25-biased IL-2 agonists are more efficient in comparison to CD122-biased ones. Moreover, CD25-binding was found to be essential for modifying CD8+ T cell exhaustion program via combined therapy using IL-2 and PD-1 blockade in a model of chronic viral infection.

Immune checkpoint inhibitors (ICIs) are typically mAbs blocking receptor-ligand interactions providing inhibitory signals to T cells. The use of ICIs in clinical practice, particularly αCTLA-4 and αPD-1 mAbs, has revolutionized cancer immunotherapy of malignant diseases, e.g. melanoma and lung carcinoma. Unfortunately, not all patients respond to ICI therapy and thus there is an urgent need to find a combination therapies to improve the overall response rate.

Strong stimulatory activity for Treg cells and vascular toxicity have been most important reasons why CD25-biased IL-2-based therapeutics including IL-2co were considered not to be suitable for cancer immunotherapy. Instead, CD122-biased IL-2 agonists were believed to be more promising. Here we show that CD25-biased IL-2co or ICs synergize with ICIs to completely eradicate large established tumors despite robust expansion of Treg cells. However, the proper timing is crucial since only administration of IL-2co after ICIs but not vice versa leads to profound antitumor effect. Mechanistically, CD25-biased IL-2co or ICs potently and selectively stimulate expansion of tumor-specific CD8+ T cells and expression of their effector functions in CD25-dependent manner and overcome Treg cell mediated suppression. Moreover, CD25-biased IL-2co possess much lower toxicity enabling much higher dosage than CD122-biased ones. These findings support the use of CD25-biased IL-2 therapeutics in combination with ICIs for cancer immunotherapy.

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Exploring fibroblast activation protein (FAP) expression in brain metastases and novel alpha-ketoamide FAP inhibitors for theranostic applications

Petr Vymola[1], Adela Simkova[2], Miroslav Vetrik[3], Barbora Vymolova1, Nathan Sidej[2], Jan Kucka[3], Tadeas Smetana[1], Tereza Ormsby[2], Filip Trajhan[2], Jan Konvalinka[2], Martin Hruby[3], Pavel Sacha[2], Aleksi Sedo[1], Petr Busek[1]

[1]First faculty of medicine, Charles University, Prague, Czech Republic.
[2]Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Prague, Czech Republic.
[3]Institute of Macromolecular Chemistry, Czech Academy of Sciences, Prague, Czech Republic

Brain metastases are a frequent and severe complication of various solid cancers, representing the most common malignant tumors of the central nervous system in adults with a poor prognosis and limited therapeutic options. This underscores the need for discovering new therapeutic targets and devising novel treatment strategies.

Fibroblast activation protein (FAP), a serine protease expressed in cancer associated fibroblasts (CAF) and cancer cells, represents a promising target for tumor visualization and therapy. This study aimed to evaluate FAP expression in brain metastases and to design and test novel FAP targeting compounds (FAPi) utilizing recently developed alpha-ketoamide FAP inhibitors.

FAP expression in brain metastases and control non-tumorous brain tissue was determined by an enzymatic assay, ELISA, and immunohistochemistry. Cancer and CAF cell cultures were derived from brain metastases and characterized by imunnocytochemistry. Novel alpha-ketoamide FAPi were labeled with 99mTc and their binding to FAP-expressing cells *in vitro* and biodistribution *in vivo* was evaluated.

FAP expression was significantly higher in brain metastases of various origins compared to non-tumorous brain tissue both at the protein and enzymatic activity level. FAP was expressed in CAFs and in addition in subsets of tumor cells in some brain metastases. A similar expression pattern was observed in patient-derived cancer cells and CAF cultures obtained from brain metastases. The 99mTc-labeled alpha ketoamide FAPi demonstrated specific binding to FAP-expressing cells *in vitro* and accumulated in tumors in an animal model.

FAP is expressed in brain metastases originating from various solid tumors, highlighting its potential as a theranostic target. We are the first to prepare novel FAP radioligands utilizing an alpha ketoamide inhibitor. Ongoing work focuses on establishing relevant *in vivo* models of brain metastases to advance the clinical translation and efficacy testing of these compounds.

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Experimental targeting of tumor microenvironment by nano-constructs. Our experience

Luca Vannucci[1], Lenka Rajsiglová[1,2], Pavol Lukáč[1,2], Paolo Tenti[1,2], Michal Babič[3], Pierpaolo Ceci[4,5], Elisabetta Falvo[4], Dmitry Stakheev[1,6], Petr Šíma[1], Daniel Smrž[1,6]

[1]Institute of Microbiology, Czech Academy of Sciences, Prague, Czech Republic.
[2]Faculty of Science, Charles University, Prague, Czech Republic.
[3]Institute of Macromolecular Chemistry, Czech Academy of Sciences, Prague, Czech Republic.
[4]Institute of Molecular Biology and Pathology, Italian National Research Council IBPM-CNR, Rome, Italy.
[5]Thena Biotech, Latina, Italy.
[6]Department of Immunology, 2nd Medical Faculty, Charles University, Prague, Czech Republic

Cancer is a complex illness with local to systemic expression. Cancer cells in relation with the constitutive components of the tissue from which they develop and the immune system elements, that interact with them to try their ablation and tissue repair, form the tumor microenvironment.

The interactions between all these elements decide the tumor evolution either toward its elimination (inflammation, immune recognition and direct cytotoxicity) or its establishment and progression (inversion of the immune response, chronic inflammation, immune cell exhaustion and establishment of an immune suppressive and tissue remodeling environment). The altered immune environment can bias the efficacy of the treatments and favors the tumor heterogeneity. Many therapeutic approaches were developed, from surgery to chemo- and radiotherapy until the recent advances in immunotherapy. However, if the therapeutic approach is systemic, sensible side effects can accompany the treatment because of the involvement also

of non-tumoral tissues. Therefore, from the last years of the XX century, a progressive interest and improvement in technical possibilities started to focus on targeting therapies using monoclonal antibodies (e.g. against cellular pathways, growth-factors, immune check-point molecules) and/or organic/inorganic nano-constructs (e.g. ferritin-based, iron-based nanoparticles) studied for directly affect the cancer cells or other microenvironment components (immune cells), focusing the intervention to the pathological component and selectively foster the anticancer response. In this presentation, we review our experience of tumor targeting by nanoparticles (organic and inorganic) in experimental models and the possibilities to enhance the tumor immunogenicity to increase the anti-cancer immune response.

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Cytoskeletal crosslinkers as a target for inhibiting hepatocellular carcinoma growth and metastasis

Martin Gregor

Institute of Molecular Genetics, Prague, Czech Republic

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver. HCC is a heterogeneous tumor with high metastatic potential and complex pathophysiology. There is increasing evidence that tissue mechanics plays a critical role in tumor onset and progression. Here we hypothesize that plectin, a major cytoskeletal crosslinker protein, plays a crucial role in mechanical homeostasis and mechanosensitive oncogenic signaling that drives hepatocarcinogenesis. Our expression analyses revealed elevated plectin levels in liver tumors, which correlated with poor prognosis for HCC patients. Using autochthonous and orthotopic mouse models we demonstrated that genetic and pharmacological inactivation of plectin potently suppressed the initiation and growth of HCC. Moreover, plectin targeting potently inhibited the invasion potential of human HCC cells and reduced their metastatic outgrowth in the lung. Proteomic and phosphoproteomic profiling linked plectin-dependent disruption of cytoskeletal networks to attenuation of oncogenic FAK, MAPK/Erk, and PI3K/AKT signatures. Importantly, by combining cell line-based and murine HCC models, we show that plectin inhibitor plecstatin-1 (PST) is well-tolerated and capable of overcoming therapy resistance in HCC. In conclusion, our study demonstrates that plectin-controlled cytoarchitecture is a key determinant of HCC development and suggests that pharmacologically induced disruption of mechanical homeostasis may represent a new mode for HCC targeting.

Modulation of Lck in immune and tumors cells and spheroids. The importance of specificity

Juan De Sanctis, Jenny Valentina Garmendia, Viktor Valentini, Hana Duchová, Marián Hajdúch

Institute of Molecular and Translational Medicine. Palacky Universitye, Olomouc, Czech Republic

Lymphocyte cell-specific protein-tyrosine kinase (Lck) is a key enzyme in T cell function, and its activity has been related to cytotoxic efficiency. On the other hand, Lck expression in tumor cells has been linked to hematologic tumors; however, Lck can be overexpressed in solid tumors, mammary cells, colon carcinoma, and other tumor types. Tyrosine kinase inhibitors have been used to inhibit tumor proliferation and sensitize certain tumors to other compounds such as cisplatin. However, inhibition of Lck may hamper cell cytotoxic activity. The aim of the study is to define inductors of Lck activity on immune cells, its effect on the cytotoxic efficiency of CD8 and NK cells, and the possible effect on tumor cells growing in normal conditions and spheroids. Some Lck activators can enhance cytotoxic activity at a short period but induce cell exhaustion after 24 hr. The cytotoxicity efficiency is comparable in adherent conditions or spheroids. Inhibition with dasatinib, imatinib, or specific Lck inhibitor blocks cytotoxic response. On the other hand, in tumor cells, there is no effect on the activators, but the inhibitors significantly increased cell death in standard culture conditions, but not in spheroids at the documented IC50. There is a difference in Lck activity between lymphocyte physiologic function and tumor proliferation and adherence which may account for the low effect of tyrosine kinase inhibitors in solid tumors.

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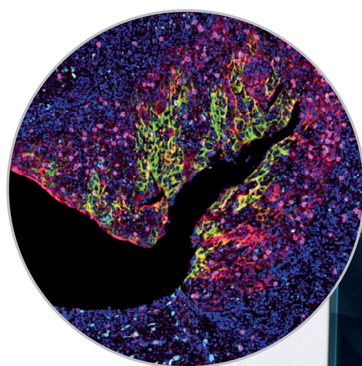

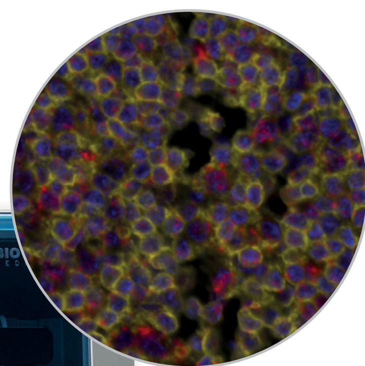

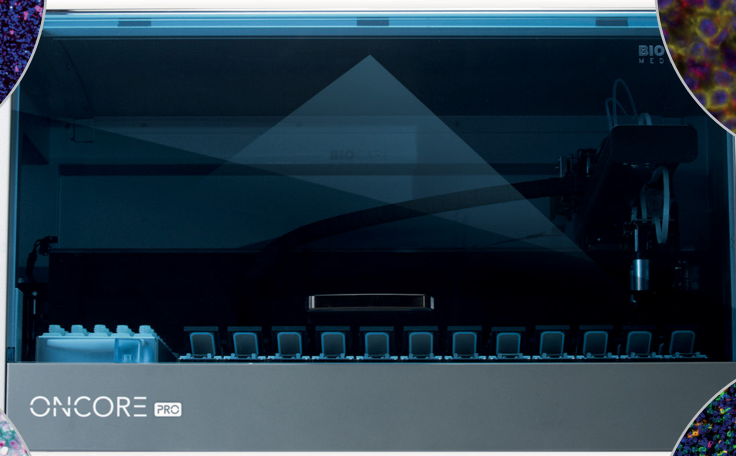

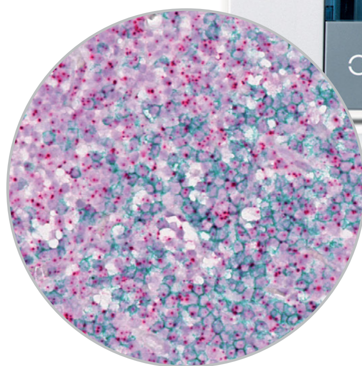

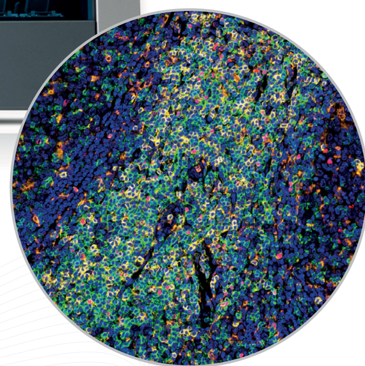

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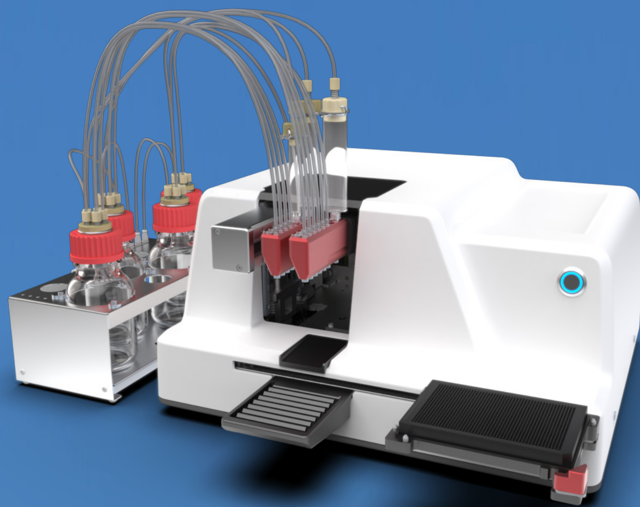

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Certified international CB-conformity	✗	✓
Latest gen. electronics at the core	✗	✓
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Angled dispensing head	✓	✓
Mixed (straight/angled) dispensing head	✗	✓
Stirring dispensing head	✓	✓
Stirring control	manual	integrated
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Wide range of viscosities	✓	✓
All SBS well plate format	✓	✓
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Improved chemical resistance case and waste tray	✗	✓
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Built-in air supply	✗	✓
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On-the-fly dispensing	✗	✓
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Valve detection	✗	✓
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Absolut timer	✗	✓
Improved sensory *	✗	✓
Bus compatibility for improved modularity *	✗	✓
Dispensing head ID detection	✗	✓
Back flush	✗	✓

* upgradeable after product launch

The effects of Andrographis paniculate extract induced lung cancer cells apoptosis and autophagy through the activation of ROS formation

Benjaporn Buranrat[1], *Supavadee Boontha2*

[1]*Mahasarakham University, Muang, Thailand.*
[2]*University of Phayao, Muang, Thailand*

Background: Andrographis paniculata is a local medicinal plant that is widely cultivated in Thailand and it has reported with several pharmacological activities, including cancer treatment.

Aim: The present study aimed to determine the underlying mechanism of A. paniculata effect on the proliferation, migration, apoptosis, and autophagy of lung cancer A549 cells.

Materials and Methods: The cancer cells viability was assessed by Sulforhodamine B and colony formation. Cell migration was observed via wound healing and Matrigel migration assay. Apoptosis, autophagy, ROS production and mitochondrial function were analyzed by flow cytometric assay.

Results: The results indicated that A. paniculata extract induced lung cancer cell death and inhibited colony formation in a dose dependent manner, with IC50 values of 46.39±7.89 µg/mL at 24 h, 3.66±0.45 µg/mL at 48 h, and 2.12±0.25 µg/mL at 72 h of SRB method. Furthermore, A. paniculata extract suppressed A549 cells migration with IC50 values of 58.08±14.06 µg/mL for Wound healing assay and 41.42±2.96 µg/mL for Matrigel migration assay. Next, A. paniculata extract decreased the cancer cells numbers and changed the morphology by dose-dependent manner. Interestingly, these extracts caused induction of lung cancer cells apoptosis and autophagy with high dose of the extract (100 µg/mL). The mechanism of cell death was determined to potentially be associated with increased ROS generation and decreased the function of mitochondria, and further leading to the induction of cell death, apoptosis, and autophagy.

Conclusion:

A. paniculata extract may therefore be beneficial for developing a novel chemotherapeutic method for lung cancer.

Therapeutic Efficacy of the Combinatorial Regimen of Temozolomide and Bacoside A on U87MG Glioblastoma cell lines

Mohammed Unais

Bharathiar University, Coimbatore, India

Glioblastoma multiforme (GBM) is the most common primary brain tumour in adults accounting for 45.2% of malignant primary brain and CNS tumours. The conventual treatment modality for GBM includes surgery followed by radiation and chemotherapy. The effect of currently used chemotherapeutic drug Temozolomide is not satisfactory because of tumour resurrection, toxicity to non-cancerous cells and chemoresistance all together compromising patient survival. Furthermore, glioblastoma multiforme (GBM) pathogenesis involves numerous genetic alterations, including mutations in genes associated with various cell signalling pathways. These genetic changes can lead to dysregulation of critical pathways such as the PI3K/AKT/mTOR pathway, the RAS/RAF/MEK/ERK pathway, and the p53 tumor suppressor pathway, among others. Understanding these alterations is crucial for developing targeted therapies and improving treatment outcomes for GBM. Numerous studies have documented various herbal products as novel anticancer drugs for efficient cancer treatment. Currently used chemotherapeutic agents are systemic and cause harmful side effects. Compared to highly toxic and expensive chemotherapeutic agents, herbal products have enabled the development of safer, nontoxic, and affordable treatment for cancer. In this context, we chose to study the efficacy of the combinatorial regimen of Bacoside A and Temozolomide for treating glioblastoma. Bacoside A is a triterpenoid saponin isolated from Bacopa monnieri. It is a mixture of chemical compounds, known as bacosides. It has shown a promising effect in anti-cancer treatments in different types of cancers. In this study, we evaluated the chemotherapeutic efficacy of a combinatorial regimen of Bacoside A and Temozolomide on the U87MG glioblastoma cell line. Various assays were employed to determine cytotoxicity, including the LDH assay, NO assay, and ROS estimation assay. Additionally, the apoptotic potential and autophagy were assessed by analysing the expression profiles of key genes involved in these processes. The results demonstrated that the combination of Bacoside A and Temozolomide significantly enhanced chemotherapeutic efficacy compared to individual treatments.

b-AP15 bound CDK4/6 and decreased colon cancer cells proliferation

Thidarath Rattanaburee[1], *Thanet Sophonnithiprasert*[1], *Napat Prompat*[2], *Soottawat Benjakul*[3], *Jirakrit Saetang*[3]

[1]*Biochemistry Unit, Department of Medical Sciences, Faculty of Science, Rangsit University, Pathum Thani, Thailand.*
[2]*Faculty of Medical Technology, Medical of Technology Service Center, Prince of Songkla University, Songkhla, Thailand.*
[3]*International Center of Excellence in Seafood Science and Innovation, Faculty of Agro-Industry, Prince of Songkla University, Songkhla, Thailand*

Colon cancer is one of the most common cancers worldwide. CDK4/6 inhibitors are efficient in decreasing colon cancer cell proliferating by evaluating cell cycle arrests. The study was performed by screening CDK4/6 inhibitors

from a library of one thousand commercial compounds using molecular docking. The anti-colon cancer activity was evaluated by MTT and colony formation assays. b-AP15 (NSC687852) showed binding energy at -10.75 kcal/mol compared to all tested compounds. Flavopiridol (a CDK4/6 inhibitor) represented the binding energy at -9.45 kcal/mol on the CDK4/6 protein. b-AP15 interacted with Asp163 (polar interaction site) and Tyr24 (hydrophobic interaction site), which are crucial for ATP binding. In addition, b-AP15 bound amino acid residue including Lys147, Ala23, His143, Val142, Arg144, Leu166 and Glu61. Flavopiridol interfered Val27, Ala41, Leu152, Ile19 (hydrophobic interactions site), His100, Val101 (hinge interactions site) and Asp163 (polar interactions site). b-AP15 and flavopiridol exhibited IC50 values of 1.77±0.19 µM and 1.62±0.28 µM on SW620 cells, respectively. b-AP15 inhibited colony formation on SW620 cells compared with non-treated cells. These results suggest that b-AP15 has potential as an anticancer agent for colon cancer.

Antiviral Activity of Selected Lamiaceae Essential Oils and Their Monoterpenes Against SARS-Cov-2

Ermin Schadich[1], Sanja Ćavar Zeljković[2,3], Marián Hajdúch[1,3], Petr Džubák[1,3], Petr Tarkowski[3,2]

[1]Institute of Molecular and Translational Medicine, Palacky University, Olomouc, Czech Republic.
[2]Crop Research Institute (CRI), Palacky University, Olomouc, Olomouc, Czech Republic.
[3]Czech Advanced Technology and Research Institute, Palacky University, Olomouc, Czech Republic

Plant essential oils are known to have the different pharmacological properties including anti-inflammatory and antiviral activity. The antiviral activity of plant essential oils is attributable to their monoterpene and sesquiterpene content and composition. Our study focused on the *in vitro* antiviral property of selected essential oils of Lamiaceae plant species and their monoterpenes against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Nineteen essential oils were extracted from dried material using hydrodistillation procedure and of, and their monoterpene profiles were determined using GC/MS analyses. Both essential oils and their monoterpene components were tested for cytotoxicity to Vero76 cells, and their antiviral activity against SARS-CoV-2 was tested in infected Vero 76 cells. The essential oils of four Mentha species, i.e., M. aquatica L. cv. Veronica, M. pulegium L., M. microphylla K.Koch, and M. x villosa Huds., but also Micromeria thymifolia (Scop.) Fritsch and Ziziphora clinopodioides Lam., and five different monoterpenes, i.e., carvacrol, carvone, 1,8-cineol, menthofuran, and pulegone, inhibited the SARS-CoV-2 replication in the infected cells. However, the differences were also in antiviral activity of essential oils and monoterpenes. The IC50 concentrations of carvone and carvacrol were 80.23 ± 6.07 µM and 86.55 ± 12.73 µM, respectively, and the other monoterpenes were less active (IC50 > 100.00 µM). Structure-activity relations analyses of related monoterpenes showed that the presence of keto and hydroxyl groups is indispensable for activity of carvone and carvacrol, respectively. Furthermore, the carvone-rich essential oil of M. x villosa had the greatest activity among all active essential oils (IC50 =127.00 ± 4.63 ppm) while the other active oils exhibited mild (140 ppm < IC50 < 200 ppm) to weak antiviral activity (IC50 > 200 ppm). Essential oils and their monoterpenes had limited or no cytotoxicity against Vero 76 cells. The results of hierarchical cluster analysis revealed that the differences in the antiviral activity of essential oils were directly associated to the antiviral efficacies of their distinct single monoterpenes. The findings from this study showed the novel antiviral property of plant essential oils and monoterpenes that might be used in the development of different measures against SARS-CoV-2. This study was published in Ćavar Zeljković S, Schadich E, Džubák P, Hajdúch M, Tarkowski P. Antiviral Activity of Selected Lamiaceae Essential Oils and Their Monoterpenes

Analysis of gliomas using portable Raman spectroscopy

Václav Ranc[1], Ondrej Pavlacka[2], Ondrej Kalita[3], Maroslav Vaverka[3]

[1]Institute of molecular and translational medicine, Palacký University in Olomouc, Olomouc, Czech Republic.
[2]Department of mathematical analysis, Faculty of Science, Palacký University in Olomouc, Olomouc, Czech Republic.
[3]Department of neurosurgery, Faculty hospital in Olomouc, Olomouc, Czech Republic

Gliomas stand still as one of the prevalent malignant tumors that are related to the human brain. Diagnosis and follow-up classification of gliomas rely considerably on a performed histopathologic examination, which provides limited information on follow-up therapy or patient prognosis. Surgical extrication is currently a preferred route to treat gliomas. Neurosurgeons, thus, must compete with a considerable challenge to correctly identify the existing margins of the glioma tissue. The residues of the primary tumor or eventually formed small satellites being left aside are one of the main reasons leading to a recurrence of the disease.

Using Raman spectroscopy, we developed a new systematic approach for spectrally characterizing the glioma and its surrounding tissue in real time during brain surgery. An optical-fiber-equipped instrument allows one to probe the targeted tissue spectrally before its extraction. It

correctly discriminates between gliomas and surrounding healthy tissue, allowing surgeons to perform a more precise surgery. The spectral analysis of Raman data consists of removing the spectral background, data smoothing, and a machine-learning-based discrimination model based on the decision tree learning approach. The developed approach was first tested on more than 100 Raman spectra obtained from 30 patients and showed statistically relevant results.

Advancing Transcriptome Analysis in Oncological Research: Comparative Evaluation of Total RNA-Seq Library Preparation Kits for Comprehensive Transcriptomic Profiling

Tereza Tesařová[1,2], Karolína Šeborová[1,2], Pavel Souček[1,2], Radka Václavíková[1,2]

[1]National Institute of Public Health in Prague, Prague, Czech Republic.
[2]Laboratory of Pharmacogenomics, Biomedical Center, Faculty of Medicine in Pilsen, Charles University, Pilsen, Czech Republic

Total RNA sequencing (total RNA-Seq) offers several advantages over mRNA sequencing (mRNA-Seq) by providing a comprehensive view of the transcriptome. While mRNA-Seq focuses only on the polyadenylated transcripts, total RNA-Seq includes long non-coding RNAs, small RNAs, and other RNA species, allowing for a more complete and detailed understanding of gene expression, regulatory mechanisms, and transcriptomic complexity. This broader scope is particularly valuable in studying oncological malignancies, where non-coding RNAs play critical roles.

In our study, we aimed to extend the scope of transcriptome analysis to encompass the entire transcriptome, moving beyond just the coding regions. We evaluated three total RNA-Seq library preparation kits with ribodepletion in solid tumor samples (n=3): Lexogen's Total RNA-Seq Library Prep Kit with RiboCop rRNA Depletion Kit V1.2, Kapa Biosystems's KAPA RNA HyperPrep Kit with RiboErase (HMR), and New England BioLabs's NEBNext Ultra II RNA Library Prep Kit. The quality of the prepared libraries was assessed and compared using the High Sensitivity DNA kit on the Bioanalyzer 2100.

Based on these assessments, we focused on the Lexogen and Kapa Biosystem kits and proceeded to sequence test samples on Illumina's NextSeq 2000, aiming for 16 million reads per sample. The ultimate selection of the most suitable kit was based on the quality control analysis of the raw sequenced data using the FastQC package and alignment quality by RSeQC package. This comprehensive approach aims to provide a more complete understanding of the transcriptomic landscape.

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HPV-related oropharyngeal cancer: Circulating and salivary DNA-based biomarkers for early diagnosis and recurrence monitoring

Ondrej Bouska[1], Vladimira Koudelakova[1,2], Zuzana Horakova[3], Marian Hajduch[1,2]

[1]Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University Olomouc, Olomouc, Czech Republic.
[2]Laboratory of Experimental Medicine, University Hospital Olomouc, Olomouc, Czech Republic.
[3]Department of Otorhinolaryngology and Head and Neck Surgery, University Hospital Olomouc, Olomouc, Czech Republic

Background: The incidence of oropharyngeal squamous cell carcinoma (OPSCC) has significantly increased over the past decades, even as the overall incidence of head and neck cancers has decreased. In the Czech Republic, the number of OPSCC cases diagnosed annually has more than tripled over the last three decades. In 2021 alone, nearly 800 new cases of OPSCC were diagnosed, surpassing the number of cervical cancer cases. Etiologically, OPSCC can be divided into two distinct categories: HPV-related OPSCC and non-HPV OPSCC. The proportion of these categories has also shifted significantly, with HPV-related OPSCCs now constituting the majority of newly diagnosed cases. Approximately 25% of OPSCC patients experience a recurrence within five years. Liquid biopsies, such as plasma or salivary samples, are being evaluated for their potential in cancer diagnosis, treatment monitoring and recurrence detection through DNA-based biomarkers.

Methods: In this study, both newly diagnosed OPSCC patients and those in remission were enrolled. HPV tumour status was assessed using HPV DNA detection in fresh or FFPE tissue samples and p16 immunohistochemistry. Only double positive (HPV-pos./p16-pos.) were evaluated as HPV-related OPC. Pre- and post-treatment HPV testing was conducted using gargle lavage (GL), oropharyngeal swabs (OPS), and plasma samples, followed by regular sampling according to standard follow-up protocols.

Results: A total of 137 OPSCC patients have been enrolled in the study. Among the 100 evaluated patients, 83% (83/100) were diagnosed with HPV-related OPSCC (37 cases have not yet been evaluated), with the HPV16 genotype being the most prevalent, detected in 97.6% of these cases. Non-HPV OPSCC patients were more frequently diagnosed with late-stage carcinomas compared to HPV-related OPC patients. Pre-treatment analysis for oral HPV DNA in gargle lavage (GL), oropharyngeal swabs (OPS), and circulating tumour HPV DNA (ctHPV DNA) in plasma showed sensitivities of 91.7%, 96%, and 95.8%, respectively, in newly diagnosed OPSCC cases. CtHPV

DNA was detected in 75% (3/4) of recurrent HPV-related OPSCC cases, with two of these also testing positive for oral HPV DNA.

Conclusion: This study aims to validate the collection of liquid biopsies and DNA-based biomarkers detection for early diagnosis and recurrence monitoring in OPSCC patients. Preliminary results of biomarkers analysis showed promising results for early detection of even early-staged OPSCC and its applicability in clinical practice. This study is ongoing, including new patient enrollment.

Characterization of growth and spontaneous regression of cutaneous melanoma

Salim Abuhajiar[1,2,3], Vratislav Horák[1], Marek Šinkora[4], Helena Kupcová Skalníková[1,3]

[1]Laboratory of Applied Proteome Analyses and Research Centre Pigmod, Institute of Animal Physiology and Genetics of the Czech Academy of Sciences, Liběchov, Czech Republic.
[2]Department of Cell Biology, Faculty of Science, Charles University, Prague, Czech Republic.
[3]Institute of Biochemistry and Experimental Oncology, First Faculty of Medicine, Charles University, Prague, Czech Republic.
[4]Laboratory of Gnotobiology, Institute of Microbiology, Czech Academy of Sciences, Novy Hradek, Czech Republic

Cutaneous melanoma is one of the deadliest and most aggressive cancers of the skin. It arises from malignant transformation of melanocytes (melanin-producing cells). The immune system plays a significant role in controlling melanoma progression. About 30% of human melanomas develop partial spontaneous regression, i.e. partial tumor disappearance without any medical intervention, and is accompanied by tumor-infiltrating immune cells, tumor destruction and replacement of tumor tissue by fibrosis or fibrous tissue. However, the immune cell infiltrates in regression are only partially characterized in human melanomas due to the malignant potential of the disease and the need of immediate therapy.

Melanoma-bearing Liběchov minipig (MeLiM) is a large animal model of hereditary melanoma suitable to study spontaneous regression and progression of melanoma. Spontaneous regression in MeLiM occurs during the first year of life. We hypothesize that specific subpopulations of tumor-infiltrating immune cells, such as T lymphocytes (CD4+ and CD8+), B lymphocytes, macrophages, granulocytes (mainly neutrophils) are infiltrating the tumors undergoing regression. We aimed to map the presence of such cells and their localization in tumors in early and advanced stages of regression. The infiltrating cells are studied by immunohistochemistry. Preliminary results from immunohistochemistry show a trend with increasing and/or decreasing patterns between different tumor-infiltrating immune cells in MeLiM piglets at different time points; 8, 12, 16 weeks of age.

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Reactivation of the embryonic genes Trop2 and Sca1 correlates with the cellular zonation of the intestinal tumors

Jakub Onhajzer, Lucie Janeckova, Monika Stastna, Dusan Hrckulak, Vladimir Korinek

Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic

Colorectal cancer (CRC) is one of the leading causes of cancer deaths worldwide. Recent studies have shown that some tumor cells re-express oncofetal genes that are normally active during embryonic development but are repressed in the healthy adult intestines. These include trophoblastic cell surface antigen 2 (Trop2) and stem cell antigen-1 (Sca1), which often re-emerge during epithelial regeneration after damage by radiation, chemical inflammation or infection. These genes may influence the development of intestinal tumors.

In our study, early activation of Sca1 and Trop2 was observed in intestinal hyperplasia and tumors due to inactivation of the tumor suppressor gene adenomatous polyposis coli (Apc). We found heterogeneous re-expression of these oncofetal markers in early-stage hyperplasia, that continued in advanced adenomas. Techniques such as flow cytometry and high-resolution 3D microscopy confirmed significant tumor cell heterogeneity, resulting in tumor zonation characterized by stem cell and fetal gene expression patterns.

In addition, RNA sequencing and organoid assays revealed distinct molecular pathways, biological processes and cellular plasticity in Sca1+ and Trop2+ tumor cells, regardless of whether they were single or double positive. Interestingly, these embryonic markers were not expressed after tissue regeneration following the loss of T cell factor 4 (Tcf4), a crucial nuclear effector of Wnt signaling in the intestine. This suggests that the reactivation of embryonic markers is associated with specific tissue regeneration processes.

Currently, some oncofetal antigens are therapeutic targets in cancer treatment. Therefore, understanding the molecular mechanisms of their re-expression is crucial for the effective selection and application of targeted therapies.

Investigating the impact of nuclear PIP2 manipulation on transcriptional regulation in cancer cells

Rabia Gonul, Martin Sztacho

Laboratory of Cancer Cell Architecture, Institute of Biochemistry and Experimental Oncology, First Faculty of Medicine, Charles University, 128 00, Prague, Czech Republic

PTEN and PIK3KA pathways are frequently mutated in variety of cancer types. These pathways control Phosphatidylinositol 4,5-bisphosphate (PIP2) metabolism, making these pathways important targets for cancer research and potential therapies. The role of PIP2 in the inner leaflet of the plasma membrane is well documented. However, its function in nuclear environment, where it is involved in the regulation of gene expression, remains poorly understood. Recent indications suggest that nuclear PIP2 plays a crucial role in modulating the phase separation of interacting proteins, thereby influencing condensates formation. The formation of RNA Polymerase 2 (Pol2) initiation condensates has been shown to be a key step in transcriptional regulation.

This project aims to investigate the relationship between increased PIP2 levels in cancer cells and changes in the nuclear localization and condensation capacities of Pol2 and transcriptional regulators. In order to study this phenomenon, we will develop a system to specifically manipulate nuclear PIP2 levels in cancer cells and thus study this relationship in more detail.

In this project we will combine molecular biology, biochemistry, proteomics, and cell biology techniques. A GFP-labeled PIP2-binding peptide, modified for nuclear targeting in a controlled inducible system, will be used to attenuate PIP2 levels in cancer cell lines. The efficacy of the system will be evaluated by assays measuring cell transformation, proliferation and invasiveness. Confocal microscopy will be used monitor the expression and localization of the peptide and its impact on nuclear PIP2 levels.

We hypothesize that manipulation of PIP2 levels will reveal a casual relationship between nuclear PIP2 levels and changes in the localization, condensation and thus efficacy of Pol2 transcription. Reducing nuclear PIP2 is expected to disrupt the phase separation of Pol2 machinery proteins, leading to a transcriptional dysregulation and providing insights into how PTEN and PIK3KA mutations contribute to the cancer cell development and progression.

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Investigation of changes in protein phase separation induced by metabolic adaptation to hypoxia in glioblastoma cells

Elena Herencia, Martin Sztacho

Laboratory of Cancer Cell Architecture, Institute of Biochemistry and Experimental Oncology, First Faculty of Medicine. Charles University, 128 00, Prague, Czech Republic

Glioblastoma (GBM) is the most common primary malignant brain tumor. The rapid growth of this tumor leads to the development of hypoxic conditions. One of the measures these cells take to adapt to these low oxygen conditions is the concentration of glycolytic enzymes in molecular condensates called G-bodies. The mechanism of their formation is poorly understood. It is known that hypoxic stress forces some of these enzymes to re-localize into biomolecular condensates by liquid-liquid phase separation (LLPS). A good example is phosphofructokinase and aldolase, both of which interact to recruit to condensates. Thus, we hypothesize that these domains are formed by phase separation process is induced by hypoxia which helps these cells to cope with this stress condition increasing the glycolytic rate.

Biomolecular condensates are membrane-less compartments formed by LLPS, more specifically by multivalent interactions between protein-protein or protein-RNA. It is usually a reversible process that depends on environmental conditions and molecular concentration. A key factor in this process is that proteins contain intrinsically disordered regions (IDR). IDRs lack a defined three-dimensional structure, allowing proteins to participate in a weak multivalent interaction that facilitate phase separation. Membrane-less compartments are used by cells to increase the molecular concentration and consequently the rate of biochemical reactions.

In this project we will use a glioblastoma cell line under hypoxia to generate molecular condensates. Further, we will precipitate, identify and analyze the hypoxia induced condensate-participating proteins by combination of mass spectrometry, bioinformatics and advanced microscopy.

Understanding the molecular mechanisms of glycolytic enzyme condensation will help to understand the principles driving cancerogenesis.

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High-Contrast PET Imaging of the „Cancer Integrin“ αvβ6 with Ga-68-D0103

Kateřina Dvořáková Bendová[1], Barbora Neužilová[1], Zbyněk Nový[1], Kristýna Krasulová[1], Falco Reissig[2], Jakub Šimeček[2], Marián Hajdúch[1,3], Johannes Notni[2], Miloš Petřík[1,3]

[1]Palacky University Olomouc, Faculty of Medicine and Dentistry, Institute of Molecular and Translational Medicine and Czech Advanced Technology and Research Institute, Olomouc, Czech Republic.
[2]TRIMT GmbH, Radeberg, Germany. 3University Hospital Olomouc, Olomouc, Czech Republic

Purpose

αvβ6-Integrin is frequently overexpressed by carcinoma cells and is upregulated in various malignant cancers, for example, in pancreatic ductal adenocarcinoma (PDAC), head-and-neck squamous cell carcinoma (HNSCC), ovarian and cervical cancer, triple-negative breast carcinoma (TNBC), and non-small cell lung cancer (NSCLC) and its brain metastases. Recently, αvβ6-integrin PET imaging with Ga-68-Trivehexin [1] has demonstrated the potential of this „cancer integrin“ as a theranostic target, e.g., for PDAC, HNSCC, NSCLC, TNBC, or parathyroid adenoma (PTA). Since the sensitivity of clinical PET imaging benefits from low nonspecific uptake and low background, we investigated Ga-68-D0103 as a novel αvβ6-integrin PET agent with accelerated biokinetics.

Methods

Ga-68 labeling of D0103 was done manually in a kit-like fashion. Preclinical characterization of Ga-68-D0103 was performed in SCID mice bearing subcutaneous xenografts of lung adenocarcinoma - H2009 (αvβ6-positive) or breast adenocarcinoma - MDA-MB-231 (αvβ6-negative), by ex-vivo biodistribution and PET imaging at different timepoints p.i.

Results

Ga-68-D0103 showed a fast blood clearance and a low uptake and retention in αvβ6-negative organs and tissues in preclinical experiments. Ex-vivo biodistribution of Ga-68-D0103 in H2009 xenografted mice after 30, 90, and 180 min showed tumor-to-blood ratios of 6.8, 37, and 124, respectively; tumor-to-muscle ratios of 12, 14, and 36, respectively; tumor-to-liver ratios of 10, 17, and 14, respectively; and tumor-to-pancreas ratios of 20, 47, and 56, respectively. Co-administration of gelofusine (succinylated gelatin, a plasma expander) reduced the kidney uptake by 89% (from 178 %ID/g to 19.1 %ID/g, 90 min p.i.). µPET imaging in H2009 xenografted mice confirmed a high tumor uptake and low background already 30 min p.i.. Blockade biodistribution and µPET in αvβ6-negative MDA-MB-231 mice demonstrated target specificity.

Conclusions

Ga-68-D0103 µPET images showed an excellent tumor-to-background ratio already at early time points after administration in αvβ6-positive H2009 mice. Thus, it is a promising agent for rapid and sensitive mapping of αvβ6-integrin expression by PET, particularly in tissues and lesions with comparably low αvβ6-integrin density, with high potential for clinical translation.

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Deciphering the role of desmoglein 2 in maintaining the epithelial phenotype of breast cancer cells

Ondřej Vacek[1,2,3], Lucia Knapeková[1,3], Jana Ondřejová[1,3], Pavla Jirgalová[1,3], Radek Fedr[1], Petr Beneš[2,3], Karel Souček[1,2,3]

[1]Department of Cytokinetics, Institute of Biophysics of the Czech Academy of Sciences, Brno, Czech Republic.
[2]International Clinical Research Center, St. Anne‘s University HospitaL, Brno, Czech Republic.
[3]Department of Experimental Biology, Faculty of Science, Masaryk University, Brno, Czech Republic

Desmoglein 2 (DSG2) is a surface protein with a physiological role in desmosomal cell-cell adhesion and maintaining epithelial tissue integrity. In cancer, the role of DSG2 is controversial, as it has been reported to have both pro-tumorigenic and tumor-suppressive effects. Since the connection of DSG2 with the metastatic cascade in cancer is understudied, we focused on its association with epithelial-mesenchymal transition (EMT) traits in breast cancer cells.

To assess the effect of DSG2 loss *in vitro*, we established a T-47D DSG2 CRISPR KO model and successfully confirmed DSG2 deletion. We examined the ability of DSG2 KO cells to adhere to various extracellular matrix (ECM) proteins and observed an overall decrease in cell adhesion to most of the individual ECM components. Measurement of the in-house developed and validated EMT surface panel revealed the upregulation of multiple mesenchymal markers in DSG2 KO cells and the downregulation of epithelial markers.

Our study demonstrates that in the T-47D breast cancer cell model, the loss of DSG2 leads to a decreased ability to adhere to various ECM components. These deregulations are also reflected in the overall mesenchymal-like cell surface

fingerprint of DSG2 KO cells. We conclude that DSG2 in the tested model associates with EMT and thus might have a role in the repression of the metastatic process.

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Interplay between miRNA and adaptive immune cells in tumor microenvironment and their prognostic value in non-viral hepatocellular carcinoma

Filip Ambrozkiewicz[1], Marie Rajtmajerová[1], Venkata Ramana Mallela[1], Esraa Ali[1], Andriy Trailin[1], Lenka Červenková[1], Richard Pálek[2,1], Ondřej Daum[1], Václav Liška[2,1], Kari Hemminki[1]

[1]Charles University, Faculty of Medicine in Pilsen, Plzen, Czech Republic.
[2]University Hospital in Pilsen, Plzen, Czech Republic

Background and Aims: Hepatocellular carcinoma (HCC) is the third deadliest cancer worldwide. Its high mortality is primarily attributed to late-stage diagnosis. The dysregulation of miRNA and their interaction with tumor environment is poorly understood. As a novel approach, we wanted to assess interplay between miRNA expression and abundance of adaptive immune cells and their combined impact on patient outcome.

Method: Tissue samples were obtained from 45 non-viral HCC patients who had undergone resection and did not received neo-adjuvant treatment prior to operation. Total RNA from paired tumour and non-tumour adjacent tissue was extracted. miRNA profiling was performed using Agilent microarrays. Differential expression analysis was performed in GeneSpring. After immunohistochemical staining, cell densities of CD3+ T cells, CD8+ T cells and CD20+ B cells were assessed in two regions of interest (tumor center (TC), invasive margin (IM). These variables were evaluated as predictors for time to recurrence (TTR), disease free survival (DFS) and overall survival (OS) with Cox regression and Kaplan-Meier method.

Results: We identified 23 differentially expressed miRNAs ($p \leq 0.05$, fold change ≥ 2). High expression of miRNA-1972 in tumor was associated with longer TTR (HR: 0.35, p=0.039) and DFS (HR: 0.35, p=0.012). Among the analysed immune cells high density of CD8 + T cells in TC was associated with longer TTR (HR: 0.30, p=0.01) and DFS (HR: 0.43, p=0.03). CD8+ T cells in IM presented even stronger associations with longer TTR (HR: 0.16, p=0.001) and DFS (HR: 0.17, p<0.001). High density of CD20 + B cells in TC were significantly associated with longer DFS (HR: 0.37, p=0.01). Variables significant in univariate analysis were next combined to assess their predictive values. Combination of high expression of miR-1972 and high CD8+T cell densities TC and IM were associated with longer TTR (HR: 0.14, p=0.003 and HR: 0.06, p=0.001) and DFS (HR: 0.19, p=0.003 and HR: 0.08, p=0.001). High densities of CD20+ B cells combined with high expression of miR-1972 in TC was associated with longer DFS (HR: 0.17, p=0.005).

Conclusion: Combination of miRNA-1972 expression and CD8+ T cells and CD20+ B cells refined their individual predictive values. The results show that factors both in the tumor and in its microenvironment may influence patient outcome in HCC and could be used for better therapy implementation.

Third-Generation Stony Brook Taxanes Overcome Taxane Resistance and Modulate NOTCH Signaling Pathway in Ovarian Cancer Models

Alzbeta Spalenkova[1,2], Karolina Seborova[1,2], Tereza Tesarova[1,2], Marie Ehrlichova[1,2], Kamila Koucka[1,2], Tomas Sychra[3,4], Hersh Bendale[5], Iwao Ojima[5], Pavel Soucek[1,2], Radka Vaclavikova[1,2]

[1]Toxicogenomics Unit, National Institute of Public Health, Prague, Czech Republic.
[2]Laboratory of Pharmacogenomics, Biomedical Center, Faculty of Medicine in Pilsen, Charles University, Pilsen, Czech Republic.
[3]Third Faculty of Medicine, Charles University, Prague, Czech Republic.
[4]Department of Surgery, University Hospital Kralovske Vinohrady, Prague, Czech Republic.
[5]Institute of Chemical Biology & Drug Discovery, Stony Brook University - State University of New York, Stony Brook, NY, USA

Resistance to taxane derivatives is a significant issue in the treatment of ovarian cancer. To counteract this resistance, new experimental taxanes, such as Stony Brook taxanes (SB-Ts), have been developed. In this study, we analyzed the impact of third-generation SB-Ts on cell viability, cell cycle, and migration in the SKOV-3 sensitive (SKOV-3/SEN) ovarian cancer cell line and its resistant subclone, SKOV-3/RES.

Based on our findings, both SB-T-121605 and SB-T-121606 significantly inhibited cell growth compared to paclitaxel (PTX). Regarding the cell cycle, we observed the highest proportion of cells in the G2/M phase in both SKOV-3/SEN and SKOV-3/RES cell lines when incubated with third-generation SB-Ts, in comparison to control cells and cells treated with PTX. In the cell migration analysis, we observed notable suppression of metastatic activity in both cell lines when incubated with SB-T-121605 and SB-T-121606.

To confirm these effects, we also conducted *in vivo* experiments using cell-line derived xenografts (CDX) prepared from both the sensitive and resistant SKOV-3 cell lines. For this experiment, the most potent Stony Brook taxane, SB-T-121606, was employed, which effectively suppressed tumor growth in both types of CDX.

Given that the NOTCH signaling pathway is critical in the context of cancer cell growth and resistance, we examined the expression of

selected factors of this pathway, specifically the NOTCH3 and NOTCH4 receptors. Using Western blot analysis in both cell lines and CDX models, we found that SB-T-121606 effectively suppressed the expression of the NOTCH4 protein. Additionally, in the cell lines (but not in the CDX models) it also suppressed the expression of NOTCH3.

The study has demonstrated that third-generation SB-Ts, particularly SB-T-121606, are effective in inhibiting cell growth and migration in both sensitive and resistant subclones of ovarian cancer cell lines. The suppression of the NOTCH signaling pathway, specifically the reduction in NOTCH4 and NOTCH3 protein expression, which suggests that SB-T-121606 counteracts resistance mechanisms in cancer cells, and potentially offers a more effective treatment option compared to conventional taxanes like paclitaxel. Thus, SB-T-121606 is a highly promising candidate for overcoming taxane resistance in ovarian cancer chemotherapy.

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2-Substituted triterpenoids inhibit Hedgehog signalling pathway in cancer cell lines with GLI1 overexpression

Ivo Frydrych[1], Barbora Vránová[2], Lucie Borková[1,2], Jan Pokorný[1,2], Nikola Jakubcová[2], Sandra Benická[2], Soňa Gurská[1], Jiří Řehulka[1], Barbora Lišková[1], Petr Džubák[1], Marián Hajdúch[1], Milan Urban[1,2]

[1]Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacký University, Olomouc, Czech Republic.
[2]Department of Organic Chemistry, Faculty of Science, Palacký University, Olomouc, Czech Republic

The Hedgehog (HH) signaling pathway is essential for normal embryonic development and adult tissue homeostasis. On the other hand, deregulation of the HH signaling pathway is implicated in initiation or progression of various human cancers. In this study, we evaluated the effect of small library of 2-substituted triterpenoids on proliferation and HH pathway activity in relevant cancer cell lines. We identified 2 highly active compounds inhibiting proliferation and inducing cell death of non-small cell lung cancer (NSCLC) and prostate cancer cell lines with hyper-activated HH signaling. Furthermore, they significantly reduced GLI-mediated transcription in two different cell-based GLI responsive luciferase reporters. Detailed immunoblot analysis revealed decreased GLI1 expression and expression of GLI1 target genes associated with tumor progression and proliferative potential in non-small cell lung cancer (NSCLC) and prostate cancer cell lines.

Investigation of Phospholipidation as a Novel Post-Translational Protein Modification in Cancer Cells

Soodabeh Abbasi Sani, Martin Sztacho

Laboratory of Cancer Cell Architecture, Institute of Biochemistry and Experimental Oncology, First Faculty of Medicine, Prague, Czech Republic

Recent advances in cancer biology have revealed novel post-translational modifications (PTMs) in cancer cells. Phospholipidation involves the covalent attachment of phosphoinositides such as phosphatidylinositol 4,5-bisphosphate (PIP2) and is thought to significantly affect protein function, particularly in cancer cells. While PTMs such as phosphorylation and ubiquitination are well studied, the role and mechanisms of phospholipidation in cancer remain largely unexplored.

This project aims to identify the molecular mechanism of phospholipidation in cells and its implications for cancer progression. Our hypothesis is that phospholipidation plays a critical role in modulating protein confotmation and localization, thereby profoundly affecting its function. To achieve our goals, we are using a combination of biochemical, molecular biological, and proteomic approaches.

Our study aims to elucidate the functional impact of phospholipidation on protein function and its contribution to cancer progression. Identifying the enzymes that catalyze phospholipidation will improve our understanding of how this PTM affects protein function and its role in cancer. Understanding these mechanisms will significantly advance our knowledge of cancer biology and may lead to innovative diagnostic and therapeutic strategies.

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CARMIL1 as a novel interaction partner of the PKN3 kinase

Petra Novotná, Tereza Volfová, Michal Dibus, Jan Brábek, Daniel Rösel

Department of Cell Biology, BIOCEV, Faculty of Science, Charles University, Prague, Czech Republic

Cell motility plays a crucial role in cancer progression and metastasis formation. This process is regulated by a myriad of signalling pathways and cellular processes. PKN3, an AGC-type Ser/Thr kinase, has emerged as a key regulator in the malignant progression of certain aggressive cancer types, particularly breast and prostate cancer. Notably, while PKN3 is not physiologically

expressed in most tissues, its expression has been detected in endothelial cells, osteoclasts, and trophoblast cells, where it exerts a positive effect on cell motility and functionality, likely through the regulation of actin cytoskeleton dynamics. Despite the well-documented influence of PKN3 on cancer progression, the underlying mechanism remains elusive. In our research, we identified CARMIL1 (CP, Arp2/3, myosin-I linker 1) as a potential substrate of PKN3. CARMIL1 is known to regulate actin cytoskeleton dynamics and cell motility by interacting with actin capping protein, thereby diminishing its inhibitory effect on actin polymerization. Importantly, our recent findings confirmed a direct interaction between PKN3 and CARMIL1. We propose that CARMIL1 may serve as an unknown mediator of PKN3's effects on actin cytoskeletal rearrangements, thereby regulating cell motility. Further elucidation of this molecular interaction may provide new insight into the underlying mechanism of cancer progression and unveil potential therapeutic targets for inhibiting metastasis.

Synergistic role of mechanical tension and tyrosine phosphorylation in regulation of p130Cas substrate domain

Lenka Koudelková[1], Veronika Kolomazníková[1], Marie Hejnarová[1], Jana Šmigová1, Radoslav Janoštiak[2], Ondřej Tolde[1], Markéta Pelantová[1], Michal Dibus[1], Jan Brábek[1], Daniel Rösel[1]

[1]Department of Cell Biology, BIOCEV, Faculty of Science, Charles University, Prague, Czech Republic.
[2]BIOCEV, First Faculty of Medicine, Charles University, Vestec, Czech Republic

The p130Cas protein is an important adaptor, mechanosensor, and mechanotransducer of cell adhesive structures such as focal adhesions and podosomes. Despite lacking its own enzymatic activity, p130Cas forms a key node for multiple signalling pathways and thus exerts a profound effect on overall cellular behaviour. Besides proliferation and differentiation, p130Cas also affects signalling, orchestrating cytoskeleton reorganization, and consequently, cell motility. Its intrinsically disordered substrate domain, housing 15 cryptic YxxP motifs recognized by Src family kinases, is essential for its mechanosensitive properties. Upon application of mechanical tension, tyrosine residues become exposed, facilitating their phosphorylation and subsequent binding of proteins containing SH2 domains. However, the precise mechanism underlying mechanically driven exposure of tyrosine residues remains not fully understood.

In our research, we aim to determine how the spatial arrangement of the substrate domain responds to mechanical stimuli, modulates the accessibility of YxxP motifs, and influences cancer cell invasiveness. Our results with a truncated mutant of p130Cas, wherein YxxP motifs remain accessible even in the absence of mechanical tension, revealed elevated tyrosine phosphorylation within the substrate domain correlating with an increased binding capacity of proteins containing SH2 domains and decreased p130Cas dynamics in focal adhesions. Additionally, we observed that expression of this p130Cas variant promotes invasive cell phenotype. Findings from this project may provide insight into the regulation of the processes behind cancer cell metastasis, which could be used to improve the targeting of migrastatic drugs.

Click estradiol dimers with novel aromatic bridgingunits: synthesis and anticancer evaluation

Jiri Rehulka

Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacký University and University Hospital in Olomouc, Hněvotínská 976/3, 779 00, Olomouc, Czech Republic

Estradiol dimers (ED) are semi-synthetic compounds that have been shown to have anticancer activity by inhibiting tubulin polymerization and disrupting mitotic spindle formation. In this study, we synthesized twelve variants of ED using copper-catalyzed azide-alkyne cycloaddition (CuAAC) with structural modifications within the aromatic bridge between two estradiol moieties and evaluated the effect of the linker on their biological activity. *In vitro* testing of the dimers (ED1-12) showed significant improvement in the anticancer selectivity. We found that simple linkers with one or two substituents at aromatic central ring were more favorable than complex linkers with multiple substituents. Two of the most active compounds, ED3 and ED5, showed cytotoxic effects on cancer cell lines that were comparable with 2-methoxy estradiol (ME), a known tubulin inhibitor. We performed cell based experiments and *in vitro* assays to confirm that estradiol dimers inhibit microtubule dynamics and interfere with mitotic spindle assembly. In addition, they also exhibited anti-angiogenic properties in an endothelial cell tube-formation model. Finally, assuming the detention of ED3 and ED5 in the colchicine binding site, an in silico model depicting highly probable binding modes of both ED3 and ED5 has been proposed. Our results suggest that estradiol dimers are promising candidates for anticancer drug development.

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Molecular Regulation of Src Kinase and Its Functional Role in Invasive Cellular Structures

Lenka Koudelková[1,2], Markéta Pelantová[1], Jana Šmigová[1], Zuzana Brůhová[1], Martin Sztacho[1], Vojtěch Pavlík[1], Dalibor Pánek[3], Jakub Gemperle[1], Pavel Talacko[4], Jan Brábek[1], Daniel Rösel[1]

[1]Department of Cell Biology, BIOCEV, Faculty of Science, Charles University, Vestec, Czech Republic.
[2]Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, Prague, Czech Republic.
[3]Imaging Methods Core Facility at BIOCEV, Faculty of Science, Charles University, Vestec, Czech Republic.
[4]Proteomics Core Facility at BIOCEV, Faculty of Science, Charles University, Vestec, Czech Republic

Src kinase is essential for various signaling pathways that regulate cellular processes such as survival, motility, proliferation, mechanosignaling, and differentiation. Tight control of Src kinase is crucial, as its aberrant activation can lead to cellular transformation and cancer development.

In the first part of this project, we explored the molecular regulation of Src kinase. Activation of Src kinase is controlled by intramolecular inhibitory interactions mediated by the SH3 and SH2 domains, maintaining it in an inactive state. The transition between inactive and active conformations is largely regulated by the phosphorylation of key tyrosines 416 and 527. We identified tyrosine 90 as a new regulatory residue, whose phosphorylation reduces the binding affinity of the SH3 domain, opens the Src structure, and renders Src catalytically active. This phosphorylation allows the SH3 and SH2 domains to serve as cooperative but independent regulatory elements.

In the second part of this project, we investigate the role of Src kinase in invasive cellular structures, such as invadopodia in cancer cells and podosome belts in osteoclasts. Using a FRET-based biosensor of Src activity, we study Src activity in living cells. This sensor allows us to closely examine the activation dynamics of Src kinase in these structures and elucidate its role in cellular invasion.

Altogether, our results shed light on how Src is regulated on the molecular level and how its activity regulates different cellular structures.

CD25-biased IL-2-based Immunocytokines In Combination With Immune Checkpoint Inhibitors Completely Eradicate Established Large Tumors Through Potent Expansion of CD8⁺ T Cells

Irfan Baki Kilic[1], Petra Weberova[1], Derek VanDyke[2,3], Milada Sirova[1], Katerina Kubesova[1], Charina S. Fabilane[3,4], Vladyslav Mazhara[1], Kathy Liu[3,5], Blanka Rihova[1], Jamie B. Spangler[2,3,5,6,7,8,9,10], Marek Kovar[1]

[1]Institute of Microbiology of the Czech Academy of Sciences, Prague, Czech Republic.
[2]Department of Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, MD 21218, USA.
[3]Translational Tissue Engineering Center, Johns Hopkins University School of Medicine, Baltimore, MD, USA.
[4]Program in Molecular Biophysics, Johns Hopkins University, Baltimore, MD, USA.
[5]Department of Biomedical Engineering, Johns Hopkins University School of Medicine, Baltimore, MD, USA.
[6]Bloomberg~Kimmel Institute for Cancer Immunotherapy, Johns Hopkins University,, Baltimore, MD, USA.
[7]Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, MD, USA.
[8]Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, MD, USA.
[9]Department of Ophthalmology, Johns Hopkins University School of Medicine, Baltimore, MD, USA.
[10]Department of Molecular Microbiology & Immunology, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD, USA

Purpose: We have recently identified that complexes of IL-2 and JES6-1A12 anti-IL-2 mAb, which selectively stimulate $CD25^+$ cells including antigen-primed $CD8^+$ T cells (CD25-biased IL-2co), surprisingly possess antitumor activity, particularly when combined with immune checkpoint inhibitors (αCTLA-4 + αPD-1 mAbs; ICIs henceforth). This potent antitumor activity is present despite the substantial expansion of Treg cells. Consequently, we aimed to evaluate the antitumor activity of a set of CD25-biased IL-2-based immunocytokines (ICs) requiring different levels of CD25 expression to be utilized. These ICs were engineered by linking IL-2 to the light chain of an anti-IL-2 mAb muteins with different affinity to IL-2 using a flexible oligopeptide $(Gly_4Ser)_7$. Control IC was created using an anti-FITC mAb. This design mimics the IL-2co structure and function while avoiding issues such as excess IL-2 or antibody and potential dissociation leading to off-target effects. The goal of this research was to examine the stimulatory effects of various ICs on antigen-primed $CD8^+$ T cells and other immune subsets, as well as their antitumor efficacy when combined with ICIs.

Methods: The stimulatory effect of CD25-biased IL-2-based ICs on antigen-primed $CD8^+$ T cells was evaluated using OT-I $CD8^+$ T cell adoptive transfer experiments. Flow cytometry was employed to

assess the expansion of different immune cell populations and their phenotypes. The antitumor efficacy of IL-2-based ICs in combination with ICIs was tested in the CT26 and MC38 tumor models.

Results: The Y33 IC based on anti-IL-2 mAb mutein with lowest affinity to IL-2 from all ICs showed a notable ability to expand antigen-primed $CD8^+$ T cells, although it was less effective than the control IC. Nevertheless, Y33 IC excelled in inducing effector molecule expression in antigen-primed $CD8^+$ T cells while minimally stimulating Treg cells. The combination of Y33 IC and ICIs significantly prolonged survival and resulted in the complete eradication of tumors in the majority of CT26 or MC38 tumor-bearing mice, without any sign of toxicity (monitored by body weight, temperature, and absence of pulmonary edema). The depletion of $CD8^+$ T cells markedly decreased the antitumor effect of Y33 IC in combination with ICIs, indicating that $CD8^+$ T cells played a crucial role in the therapeutic outcome.

Conclusion

CD25-biased IL-2-based ICs show significant promise for cancer immunotherapy, particularly when used in conjunction with ICIs.

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The Role of Nuclear Phosphoinositides in Cancer: From Biomolecular Condensates to Gene Expression Control

Agnieszka Chytła, Martin Sztacho

Laboratory of Cancer Cell Architecture, Institute of Biochemistry and Experimental Oncology, First Faculty of Medicine, Charles University, Prague, Czech Republic

Understanding the mechanisms underlying the aggressive metastatic behaviour of tumour cells is a major challenge in the development of new targeted therapies. Recent studies have highlighted the role of nuclear phosphoinositides (PIPs) and their metabolism in cancer progression. PIPs are enriched in nuclear structures where they regulate transcriptional activity, modulate the activity of transcription factors and chromatin modifiers, and thus act as key regulators in signalling pathways that control gene expression. The efficacy of these processes depends on their high concentration at specific nuclear sites, driven by the formation of biomolecular condensates. These sub-compartments are formed by liquid-liquid phase separation (LLPS). Recently, PIPs have been proposed as novel regulators of LLPS, as increased nuclear levels of PIPs lead to aberrant changes in condensate phase separation, presumably affecting gene expression patterns and consequently enhancing oncogenic processes.

Using various methods, including confocal microscopy, we study the protein composition of nuclear PIP2 condensates, with a focus on transcriptional regulators such as BRD4 and MED1. In addition, we investigate differences in the spatial organization of RNA polymerase II transcription in different cancer cell types. These findings will provide valuable insights into the role of nuclear PIPs in cancer progression.

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Novel Azaspirooxindolinone-Based PROTACs for Selective BTK Degradation and Enhanced Anticancer Activity

Naveen Kumar Rampeesa[1,2], Gopal Muddasani[2], Sudhakar T[2], Sreenivasa Anugu[2], Soňa Gurská[3], Barbora Lišková[3], Juan Bautista De Sanctis[3], Petr Džubák[3], Marián Hajdúch[3], Viswanth Das[3], Rambabu Gundla[1]

[1]Department of Chemistry, School of Science, GITAM University, Hyderabad, India.
[2]Aragen Life Sciences Ltd, Medicinal Chemistry Laboratory Division, Hyderabad, India.
[3]Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacký University and University Hospital Olomouc, Olomouc, Czech Republic

The discovery of novel targets and the development of small-molecule inhibitors have emerged as promising strategies for treating various diseases. Traditional anti-cancer drugs often face limitations due to restricted selectivity and susceptibility to drug resistance, fueling interest in cancer-targeted therapies. Proteolysis targeting chimeras (PROTACs) facilitates the degradation of specific endogenous proteins via the E3 ubiquitin ligase pathway. Recent efforts have focused on developing inhibitors targeting IL-2 inducible T cell kinase (ITK) and Bruton's Tyrosine Kinase (BTK) for treating different hematological malignancies, autoimmune diseases, allergies, and neuroinflammation. In this study, we evaluated nine PROTAC derivatives of azaspirooxindolinone, conducting docking analysis and *in vitro* anti-cancer profiling. Among the tested PROTACs, three compounds (7, 14, and 25) exhibited high cytotoxicity (IC50 < 10 μM) against BTK- and ITK-positive cancer cell lines, while showing no cytotoxicity against non-cancer cell lines and normal T/B-cell lymphocytes. Notably, PROTAC 7, despite having the highest docking score of -12.1 kcal/mol, was unable

to reduce BTK or ITK protein levels in treated cells. PROTAC 14, with a docking score of -11.2 kcal/mol, selectively reduced ITK levels in JURAKT cells but did not affect BTK levels in RAMOS cells. PROTAC 25, also with a docking score of -11.2 kcal/mol, demonstrated the most promising effect on BTK degradation, which was inhibited in the presence of bortezomib. This degradation led to the inhibition of downstream BTK-mediated signaling in IgM-stimulated RAMOS cells. In conclusion, we report a novel PROTAC derivative of azaspirooxindolinone that shows significant activity against BTK-high cells, offering a promising avenue for developing innovative therapeutics.

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New small-molecule modulators of p53 tumor suppressor isoforms expression

Karolina Kubová[1], Kateřina Koždoňová[1], Stjepan Uldrijan[1,2]

[1]Department of Biology, Faculty of Medicine, Masaryk University, Brno, Czech Republic.
[2]International Clinical Research Center, St. Anne´s University Hospital in Brno, Brno, Czech Republic

The p53 protein, encoded by the *TP53* tumour suppressor gene, is an indispensable cellular stress response regulator. It has been observed that p53 occurs in many isoforms within the human transcriptome and proteome. These variants can originate in alternative promoters of the *TP53* gene, p53 transcript alternative splicing, or the use of alternative translation initiation sites. Each isoform has a distinct function and transcriptional activity, and they differ in interactions with various cellular proteins or sub-cellular localization. The expression of p53 isoforms may serve as a prognostic marker or be used in targeted cancer therapy.

This work aims to determine the mechanism of action of a series of small-molecule compounds modulating p53 isoform expression in various cancer cell lines. The chemical structures of these modulators differ minimally among themselves, yet they have a considerably different impact on the expression of alternatively spliced p53 isoforms. Using siRNA-mediated gene knock-down, western blot analysis, PCR, and qRT-PCR, we proved which isoforms are expressed and the significant change in the ratio between them. The most distinct shift is the potent increase in p53β formation. Moreover, the results show a moderate upregulation of p53γ expression either. The impact of these small-molecule modulators on the alternative splicing pathway was not confirmed. Our latest data suggest the effect on the nonsense-mediated mRNA decay pathway.

Since p53β expression has been linked to a better prognosis in breast cancer1, melanoma2, renal carcinoma3 or acute myeloid leukemia4, the small-molecule modulators could be advantageous in the treatment of these diseases.

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Machine learning algorithms and MALDI-TOF MS: Discriminating samples from multiple myeloma and extramedullary disease patients

Monika Vlachová[1], Lukáš Pečinka[2], Lukáš Moráň[2], Tereza Růžičková[1], Martin Štork[3], Luděk Pour[3], Josef Havel[4,5], Petr Vaňhara[6,1], Sabina Ševčíková[1,7]

[1]Babak Myeloma Group, Department of Pathophysiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic.
[2]Research Centre for Applied Molecular Oncology (RECAMO), Masaryk Memorial Cancer Institute, Brno, Czech Republic.
[3]Department of Internal Medicine, Hematology and Oncology, University Hospital Brno, Brno, Czech Republic.
[4]Department of Chemistry, Faculty of Science, Masaryk University, Brno, Czech Republic.
[5]International Clinical Research Center, St. Anne's University Hospital Brno, Brno, Czech Republic.
[6]Department of Histology and Embryology, Faculty of Medicine, Masaryk University, Brno, Czech Republic.
[7]Department of Clinical Hematology, University Hospital Brno, Brno, Czech Republic

Multiple myeloma (MM) is a hematological malignancy

characterized by the clonal proliferation of malignantly transformed plasma cells in the bone marrow, resulting in the production of high levels of monoclonal immunoglobulin. MM is typically manifested by so-called CRAB symptoms, which comprise hypercalcemia, renal insufficiency, anemia, and bone lesions. Extramedullary disease (EMD) refers to a condition in which the transformed plasma cells migrate outside the bone marrow to form paraskeletal or extraskeletal lesions. EMD is associated with poor prognosis in MM patients. It often indicates a more aggressive course of disease and increased resistance to conventional therapies.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a powerful tool already used in clinical microbiology for accurate pathogen identification, and it is increasingly utilized in cancer research for biomarker discovery. The integration of machine learning algorithms for spectra analysis can further enhance MS capabilities. Our study aimed to use the aforementioned methods to distinguish peripheral blood samples from MM and EMD patients; the advantages of our approach include speed and minimal invasiveness. To this end, we also developed a two-step protocol for protein and peptide extraction that increases the intensity across the entire m/z range up to 50 times. Mass spectra were recorded using MALDI-7090 (Shimadzu) equipped with a solid-state UV laser (Nd-YAG: 355 nm) in the linear positive ion mode and mass range of 2-20 kDa. Subsequently, the spectra were evaluated in RStudio using partial least squares-discriminant analysis (PLS-DA), k-nearest neighbors (k-NN), random forest (RF), decision tree (DT), and artificial neural networks (ANN). The results demonstrate that PLS-DA predictive model can differentiate between MM and EMD samples with satisfactory sensitivity and specificity. Given further studies on larger patient cohorts, our approach could find application in clinical practice.

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Xenograft derived from circulating tumor cells as a model for preclinical research

Zuzana Kahounová[1], Markéta Pícková[1,2,3], Stanislav Drápela[4], Ráchel Víchová[1], Jiřina Procházková[1], Jiří Navrátil[5], Pavel Fabian[6], Karel Souček[1,2,3]

[1]Department of Cytokinetics, Institute of Biophysics of the Czech Academy of Sciences, Brno, Czech Republic.
[2]International Clinical Research Center, St. Anne´s University Hospital, Brno, Czech Republic.
[3]Department of Experimental Biology, Faculty of Science, Masaryk University, Brno, Czech Republic.
[4]Department of Molecular Oncology, H. Lee Moffitt Cancer Center & Research Institute, Tampa, Florida, USA.
[5]Department of Comprehensive Cancer Care, Masaryk Memorial Cancer Institute, Brno, Czech Republic.
[6]Department of Oncological Pathology, Masaryk Memorial Cancer Institute, Brno, Czech Republic

Circulating tumor cells (CTCs) are mediators of tumor dissemination, therefore their capture, characterisation and further expansion can increase our knowledge of the metastatic process. Novel preclinical models, whether *in vivo* CTCs-derived xenografts (CDX) or *in vitro* cell cultures, are urgently needed to understand the biology of CTCs, their role in dissemination, and to identify potential drugs targeting CTCs.

In this work we present the derivation and characterisation of *in vivo* CDX and CDX-derived *in vitro* cell culture as models of progressive breast cancer. The CTCs-enriched fraction was able to form a tumor under the renal capsule, and the generated CDX was propagated subcutaneously by re-transplantation in several passages. The metastatic potential of CDX-derived tumor cells was confirmed *in vivo*. To extend this preclinical model, CTCs-derived tumor cells were seeded and propagated *in vitro*. The stability of the phenotype of CDX and cell cultures was investigated using previously established multicolor protocol detecting selected surface markers by spectral flow cytometry. Finally, the potential of established CDX as a model for preclinical drug testing *in vivo* was examined. We compared the effect of drugs previously given to the patient and new drugs selected based on the genomic and transcriptomic profiling of CDX. Overall, we present here novel preclinical model of progressive breast cancer derived from CTCs that may serve as usefull model to understand the plasticity and behaviour of CTCs during tumor progression and to test therapeutics potentially targeting CTCs.

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Novel casein kinase 1 alpha inhibitors for acute myeloid leukemia therapy – tolerability, efficacy and biomarkers study

Pavlina Janovska[1,2], Ingrid Prišticová[1], Hana Plešingerová[1], Petra Procházková[1,2], Martin Čulen[3,4], Michal Šmída[3,5], Daniel Buša[3], Adrika Ladungová[5], Lukáš Maier[2,6], Štěpán Havel[2,6], Jiří Mayer[3,5,4], Kamil Paruch[2,6], Vitezslav Bryja[1]

[1]Department of Experimental Biology, Faculty of Science, Masaryk University, Brno, Czech Republic.

[2]CasInvent Pharma, Brno, Czech Republic.
[3]Department of Internal Medicine, Hematology and Oncology, Faculty of Medicine, Masaryk University, Brno, Czech Republic.
[4]Department of Internal Medicine, Hematology and Oncology, University Hospital Brno, Brno, Czech Republic.
[5]Central European Institute of Technology, Masaryk University, Brno, Czech Republic. [6]Department of Chemistry, Faculty of Science, Masaryk University, Brno, Czech Republic

Background:

Inhibition or degradation of Casein kinase 1 alpha (CK1α) alpha isoform represents an attractive option for targeted therapy of acute myeloid leukemia (AML). The high sensitivity of AML cells to CK1α inhibition/depletion is directly connected to the kinase role in p53 regulation – triggering p53-dependent apoptosis in TP53 wt AML cells. None of the heretofore reported small-molecule inhibitors of CK1α possess sufficient kinome-wide selectivity, thus the therapeutic potential of selective CK1α inhibition still remains to be assessed. Similarly, efficacy and safety of CK1α degradation are the subject of the ongoing Phase 1 clinical study focused on R/R AML and HR-MDS.

We have developed a series of potent and highly selective small-molecule inhibitors of CK1 kinases, including the most potent CK1α inhibitors known to date. They inhibit all CK1δ, ε and α isoforms, which brings the advantages of blocking both CK1δ, ε- and CK1α-dependent processes, and leads to higher efficacy and good tolerability. Part of this series was published in 2023 (Nemec et al. doi: 10.1002/anie.202217532).

Aims: We characterize a novel series of triple CK1α/δ/ε inhibitors created by lead optimization process using a unique set of assays. We further describe their effects on AML cells *in vitro* and *in vivo*, demonstrating for the first time the potential of selective CK1α inhibition. We also investigate biomarkers that would determine the sensitivity towards CK1α inhibition, leading to selection of patient subgroups that would benefit from the single-agent treatment or combined therapy.

Methodology: Cellular target engagement assays, *in vitro* kinase assays, ADME profiling, testing of *in vitro* sensitivity to CK1 inhibition and standard-of-care AML treatments in cell cultures (cell lines, primary AML samples), combination index calculations, TP53 CRISPR/Cas9 knockout cell lines production, *in vivo* validation - efficacy and tolerability.

Streamlining the processing of multi-batch LCMS data with an easy to use offline Python pipeline

Jan Machàň, Lukáš Najdekr

Institute of Molecular and Translational Medicine, Olomouc, Czech Republic

In this work, we present an open-source off-line Python pipeline that is designed to simplify the processing of large „multi-batch" LCMS data. This tool includes various filters, correction methods, and statistical tools that allow users to customize settings to the specific needs of their data. We conceived the pipeline as an all-in-one solution for data processing following the use of „peak-picking" software such as MZmine. Pipeline effectively addresses the problems of batch effect and other analytical errors. In addition to data processing, the main advantages are the ability to generate images suitable for presentations or publications and the subsequent creation of a reporting PDF file that allows users to follow each process applied onto the data step by step. The ability to work off-line with „multi-batch" LCMS data, including those with many missing values, is particularly useful for cases of confidential or excessively large files that are not suitable for uploading to popular online tools. The user can run the workflow locally and interpret the data very quickly by themselves. The code of the pipeline is written entirely in Python, ensuring accessibility and easy implementation of new filters and data transformations. With Python as the main machine learning language, it is also ready to integrate various AI models for feature prediction, data classification, or other correction methods such as SERRF, for example. This pipeline can contribute not only to accelerate data analysis and evaluation, but also to improve reproducibility and unify approaches across scientific teams thanks to its fully accessible code.

An Influence of the Cryopreservative Medium on the Methylation Pattern of Semen Samples

Lucie Kotková, Rastislav Slavkovský, Jana Vrbková, Jiří Drábek

Institute of Molecular and Translational Medicine, Palacky University, Olomouc, Czech Republic

Development of age prediction models based on the DNA methylation pattern suitable for semen traces is limited by the low number of available samples. Due to an inherent nature of germinal tissue as the most private human part capable of *in vitro* impregnation it is comparatively difficult to acquire such samples from volunteers. The obvious solution would be to use the archived specimen from previous studies. However, most samples that were collected previously and available through biobanking systems are stored using cryoprotective media.

It is not clear from the literature, whether cryopreservation media disrupt methylation signal, preventing the use of archived samples for age prediction. Thus, we want to test by pairwise comparison whether cryoprotective medium PreservCyt affects DNA methylation pattern at the loci, used in our age prediction model.

Ten paired semen samples were stored as fresh frozen samples and stored with PreservCyt. We isolated DNA and obtained the methylation

data for individual CpGs using bisulphite conversion and amplicon methyl-specific sequencing Meth-Age on Illumina MiSeq.

Here we present the comparison of methylation level results between fresh frozen and cryoprotective stored samples and its influence on the final results of age prediction.

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Prognostic value of MALAT1 and ANRIL in epithelial ovarian carcinoma: Expression analysis and serum RNA isolation techniques

Karolina Seborova[1,2], Katerina Elsnerova[1], Lukas Rob[3], Martin Hruda[3], Jiri Bouda[4], Vendula Smoligova[4], Alena Bartakova[4], Pavel Soucek[1,2], Radka Vaclavikova[1,2]

[1]Toxicogenomics Unit, National Institute of Public Health in Prague, Prague, Czech Republic.
[2]Laboratory of Pharmacogenomics, Biomedical Center, Faculty of Medicine in Pilsen Charles University, Pilsen, Czech Republic.
[3]Department of Gynecology and Obstetrics, University Hospital Kralovske Vinohrady and Third Faculty of Medicine, Prague, Czech Republic.
[4]Department of Gynecology and Obstetrics, University Hospital in Pilsen, Pilsen, Czech Republic

Introduction: Of all gynecological carcinomas, epithelial ovarian carcinoma (EOC) has the greatest fatality rate. Two main causes account for this higher mortality: the fact that the diagnosis usually comes at an advanced stage and subsequently, tumors are frequently resistant to anticancer therapy regimens based on taxanes and platinum derivatives. Among the numerous target genes in the extensive list of molecular processes linked to cancer, long non-coding RNAs (lncRNAs) are significant epigenetic regulators of gene expression of these genes. The purpose of this study was to establish the methods for estimating lncRNAs expression in serum samples and investigate the relationship between lncRNAs levels in tumor tissue of EOC patients with their clinical data.

Methods: In this study, a total of 101 EOC patients and 14 heathy controls were analyzed (pilot set – 20 EOC patients and 5 controls). lncRNAs expression was estimated by qPCR (ViiA7 RT-PCR System). For RNA isolation from serum three options were tested (TRIzol LS Reagent with/without Phasemaker Tubes (Invitrogen) and miRNeasy Serum/Plasma Kit (Qiagen)). Further, optimization for lncRNA expression in RNA from serum was performed (TaqMan Non-coding RNA Assay and TaqMan Fast Advanced Master Mix for qPCR, both Applied Biosystems).

Results: Comparison of lncRNAs expression in pilot set of EOC patients, showed significant down-regulation of MALAT1 (p=0.013) and ANRIL (p=0.007) in tumor tissue compared to healthy control tissue. EOC patients with high-grade serous subtype had higher expression of MALAT1 (p=0.039) and ANRIL (p=0.007) compared to other subtypes. Patients with residual tumor has significantly higher expression of MEG3 (p=0.015) and MALAT1 (p=0.034). No associations with stage, grade, or resistance status were found. Also, strong correlation between MALAT1 and ANRIL lncRNAs was observed (R=0.406; p<0.001). MALAT1 down-regulation in tumors after neaodjuvant therapy was associated with shorter time to progression (p=0.035). The best performance for serum RNA isolation showed miRNeasy Serum/Plasma Kit from Qiagen, where we found the best quality and quantity of isolated RNA. The worst quality and quantity was observed for TRIzol LS Reagent with Phasemaker Tubes. The quality of isolated serum RNA was also supported by results from qPCR – expression of candidate lncRNA MALAT1, where the expression in RNA samples isolated by Qiagen kit showed the best efficiency and results.

Conclusion: Our study showed significant associations of intratumoral lncRNAs – MALAT1 and ANRIL with clinical parameters of EOC patients. Optimization of serum RNA isolation and lncRNA expression for one the candidates (MALAT1) in serum was done. Study was supported by the Czech Health Research Council grant AZV no. NU20-09-00174, Cooperatio program no. 207035, “Maternal and Childhood Care” by 3rd Faculty Medicine, Charles University and the project National Institute for Cancer Research – NICR (Programme EXCELES, ID Project No. LX22NPO5102) - Funded by the European Union - Next Generation EU, and Institutional Support from National Institute of Public Health in Prague to K.S..

Morphological profiling of JUMP compounds by high throughput screening using cell painting assay

Pavel Polishchuk[1], Alzbeta Srovnalova[1], Anna Siskova[2], Aleksandra Ivanova[1], Marian Hajduch[1], Petr Dzubak[1]

[1]Institute of Molecular and Translational Medicin, Faculty of Medicine and Dentistry, Palacký University, Olomouc, Czech Republic.
[2]Faculty of Science, Masaryk University, Brno, Czech Republic

INTRODUCTION

Morphological or cytological profiling using the Cell Painting assay relies on high-content and multiplexed image-based analysis. This technique combines fluorescent staining of cellular organelles (nucleus, endoplasmic reticulum, mitochondria, Golgi apparatus, nucleolus, F-actin, cytoplasmic RNA, and plasma membrane) with automated image analysis to provide comprehensive quantitative and qualitative information about cellular organization and morphology. The

primary advantage of this assay is the visualization of various organelles using high-content imaging, which derives multiple phenotypic parameters at the single-cell level, leading to the identification and enhanced understanding of the effects of chemical compounds. Phenotypic parameters, such as shape (e.g., width, roundness), threshold compactness, radial distribution, symmetry, and texture, are extracted using image analysis platforms. This phenotypic drug discovery approach facilitates the evaluation of therapeutic candidates and the elucidation of mechanisms of action for small molecules without prior knowledge of the drug target. Morphological profiling is also crucial in identifying disease-specific phenotypes and predicting the biological impact and toxicity of compounds.

METHODS

In this study, six fluorescent probes were employed to tag eight cellular organelles, followed by acquisition in four channels using high-throughput microscopy (CellVoyager 8000). Six cell lines were utilized: A549, BJ, CT26, HCT116, HT29, and U2OS. Cells were treated in quadruplicate with a library of 305 bioactive compounds with annotated modes of action. Post-chemical perturbation, cells were fixed, stained, and analyzed using a robotic platform and automation. Over 1500 morphological features were extracted using image analysis software. All features were scaled by z-score normalization using DMSO as a reference. The most informative features were subsequently selected using the standard Cytominer pipeline. Pearson correlation was employed to identify similar profiles.

RESULTS AND CONLUSION

Data extracted from the image analysis, followed by quantitative and qualitative analysis, provided the morphological profiles of 305 bioactive compounds. Compounds with similar modes of action exhibited highly similar profiles and were clustered together. These findings validate the utility of morphological profiling in predicting the modes of action for novel compounds. Moving forward, the dataset consisting of 305 known compounds will serve as a reference to identify the modes of action for newly investigated compounds.

Comparative Biodistribution Profiles of 161Tb-PSMA-I&T and 161Tb-PSMA-617 in LNCaP Tumor-Bearing Mice

Katarína Hajduová[1], Martin Vlk[2], Ján Kozempel[2], Zbyněk Nový[1], Miloš Petřík[1], Marián Hajdúch[1]

[1]Institute of Molecular and Translational Medicine, Olomouc, Czech Republic.
[2]Faculty of Nuclear Sciences and Physical Engineering, Prague, Czech Republic

Introduction: Prostate-specific membrane antigen (PSMA)-targeted radioligand therapy has been a promising approach for the treatment of prostate cancer. The radioligands 161Tb-PSMA-617 and 161Tb-PSMA-I&T demonstrate higher linear energy transfer and additional Auger electron emission compared to established 177Lu-PSMA-617 and 177Lu-PSMA-I&T, potentially offering enhanced therapeutic efficacy. Therefore we performed this study which aims to compare biodistribution profiles of 161-Tb-PSMA I&T and 161Tb-PSMA617 in LNCaP tumor bearing mice to evaluate their potential as therapeutic agents.

Methods: LNCaP tumor-bearing mice were retroorbitaly injected with 161Tb-PSMA I&T or 161Tb-PSMA-617. Biodistribution studies were conducted at various time points post-injection (1, 4 and 24 hours). Mice were euthanized, and tissues including blood, tumor, liver, kidneys, spleen, lungs, heart, bone, intestines, pancreas, lungs and muscle were collected. The radioactivity of each sample was measured using automatic gamma counter. The percentage of injected dose per gram of tissue (%ID/g) was calculated to determine the uptake and retention of the radioligands in different organs.

Results: Both 161Tb-I&T and 161-PSMA-617 demonstrated preferential uptake in LNCaP tumors, confirming their targeting specificity for PSMA-expressing cells. 161Tb-PSMA-617 showed higher tumor-to-blood ratios (TBR) at all time points compared to 161Tb-PSMA I&T. Maximum tumor uptake for 161Tb-PSMA-617 was observed at 24 hours post-injection (671.9 %ID/g), likewise 161Tb-PSMA I&T reached its maximum at 24 hours post-injection (161.3 %ID/g). Renal uptake was substantial for both radioligands, with 161Tb-PSMA-I&T exhibiting a higher kidney-to-blood ratio, suggesting a higher renal clearance. Hepatic uptake was minimal for both agents, indicating favorable biodistribution profiles with limited off-target accumulation.

Conclusion: The biodistribution profiles of 161Tb-PSMA I&T and 161Tb-PSMA-617 in LNCaP tumor-bearing mice reveal distinct differences in tumor uptake and retention. 161Tb-PSMA-617 demonstrates superior tumor accumulation and TBR, suggesting higher efficacy in targeting PSMA-expressing prostate cancer cells. However, the renal clearance observed for 161Tb-PSMA-617 warrants further investigation to mitigate potential nephrotoxicity as we aim to do in further therapeutic studies. These findings support the continued development and optimization of 161Tb-PSMA-617 as a potent therapeutic radioligand for prostate cancer, while 161Tb-PSMA I&T offers an alternative with different pharmacokinetic properties. Oncoming therapeutic study will explore these effects further.

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Microbial Diversity in Non-Small Cell Lung Cancer Patients

Aneta Brisudová[1], Jozef Škarda[1,2], Hana Bielníková-Kryštofová[2,1], Vladislav Raclavský[3], Mária Janíková[1], Oldřich Motyka[4], Zdeněk Kolář[1], Jan Bouchal[1]

[1]Department of Clinical and Molecular Pathology, Palacky University Olomouc, Olomouc, Czech Republic.
[2]Institute of Molecular and Clinical Pathology and Medical Genetics, University Hospital Ostrava, Ostrava, Czech Republic.
[3]Department of Microbiology; Faculty of Medicine and Dentistry, Palacky University Olomouc, Olomouc, Czech Republic. [4]Faculty of Mining and Geology, VŠB – Technical University of Ostrava, Ostrava, Ostrava, Czech Republic

INTRODUCTION

Lung carcinoma (LC) is a heterogeneous disease and leading cause of death in both men and women worldwide. LC is classified mainly into two types, small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). Our study was interested in interactions between human microbiome with adenocarcinoma (ADC) and squamous cell carcinoma (SCC), subgroups of NSCLC. It is well known that microbiome influences systemic metabolic, endocrine and immune systems. Recent studies have revealed that the microbiota of healthy and tumor-affected lung tissue differ in numerous ways. It is generally known that the complex interactions between human cells and microbes may form cancer. Many of processes taking place in orgamisms, including the immune response, tumor growth, and responsiveness to anticancer therapy, are influenced by microbiota from our gut, skin, oral, respiratory, and genital tracts. In our study we characterized culturable microorganisms associated with adenocarcinomas (ADC) and squamous cell carcinomas (SCC) that can be recovered from mouth wash and rectal swab. Altogether 141 patients of all stages of illness were included in the study (86 ADC and 55 SCC cases). The highest significance of relative frequency in mouth wash samples was observed in Lactobacillus fermentum (represent in 10% ADC vs 30% SCC, p=0,002), followed by streptococcal species and Neisseria macacae. In rectal swab samples, the highest significance was observed in Streptococcus anginosus (represent in 50% ADC vs 22% SCC, p=0,002), followed by Klebsiella oxytoca and Corinebacterium aurimucosum. Ïn comparison with quasicontrol group, overrepresentation of streptococcal species and underrepresentation of Lactobacillus fermentum was confirmed. The association between microbial dysbiosis and lung cancer is not clearly understood. Future studies may elucidate the correlations between gut microbiota and lung cancer development.

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Functional validation of somatic variability in KRAS and TP53 for prediction of platinum sensitivity and prognosis in epithelial ovarian carcinoma patients

Ivona Krus[1], Mohammad Al Obeed Allah[2], Karolina Šeborová[1,2], Esraa Alli[2], Petr Holý[1,2], Vojtěch Haničinec[2], Filip Ambrozkiewicz[2], Veronika Boušková[1,2], Lukáš Rob[3], Martin Hruda[3], Marcela Mrhalová[4], Kateřina Kopečková[5], Alena Bartáková[6], Jiří Bouda[6], Pavel Souček[1,2], Radka Václavíková[1,2]

[1]Toxicogenomics Unit, National Institute of Public Health, Prague, Czech Republic.
[2]Biomedical Center, Faculty of Medicine in Pilsen, Charles University, Pilsen, Czech Republic.
[3]Department of Gynecology and Obstetrics, Third Faculty of Medicine, Charles University and University Hospital Královské Vinohrady, Prague, Czech Republic.
[4]Department of Pathology and Molecular Medicine, Second Faculty of Medicine and Motol University Hospital, Charles University, Prague, Czech Republic.
[5]Department of Oncology and Molecular Medicine, Second Faculty of Medicine and Motol University Hospital, Charles University, Prague, Czech Republic.
[6]Department of Gynecology and Obstetrics, Faculty of Medicine and University Hospital in Pilsen, Charles University, Pilsen, Czech Republic

Epithelial ovarian cancer (EOC) is among the most lethal gynecological malignancies. It is often diagnosed at advanced stages with poor prognosis. Early detection of EOC is a challenge, because, in most cases, it is asymptomatic at its beginning. Understanding the genetic mutations driving this cancer is crucial for early detection and for developing effective treatments. Concerning the dismal prognosis of chemoresistant patients with EOC, we aimed to validate the findings of a previous whole exome sequencing study on 50 patients using an orthogonal Sanger sequencing method on the same patients and a separate set of 127 EOC patients (complete set, N=177). We focused on TP53 as a frequently mutated gene relevant for chemosensitivity, included KRAS as an additional therapeutically relevant target, complemented study with transcript levels of both genes, and compared results with clinical parameters. All variants in TP53 and KRAS detected by exome sequencing were confirmed. Key variants were validated using droplet digital PCR (ddPCR). KRAS mutated patients had significantly more frequently FIGO stages I or II (p=0.007) and other than high-grade serous tumor subtypes (nonHGSCs) (p<0.001), which was connected with lower KRAS transcript levels (p=0.004). Patients with nonHGSCs harboring TP53 missense variants disrupting the DNA binding loop had significantly poorer platinum-free interval than the rest (p=0.008). Tumors bearing nonsense, frameshift, or splice site TP53 variants had a significantly lower TP53 transcript level, while

those with missense variants had significantly higher levels than wild-types ($p<0.001$). The normalized intratumoral TP53 and KRAS transcript levels were correlated, and three patients with both genes co-mutated had extremely poor survival. Taken together, our study points to KRAS as a target for future therapy of nonHGSCs and reveals the prognostic value of TP53 variants in the DNA binding loop.

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Evaluation of cyclobut-3-ene-1,2-dione-3-hydrazones with benzothiazole moiety as novel anticancer agents inducing nonapoptotic oncosis-like cell death

Alzbeta Srovnalova[1], Kaplanek Robert[2], Zdenek Kejik[3], Jan Hajduch[3], Soňa Gurska[1], Marian Hajduch[1], Petr Dzubak[1], Milan Jakubek[3]

[1]Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University and University Hospital in Olomouc, Olomouc, Czech Republic.
[2]Department of Chemistry of Natural Compounds, University of Chemistry and Technology, Prague, Czech Republic.
[3]BIOCEV, First Faculty of Medicine, Charles University, Prague, Vestec, Czech Republic

INTRODUCTION

Molecular hybridization of 3 pharmacophores (benzothiazole, cyclobut-2-ene-1,2-dione, and hydrazone) provides a set of novel active compounds can potential interact with multiple biological targets and utilize their effects through a combination of various mechanisms of action. All of 3 pharmacophores exhibit anticancer, antimicrobial, anti-inflammatory, antiviral and other therapeutic properties. Several derivatives demonstrated potent anticancer activity and high selectivity towards T-lymphoblastic leukaemia (CCRF-CEM) and colorectal cancer (HCT116) cell lines, with selectivity indexes up to 24.5. Certain derivatives emitted green fluorescence, enabling the use of fluorescence microscopy for target organelle identification. For specific derivatives that exhibited fluorescent properties, cytoplasmic vacuoles were observed after 24 hours of treatment of the U2OS cell line. Cytoplasmic vacuolization prompted further investigation into the mechanism of action, indicating the involvement of non-apoptotic cell death. Given that some benzothiazole-containing compounds have been reported to induce oncosis, our focus shifted in this direction. Oncosis is characterized by energy depletion, increased plasma membrane permeability, swelling and vacuolization of the endoplasmic reticulum and other organelles, mitochondrial swelling and condensation, and concurrent protein denaturation and hydrolysis. All these features are related to the effect on cells of compound 6. Further investigations into *in situ* formed metal complexes revealed that Fe(III) complexes of some derivatives were more active than the parent ligands, while Cu(II) and Zn(II) complexes did not enhance cytotoxicity. Notably, compound 6 showed reduced cytotoxic activity with iron addition, suggesting that the balance between extracellular and intracellular metal chelation is crucial. Our findings highlight the promise of these hybrid compounds as a novel class of anticancer agents with improved potency, selectivity, and the ability to overcome drug resistance.

METHODS

In our study, we employed various microscopic techniques to utilize the fluorescent features of target organelles in the U2OS cell line. Compound 6, which exhibited the most stable green fluorescence, was selected for further investigation. MitoTracker dye was used for mitochondrial detection, and colocalization was assessed using the Pearson correlation coefficient. TMRM dye was employed to evaluate mitochondrial membrane potential, as it incorporates only into cells with normal potential. For endoplasmic reticulum detection, we used a recombinant U2OS cell line fused with m-Cherry-tagged calreticulin to assess colocalization with cytoplasmic vacuoles. Lysosomal integrity and vacuole colocalization were examined using LysoTracker dye. To determine whether the observed cytoplasmic vacuolization was dependent on caspase activation, U2OS cells were treated with compound 6 in the presence or absence of the pancaspase inhibitor Z-VAD-FMK. The cleavage of caspase-3 and its downstream target PARP-1 was then detected by western blot analysis. The fluorescent ubiquitination-based cell cycle indicator (FUCCI) reporter system was utilized for the cell cycle analysis. The fusion of Cdt1 protein with blue fluorescent protein (BFP) was achieved through the application of the lentiviral plasmid pCCL-CellCycle. Moreover, for the visualization of the S/G2/M phase, the geminin protein, responsible for the inhibition of Cdt1 and subsequent prevention of re-replication events during the cell cycle, was integrated with the red fluorescent protein (RFP) probe. To identify cells in the mitotic and G0 phases, the far-red probe (FR) was employed to label the histone H2B which is present in every nucleus. Cell morphology alterations after prolonged treatment with compound 6 were detected using bright field microscopy technique. Image acquisition was performed using a Yokogawa CV8000 high throughput microscope.

RESULTS

Compound 6 exhibited strong co-localization with mitochondria (particularly Pearsons coefficient for colocalization yields positive-corelated values of 0.85 and 0.83 for compound 6 in two concentrations), accompanied by mitochondrial dysfunction, ER vacuolization, and the induction of non-apoptotic, oncosis-like cell death. These findings suggest that compound 6 may exert its anticancer effects through the simultaneous targeting of multiple cellular compartments and the induction of ER stress and mitochondrial dysfunction. The induction of oncosis-like cell death is particularly interesting, as it represents an alternative cell death pathway that could potentially overcome resistance to apoptosis, a major challenge in cancer therapy. Furthermore, compound 6 induced a reduction in LysoTracker staining, suggesting a disruption of lysosomal integrity function. The potential contribution of lysosomal dysfunction to the cytotoxic effects of compound 6 warrants further investigation, as targeting lysosomes has emerged as a promising strategy in cancer therapy. In addition, cell cycle analysis revealed that compound 6 induced a concentration-dependent G2/M cell cycle arrest, contributing to its anticancer effects.

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Vascular endothelial growth factor A signalling pathway in human neural stem cells and glioblastoma stem cells

Jakub Červenka[1,2], Elena Garcia-Borja[3], Jiřina Tylečková[2], Kateřina Vodičková Kepková[2], Petr Vodička[2], Petr Bušek[3], Aleksi Šedo[3], Helena Kupcová Skalníková[1,2]

[1]Laboratory of proteomics, Institute of Biochemistry and Experimental Oncology, First Faculty of Medicine, Charles University, Prague, Czech Republic.
[2]Laboratory of Applied Proteome Analyses, Research Center PIGMOD, Institute of Animal Physiology and Genetics of the Czech Academy of Sciences, Libechov, Czech Republic.
[3]Laboratory of Cancer Cell Biology, Institute of Biochemistry and Experimental Oncology, First Faculty of Medicine, Charles University, Prague, Czech Republic

Signalling via secreted Vascular endothelial growth factor A (VEGF-A) is an evolutionary conserved signalling pathway present in all vertebrates. The main function of VEGF-A is regulation of angiogenesis and vasculogenesis by stimulating the survival, proliferation and migration of endothelial cells. Neural stem cells (NSCs) and neurons are known to secrete VEGF-A to promote angiogenesis during brain cortex development, and depletion of VEGF-A leads to hypoxia and apoptosis of brain cells. Moreover, secreted VEGF-A regulates survival, proliferation, differentiation and maturation of both NSCs and neurons, independently on oxygen level. Beside its physiological roles, VEGF-A signalling is highly activated in various cancer types, as hypoxia and angiogenesis are typical features of solid tumours, including glioblastoma. Moreover, gene expression of VEGFA is often associated with poor prognosis of patients.

Glioblastoma is the most aggressive and the most prevalent type of primary brain cancer affecting mainly individuals (more commonly men) between 45 to 70 years. Unfortunately, the overall prognosis is very poor as the life expectancy is only about 14 months despite treatment. VEGF-A signalling pathway in glioblastoma cells is typically stimulated through Hypoxia-inducible factor 1 (HIF-1) and Wnt/β catenin signalling pathways by activation of VEGFA expression. VEGF-A signalling then supports invasion of glioblastoma cells (via induction of proliferation and permeabilization of endothelium), affects response to treatment and correlates with poor prognosis.

The aim of this study was to assess the roles of VEGF-A signalling in differentiation of human NSCs and glioblastoma stem cells. We have analysed the proteome and secretome of human NSCs during their spontaneous *in vitro* differentiation after growth factor withdrawal. Secretome analysis by xMAP multiplex immunoassay showed increasing secretion of Interleukin-6 and VEGF-A during NSC differentiation. Analysis of cellular proteome by mass spectrometry revealed that more than 60% of proteins significantly change their abundance through the differentiation time course. Beside cellular proteome changes connected to NSC differentiation (e.g., downregulation of proteins associated with cellular growth and proliferation and upregulation of proteins associated with gliogenesis and neurogenesis), we have observed activation of HIF-1, VEGF-A and Wnt/β catenin signalling pathways. Follow-up experiments provided evidence that VEGF121 (an isoform of VEGF-A) stimulates proliferation and survival of human NSCs in conditions of spontaneous differentiation. Currently, we are working on the analysis of glioblastoma stem cells derived from patients with glioblastoma to elucidate effects of VEGF-A on *in vitro* differentiation of such cells.

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Implementation of liquid biopsy-based analyses of solid tumors

Pavel Stejskal[1], Josef Srovnal[1], Alona Rehulkova[1], Emil Berta[1,2], Lubomir Vecera[3], Tomas Gabrhelik[3], Filip Haiduk[4], Jan Maca[4], Jan Bruthans[5], Pavel Michalek[5], Pavla Kourilova[1], Marian Hajduch[1]

[1]Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Czech Advanced Technology and Research Institute, Palacky University and University Hospital in Olomouc, Olomouc, Czech Republic.
[2]Ringerike Hospital, Honefoss, Norway.
[3]Tomas Bata Hospital, Zlin, Czech Republic.
[4]University Hospital Ostrava, Ostrava, Czech Republic.
[5]General University Hospital, Prague, Czech Republic

Introduction

Although the liquid biopsy (LB) concept has gained significant attention in the last decade, its widespread implementation in clinical practice remains in progress. However, substantial progress has been made in developing and standardizing LB methods, which are crucial for its broader adoption. LB relies on detecting and analyzing tumor-derived biomarkers, such as circulating tumor cells (CTCs) and cell-free circulating tumor DNA and RNA (ctDNA, ctRNA), present in body fluids. These biomarkers hold valuable information about tumor characteristics and evolution, making them promising for diagnostic, predictive, and prognostic applications. Here, we focus on the implementation of LB biomarkers in solid tumors such as colorectal cancer (CRC), and glioblastoma multiforme (GBM).

Material and Methods

The peripheral blood samples were collected in Cell-Free DNA BCT® (Streck, Inc.) tubes. CTCs were identified using CytoTrack CT11TM instrument, a semi-automated immunofluorescence microscopy detecting pan-cytokeratin and EpCAM signals for CRC, and glial fibrillary acidic protein and vimentin signals for GBM CTC detection. Selected CTCs were isolated by micromanipulation and subjected to confocal microscopy for high-resolution imaging and whole genome amplification (WGA) of single cell-derived DNA for genetic profiling by targeted next-generation sequencing (NGS) or copy number variation analyses. CfDNA/cfRNA was extracted from plasma derived from the peripheral blood samples collected to Cell-Free DNA/RNA BCT® (Streck, Inc.) tubes and subjected to targeted genome/whole transcriptome profiling by NGS (NovaSeq 6000, Illumina).

Results and conclusions

We have analyzed the CTC presence in the samples of 200 colorectal cancer and 90 glioblastoma multiforme patients with positivity rates of about 30% and 38%, respectively. We have implemented the molecular-genetic profiling of CTCs in CRC and GBM patients. The NGS-based ctDNA/ctRNA profiling was optimized for cancer patient plasma samples. The established protocols will be applied to larger cohorts of patients whose enrolment is ongoing.

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Identification of novel specific inhibitors of carbonic anhydrase IX

Soňa Gurská[1], Pavlo Polishchuk[1], Roman Lesyk[2], Jiří Brynda[3], Pavlína Řezáčová[3], Petr Džubák[1], Marián Hajdúch[1]

[1]Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic.
[2]Dept. of Pharmacology, Danylo Halytsky National Medical University, Lviv, Ukraine.
[3]Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Prague, Czech Republic

Introduction

Hypoxia in the tumor microenvironment plays a crutial role in cancer progression, metastasis and treatment resistence. Carbonic anhydrase IX (CA IX), a key factor induced by hypoxia, is metalloenzyme that catalyzes the conversion of carbon dioxide into bicarbonate, releasing a proton and thus regulating pH and acidosis. Due to its significant overexpression in hypoxic tumors compared to normal tissues, CA IX represents a promising target for drug development.

In our previous high-throughput screening compaign aimed at identifying novel CA IX inhibitors, we designed a new fluorescence-based assay using pyranine as a pH change indicator. We analyzed the inhibition activity of over 10.000 unique compounds from the IMTM Proprietary Library. This screening led to the identification of five derivatives as lead compounds with high specificity against CA IX.

To enhance the specificity, physiochemical, and biological properties of the lead compounds, we synthesized new derivatives. This study aims to test the specific inhibition activity of a series of 46 modified hits against CA IX and determine the structural features crucial for efficient and selective inhibition of CA IX activity through structure-activity relationship sutides. The results obtained will be presented and discussed.

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Role of fibroblast activation protein expressing stromal cells in angiogenesis and vascular destabilization in glioblastoma

Eva Balážiová, Martin Chudada, Petr Bušek, Aleksi Šedo

Laboratory of Cancer Cell Biology, Institute of Biochemistry and Experimental Oncology, First Faculty of Medicine, Charles University, Prague, Czech Republic

Glioblastoma (GBM), the most malignant primary brain tumor, is characterized by rampant neovascularization and aberrant blood vessel structure. Previous studies, including our own, have shown that a transmembrane protease fibroblast activation protein (FAP), a candidate diagnostic and therapeutic target in various cancer types, is expressed on stromal cells predominantly located near blood vessels in GBM. The quantity of stromal FAP positively correlates with the extent of vascularization in GBM and patient-derived FAP-positive stromal cells enhance angiogenic sprouting *in vitro*. This study aims to elucidate the role of FAP-positive stromal cells in the destabilization of blood vessels in GBM.

Analysis of the open-access IVY glioblastoma atlas revealed that vascular regions with high FAP expression are characterized by reduced expression of proteins involved in maintaining blood-brain barrier integrity compared to those with low FAP expression. In parallel, we observed a correlation between the presence of FAP and the degree of dysmorphia in the vascular network of glioblastoma tissue. Preliminary results of our *in vitro* experiments further indicated that conditioned media from patient-derived cultures of FAP-positive stromal cells increase the permeability of endothelial monolayers. Our data suggest that FAP-positive stromal cells may be linked to formation of abnormal blood vessels and contribute to the breakdown of the blood-brain barrier in GBM.

Together, FAP-positive stromal cells in GBM not only support the formation of new blood vessels but also contribute to their destabilization. This dual role in promoting angiogenesis and increasing vascular permeability underscores the significance of FAP-positive stromal cells in the pathophysiology of glioblastoma.

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Inhibition of the PI3K/AKT Pathway and Its Impact on Vesicle Trafficking and Exosome-Mediated Intercellular Communication in Glioblastoma

Claudia Carrera Bravo, Martin Sztacho

Laboratory of Cancer Cell Architecture, Institute of Biochemistry and Experimental Oncology, First Faculty of Medicine, Charles University, Prague, Czech Republic

Glioblastoma, the most aggressive form of brain cancer, is characterized by rapid progression and resistance to standard therapies. The PI3K/AKT signaling pathway plays a pivotal role in glioblastoma cell survival and proliferation. However, the effect of this pathway on vesicle dynamics and exosome-mediated intercellular communication is not well understood, despite both being key processes in promoting tumor growth, metastasis, and resistance to therapies. We hypothesize that PI3K/AKT pathway inhibition will lead to changes in the expression and function of key genes and proteins involved in vesicle trafficking and exosome-mediated communication in glioblastoma cells, thereby affecting the tumor's growth and ability to communicate with surrounding cells.

This study aims to explore the effects of PI3K/AKT pathway inhibition on vesicle trafficking and exosome-mediated communication in glioblastoma cells. We will use a combination of molecular, biochemical, cell biology, analytical, and omics techniques. The PI3K/AKT pathway will be inhibited in the U-251 MG human glioblastoma astrocytoma-derived cell line using Wortmannin. Pathway inhibition will be validated by assessing the levels of phosphorylated AKT and total AKT via western blot. Additionally, RT-qPCR will be used to measure the expression of key genes involved in vesicle trafficking, including RAB5, RAB27A, SNAP23, and SYT1. Vesicle trafficking dynamics will be studied by tracking early and late endosomal markers through confocal microscopy. Exosomes will be isolated from the PI3K/AKT-inhibited U-251 MG cells and characterized through transmission electron microscopy, nanoparticle tracking analysis, and western blot. Furthermore, the exosome cargo will be analyzed using proteomics, lipidomics, and transcriptomics. Finally, the functional impact of the exosomes will be evaluated on healthy astrocytes and glioblastoma cells through cell proliferation and migration assays.

Understanding these mechanisms may lead to identifying novel target molecules from glioblastoma exosomes that could be incorporated into engineered liposomes to cross the blood-brain barrier effectively, delivering therapeutic agents directly to glioblastoma cells to suppress cell proliferation, migration, and vesicle trafficking.

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Live imaging of A3 adenosine receptor interactions using a fluorescent antagonist probe

Kateřina Ječmeňová, Marián Hajdúch, Petr Džubák

Institute of molecular and translational medicine, Olomouc, Czech Republic

Introduction:

Adenosine receptors (ARs), particularly the A3 adenosine receptor (A3AR), are important G-protein-coupled receptors highly expressed in cancer cells, making them significant therapeutic targets. However, the current methods for live cell imaging of ARs – visualization of cellular expression, spatial monitoring in the membrane, or observation of interactions between AR modulators (competition, competitive displacement) are limited. Increased understanding of these receptors is required to improve the success rate of adenosine receptor drug discovery. Here, we introduce the microscopic technique for A3AR visualization and functional studies using CELT-228, a novel A3AR-specific fluorescent probe. This method could be used in living cells and competitive binding assays to identify and validate novel A3AR agonists and antagonists.

Material and methods:

The reporter cell line overexpressing A3AR was treated by the A3AR-specific fluorescent probe (fluorescently labeled A3AR antagonist - CELT-228; Celtarys, Spain), and the fluorescent images were taken by spinning disc confocal microscopy (Yokogawa CV7000) at different timepoints and concentrations. Then, the localization and intensity of the signal were analyzed using Signals Image Artist (SImA) software. The competitive assay based on the CELT-228 fluorescent probe was developed to study interactions of the potential A3AR agonists or antagonists and used for the High Content Screening of newly synthesized nucleoside-based compounds. The competition assay was used to test the interaction of newly identified A3AR ant-/agonists on cancer cell lines derived from tumors of various histogenetic origins with A3AR expression.

Results:

The fluorescent signal of the bound probe was localized on the cell membrane of the reporter cell line and most of the cancer cell lines. Our data from competition assays indicated that the newly synthesized potential anticancer compound binds to the same (orthosteric) binding site on the A3 receptor and competes for it with the fluorescent-labeled probe.

Conclusion:

This technique offers a powerful tool for observing A3AR expression, monitoring small molecule competition, and confirming the binding of novel compounds under native conditions. Our method can potentially enhance understanding of adenosine receptors and improve the success rate of AR-targeted drug discovery.

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Selective inhibition of Carbonic Anhydrase IX for Cancer Diagnosis and Therapy

Linh Huong Ngô, Jiří Brynda, Klára Pospíšilová, Pavlína Řezáčová

Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Prague, Czech Republic

Carbonic anhydrase IX (CA IX) belongs to a group of 15 isoforms of the human carbonic anhydrase enzymes. Typically localized on the cell surface, CA IX is primarily found in specific tissues within the gastrointestinal tract. Its expression is induced in response to local hypoxia, aiding in the regulation of pH levels to accommodate the metabolic production of acidic by-products, thereby promoting cancer cell survival and proliferation. The overexpression of CA IX in solid tumors, coupled with its extracellular presence, suggests its potential utility in cancer diagnosis and therapy.

Primarily, most of CA IX inhibitors feature sulphur-based functional group that coordinates the Zn2+ ion in the active site. Although overexpression of CA IX is predominantly associated with tumor tissues, other isoforms are present in normal tissues that contributing critical physiological processes. The high sequence similarity and structural homology among CA isoform family causes off-target inhibition leading to unintended side effects. This underscores the need for developing highly selective inhibitors that minimize off-target effects. The project aims to address these challenges by designing novel functional group to enhance both the affinity and selectivity of CA IX inhibitors.

The active site of CA is situated within the central β-sheet, where the zinc-binding core serves as a key junction for the proposes inhibitors, which are designed with a scaffold capable of attaching enzyme moieties. This scaffold comprises a sulfonimine binding group for metal ion interaction, a functional group for interaction with the hydrophobic regions, and additional heteroaromatic moieties to improve affinity. Structural optimisation of these inhibitors has been conducted by understanding how they are fitting within the enzyme's active site, in order enhancing their affinity for tumor-specific CA IX while restricting interactions with other CA isoforms. Additionally, some potent chelators have been selected for theranostics applications, ensuring they do not compromise the binding capacity of inhibitors.

Recombinant CA IX is produced

and expressed in Escherichia coli BL21, followed by purification via several chromatographic steps to ensure high protein purity. The purified CA and a series of inhibitors are assessed for affinity using the stopped-flow method to screen a library of inhibitors. To better understand the binding modes between selective inhibitors and the enzyme, X-ray crystallography is employed to achieve high-resolution structures of the compounds. The obtained structural information will guide the modification and optimisation the anchored and sticky groups in design the selective inhibitors. This approach aims to maximize affinity for tumor-specific CA IX while minimizing interactions with other carbonic anhydrase isoforms.

Accelerating Kinase Inhibitor Identification with ECHO MS

Martina Kintlová, Petr Džubák

Institute of molecular a translational medicine, Faculty of Medicine and Dentistry, Palacký University Olomouc, Olomouc, Czech Republic

Kinases play a crucial role in various biological processes, making them key targets in high throughput screening in drug discovery. Identifying novel kinase inhibitors is essential for developing therapeutic strategies for numerous diseases. Until now, we have primarily relied on MALDI-TOF analysis for early-stage drug discovery. To enhance the speed and capacity of our workflow and address the limitations of MALDI-TOF, we have integrated the SCIEX Echo® MS System into our laboratory. This innovative platform enables rapid, chromatography-free MS/MS analysis using acoustic sample ejection. The system facilitates direct sample introduction to electrospray from the plate and supports sample dilution. The 6500+ Triple Quad can detect chemical compounds within a mass range of 5 2000 Da, even in complex matrices, making it ideal for high-throughput, quantitative studies. The Echo® MS assay has been optimized for Aurora and cyclin-dependent kinases, potential targets in cancer treatment, as well as MARK kinases, which are potential therapeutic targets for Alzheimer‘s disease.

Selective Expansion of Antigen-Primed CD8+ T Cells by CD25-Biased IL-2/ anti-IL-2 mAb Complexes and Their Resistance to Treg Cell-Mediated Suppression

Petra Weberova, Baki Irfan Kilic, Katerina Behalova, Bohumil Ptacek, Milada Sirova, Blanka Rihova, Marek Kovar

IInstitute of Microbiology of the CAS, v. v. i., Prague, Czech Republic

Introduction: Interleukin-2 (IL-2) plays a central role in the proliferation, survival, and functional maturation of activated T cells, making it a key component in cancer immunotherapy. However, its clinical application has been significantly constrained by off-target effects, including severe toxicity and the undesired expansion of T regulatory (Treg) cells, which can suppress anti-tumor immunity. The cytotoxic potential of activated CD8+ T cells is critical for successful tumor elimination, positioning them as central players in cancer immunotherapy.

Objective: This study aims to evaluate the efficacy of CD25-biased IL-2/anti-IL-2 monoclonal antibody (mAb) complexes, specifically formed by recombinant murine IL-2 (rmIL-2) and JES6-1A12 mAb (IL-2/JES6), in selectively expanding antigen-primed CD8+ T cells and augmenting their effector functions. Given the strong propensity of IL-2/JES6 to expand Treg cells, we further investigated whether this complex could neutralize Treg cell-mediated suppression of activated CD8+ T cells.

Methods: A model was established using OT-I CD8+ T cells adoptively transferred into congenic B6 mice, followed by ovalbumin priming and treatment with IL-2/JES6. The expansion and functional activation of the transferred CD8+ T cells were assessed via flow cytometry, focusing on markers such as CD25, granzyme B, and perforin. Additionally, *in vitro* Treg suppression assays were conducted to determine the impact of IL-2/JES6 on the ability of Treg cells to suppress the proliferation of activated CD8+ T cells.

Results: The CD25-biased IL-2/JES6 complex significantly expanded antigen-primed OT-I CD8+ T cells and Treg cells. Importantly, IL-2/JES6 enhanced the expression of activation markers (CD25) and effector molecules (granzyme B, perforin) in CD8+ T cells. Notably, in the presence of IL-2/JES6, Treg cells failed to suppress the proliferation of activated CD8+ T cells, suggesting that IL-2 sequestration by Treg cells is a key mechanism of their suppressive function.

Conclusion: Our findings demonstrate that CD25-biased IL-2/JES6 complexes effectively expand antigen-primed CD8+ T cells while also promoting Treg cell proliferation. However, the presence of IL-2/JES6 impairs Treg cell-mediated suppression, thereby enhancing the therapeutic potential of CD8+ T cells in cancer immunotherapy.

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FADD and executioner caspases are indispensable for cell death of Jurkat cells upon anti-cancer drugs treatment

Iva Slaninová[1], *Zuzana Mrkvová*[1,2], *Petra Kulíšková*[1,3], *Gabriela Filipová*[1]

[1]*Masaryk University, Faculty of Medicine, Department of Biology, Brno, Czech Republic.*
[2]*Masaryk University, Faculty of Medicine, Internal hematology and onkology clinic, Brno, Czech Republic.*
[3]*Masaryk University, Faculty of Medicine, Department of Clinical Immunology and Allergology, Brno, Czech Republic*

Dysregulation of programmed cell death pathways is one of the cancer hallmarks and one of the reasons of drug resistance. Programmed cell death (PCD) pathways play a crucial role in the response of cancer cells to treatment. Apoptosis is the best known cell death, but new studies show the importance of other PCDs, e.g. necroptosis. An interesting question is which cell death modality the cancer cells prefer and to what extent the signaling pathways of PCD are interconnected. In this study we investigated the significance of the individual members of apoptosis and/or necroptosis signaling pathways in response to treatment with common anti-cancer drugs using the T-cell leukemia Jurkat cells with single or multiple knockouts of necroptosis and/or apoptosis genes. We studied the effect of 6 anticancer drugs on 13 lines of Jurkat cells and identified apoptosis as the primary cell death pathway upon anti-cancer drugs treatment. The results of the study demonstrated that the knockout of the FADD or the combined knockout of all executioner caspase genes (i.e. caspase 3, 6 and 7) lead to resistance of Jurkat cells to common anti-cancer drugs. This resistance could be overcome by a combined treatment with TNF-α and LCL161 (smac mimetic, SM). The cell death triggered by TNF-α/SM was RIP1-dependent necroptosis. Despite RIP1 was essential for cellular response to TNF-α/SM treatment, it was completely dispensable in the response to anti-cancer drugs. The individual caspases, either initiation or effector, were dispensable for the cell death execution, indicating the interchangeability of the particular caspases.

Here we demonstrated the significance of FADD and executioner caspases in carrying out programmed cell death upon anti-cancer drug treatment and the ability of combined treatment with TNF-α and smac mimetic to partially overcome drug resistance of FADD and/or CASP3/7/6-deficient cells via RIP1 dependent necroptosis. Thus, a combination of TNF-α and smac mimetic could be a suitable strategy for overcoming the resistance to therapy in cells unable to trigger apoptosis.

PHPMA-*b*-PPO amphiphilic copolymers for treatment of resistant solid tumors

Alena Braunová[1], *Martin Kaňa*[2,3], *Daniil Starenko*[3], *Jan Bouček*[2], *Marek Kovář*[3], *Blanka Říhová*[3], *Milada Šírová*[3], *Tomáš Etrych*[1]

[1]*Institute of Macromolecular Chemistry, Czech Academy of Sciences, Prague 6, Czech Republic.*
[2]*Department of Otorhinolaryngology and Head and Neck Surgery, First Faculty of Medicine, Charles University and University Hospital Motol, Prague 5, Czech Republic.*
[3]*Institute of Microbiology, Czech Academy of Sciences, Prague 4, Czech Republic*

The role of ATP-binding cassette (ABC) transporters in organism is elimination of toxins and drugs out of the cells. In cancer cells, overexpression of these efflux pumps is often associated with the emergence of so-called multidrug resistance (MDR). This fact strongly influences the efficiency of tumour therapy and significantly contributes to the reducing of final therapeutic effect. In this work, we focused on the inhibition of P-glycoprotein (P-gp), one of the most famous ABC transporters, to make the used anti-tumour therapy more effective. In the last more than 50 years, carriers of biologically active compounds (e.g. drugs, hormones, antibodies, etc.) have been investigated and developed to improve undesirable side effects of drugs and increase the tumour therapy efficacy. They are predominantly based on various polymers with specific properties (non-toxicity, non-immumogenicity, mostly hydrophilicity and water-solubility). The advantage of drug delivery systems (DDS) is primarily extension of their circulation time in bloodstream in consequence of high molecular weight (HMW) and non-immunogenicity of the polymers. These carrier properties significantly contribute to protection of the drug from premature capture by reticuloendothelial system (RES) and subsequent elimination from the body. Moreover, the polymer carrier structure can be tailored for controlled transport, targeting (passive or active) and release of drugs only in the site of action.

We designed, synthesized and characterized novel amphiphilic diblock copolymers (DBs) based on the well-known hydrophilic poly[N-(2-hydroxypropyl)methacrylamide] copolymer (PHPMA) and hydrophobic poly(propylene oxide) derivative (PPO) where PPO is known to be a good HMW P-gp inhibitor. Based on amphiphilic character of prepared DB copolymers, self-assembly of copolymers into supramolecular structures, micelles, occurs in an aqueous environment, and subsequently an increase in their hydrodynamic size (Rh). In this way, passive targeting of the systems to the solid tumours by the enhanced permeability and retention (EPR) effect is ensured caused by preferential uptake of HMW compounds in tumour tissue. Thus, in resulting conjugates, we combined and utilized inhibitory properties of PPO, and simultaneously unique properties of PHPMA and favorable micellar character of whole DB conjugate. Anti-tumour antibiotic drug doxorubicin (Dox) was attached to DB copolymers through pH-sensitive hydrazone bond, degradable inside the cells, but quite stable in bloodstream.

The copolymer exclusion from the body is ensured by the micelle disintegration into unimers with a size below the limit of glomerular filtration after fulfilling its transport and protection role. The presented copolymers and drug-conjugates were characterized using physico-chemical methods (SEC, DLS, FFF, UV-VIS spectroscopy) and by both *in vitro* and *in vivo* biological evaluation.

The polymer-drug conjugates DB-Dox based on diblock copolymers of PHPMA and PPO showed inhibition of efflux membrane transporters, depletion of intracellular ATP levels, alteration of mitochondrial membrane potential, and increased ROS production. Biological evaluation *in vivo* also proved that DB-Dox inhibited tumour growth more effectively by comparison with corresponding the only PHPMA-based drug delivery system. The presented micellar copolymers and their Dox-conjugates are promising P-gp inhibitors as well as drug delivery systems, and candidates for the treatment of MDR tumours, for which traditional chemotherapy is not satisfactory.

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Accelerating the development of isogenic cystic fibrosis cell models

Lukas Lenart[1], *Martin Ondra*[1,2], *Marian Hajduch*[1,2,3]

[1]*Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic.*
[2]*Czech Advanced Technology and Research Institute, Palacky University, Olomouc, Czech Republic.*
[3]*Laboratory of Experimental Medicine, Institute of Molecular and Translational Medicine, University Hospital Olomouc, Olomouc, Czech Republic*

Cystic fibrosis (CF) is a severe autosomal recessive disorder, caused by mutations of a gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein, a member of the ATP-binding cassette (ABC) transporter family that acts as an ion channel. Mutations of the CFTR can lead to variable outcomes, including reduced CFTR function, impaired CFTR plasma membrane localization, or even total absence of CFTR. Some of the CFTR defects underlying these outcomes can now be targeted with CFTR modulators, a group of drugs that aim to restore the CFTR membrane localization and function. Although up to 85% of CF patients, mostly carrying the most frequent CFTR mutation (ΔF508) at least in one allele, are eligible for treatment with the highly efficient combination of CFTR modulators Trikafta®, others with rare CFTR mutations cannot benefit from this treatment. To identify novel modulators targeting rare CFTR mutations, the development of relevant model cell lines that carry the desired CF-causing mutations is required. Such model cell lines can be generated by CRISPR/Cas9 mediated introduction of CF-causing mutations into already developed reporter cell line with endogenous expression of HiBiT tagged WT-CFTR (Ondra et al., 2023). As the efficiency of CRISPR/Cas9 editing is not immaculate, subsequent preparation of monoclonal cell lines carrying only the desired modifications is of utmost importance. Therefore, it is necessary to develop a rapid, reproducible, and efficient genotyping platform to distinguish correctly modified clones from those carrying unspecific modifications or no modifications at all. Here, a sophisticated cell free DNA-based genotyping approach utilizing culture medium is described.

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Effects of Steroid Ligands on Pancreatic Cancer Cells in Two- and Three-Dimensional Culture

Martina Peřinová[1,2], *Jana Novobilská*[1], *Lucie Rárová*[3], *Monika Levková*[1,2], *Marie Kvasnicová*[3], *Jana Steigerová*[1,2]

[1]*Department of Clinical and Molecular Pathology, Palacký University, Olomouc, Czech Republic.*
[2]*Institute of Molecular and Translation Medicine, Faculty of Medicine and Dentistry, Palacký University and Faculty Hospital in Olomouc, Olomouc, Czech Republic.*
[3]*Laboratory of Growth Regulators, Faculty of Science, Palacký University, and Institute of Experimental Botany of the Czech Academy of Sciences, Olomouc, Czech Republic*

The study of novel steroid ligands and their molecular effects has become a significant approach in identifying new agents with anti-tumour activity in humans. One strategy to improve the success rate of new cancer drugs transitioning to the clinic is to more closely align the cellular models used in early lead discovery with pre-clinical animal models and patient tumours. For solid tumours, this requires the development and implementation of three-dimensional (3D) *in vitro* cancer models that more accurately recapitulate human solid tumour architecture and biology. 3D cell structures better reflect the *in vivo* distribution of metabolites, nutrients, oxygen, and signalling molecules, while also more accurately mimicking 3D tissue architecture, cell proliferation, motility, and migration through an artificial extracellular matrix. These natural interactions enable cells to acquire morphological and other cellular characteristics like those found in solid tumours *in vivo*. There is currently limited information on the antiproliferative and anticancer

effects of steroid derivatives in pancreatic cancer, particularly regarding their mechanism of action on nuclear receptors. For this reason, we investigated the effects of selective steroid derivatives on cell proliferation, cell cycle progression, and apoptosis in both 2D and 3D models of pancreatic cancer cells. The study of the biological properties of 3D spheroid cells and their responses to drug treatment reveals differences compared to 2D cultures. Therefore, evaluating the effects of potential anti-tumour drugs in 3D cell models could become a standard component of preclinical testing.

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Application of lipid profiles for the characterisation of cardiovascular diseases

Richard Masař[1], Štěpán Hudec[2], Martin Rada[2], Veronika Kučerová[3], Lukáš Najdekr[1]

[1]Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry Palacky University Olomouc, Olomouc, Czech Republic.
[2]Department of Cardiology, University Hospital Olomouc, Olomouc, Czech Republic.
[3]Department of Clinical Biochemistry, University Hospital Olomouc, Olomouc, Czech Republic

Cardiovascular diseases (CVDs) are the leading cause of death in developed and developing countries, taking approximately 20.5 million lives each year. Estimates are that by 2030, the annual mortality of CVDs is expected to reach 23.6 million. However, according to a report by the World Heart Federation, up to 80 % of premature heart attacks and strokes could be prevented by the correct use of more specific diagnostic tools. An untargeted lipidomic approach seems to be a suitable tool for the search for potential new biomarkers. Here in our work, we present data from analyses of lipid profiles of patients after STEMI (ST-elevation myocardial infarction) compared to the control group.

We have collected nine patient plasma samples from the Department of Cardiology and nine control plasma samples from the Department of Clinical Biochemistry. The human plasma samples were analysed using reversed-phase liquid chromatography coupled with a mass spectrometer (RP-HPLC/MS). The data matrix was processed using Compound Discoverer 3.3 SP3 (Thermo Fisher Scientific, USA).

By the approach of untargeted lipidomics, after the “peak-rating” filtering in CD 3.3 SP3, we have putatively annotated 875 compounds in positive and 631 compounds in negative ionisation modes. With the intelligent acquisition of fragmentation mass spectra, which help in the structural identification of lipids and the application of molecular networks, we could group molecules based on their structure similarity, thus revealing new biomarker candidates. Many of those compounds were successfully annotated using machine learning algorithms (SIRIUS, University of Jena, Germany). Using orthogonal partial least squares discriminant analysis (OPLS-DA) and volcano plots, ceramides (namely, Cer(d18:1/16:0), Cer(d18:1/24:1), and Cer(d18:2/24:1)) and phosphatidylcholines (specifically PC 16:0_22:5) were evaluated as the most significant compounds in patient samples. Within the Mayo Clinic network of hospitals, Cer(d18:1/16:0), Cer(d18:1/18:0), and Cer(d18:1/24:1) levels, and their ratios to Cer(d18:1/24:0) are used to assess the risk of adverse severe CVD events over one to five years.

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Novel 2-phenylethenyl-1H-benzo[e]indoles and their photo-induced anticancer activity in melanoma cell lines

Eva Řezníčková[1,2], Gabrielė Varvuolytė[3,4], Asta Žukauskaitė[5], Algirdas Šačkus[3,4], Vladimír Kryštof[1,2]

[1]Department of Experimental Biology, Faculty of Science, Palacký University, Olomouc, Czech Republic.
[2]Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacký University, Olomouc, Czech Republic.
[3]Department of Organic Chemistry, Kaunas University of Technology, Kaunas, Lithuania.
[4]Institute of Synthetic Chemistry, Kaunas University of Technology, Kaunas, Lithuania.
[5]Department of Chemical Biology, Faculty of Science, Palacký University, Olomouc, Czech Republic

A novel series of 2-phenylethenyl-1H-benzo[e]indoles has been prepared via the Knoevenagel condensation reaction and characterised by various spectroscopic methods. The compounds demonstrated cytotoxicity against human melanoma cells at submicromolar doses when irradiated with blue light (414 nm), which prompted us to investigate their photodynamic effects. The mechanism of action of the lead compound in the series was further investigated and linked to the substantial generation of reactive oxygen species leading to DNA damage. The subsequent induction of cell death, which was confirmed by several methods, was shown to be dependent on the concentration of the photosensitizer and the irradiation intensity of the blue light. These results contribute to our

understanding of the photocytotoxic properties of such compounds and suggest potential avenues for improving photodynamic therapy.

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The impact of inhibition of lactosylceramide synthases B4GALT5 and B4GALT6 on colon cancer cell death

Barbora Lujka[1,2], Tereza Šošolíková[1,2], Gerardo Vázquez-Gómez[1], Ondřej Kováč[3], Miroslav Machala[3], Alena Hyršlová Vaculová[1], Jan Vondráček[1]

[1]Department of Cytokinetics, Institute of Biophysics of the Czech Academy of Sciences, Brno, Czech Republic.
[2]Department of Experimental Biology, Faculty of Science, Masaryk University, Brno, Czech Republic.
[3]Department of Pharmacology and Toxicology, Veterinary Research Institute, Brno, Czech Republic

Glycosphingolipids (GSLs) represent an important group of bioactive lipid molecules that have been found to be deregulated in colon cancer cells, with a potential role in modulation of the disease progression. We investigated the impact of inhibition of lactosylceramide synthesis (using selective targeting of specific lactosylceramide synthases, B4GALT5 or B4GALT6 by CRISPR/Cas9 gene knockout) on control of proliferation and especially cell death in DLD-1 human colon adenocarcinoma cells. The efficacy of the downregulation/inhibition of selected enzymes was verified by decrease in respective mRNA (RT-qPCR) and protein (Western blotting) levels. We also observed significantly reduced levels of lactosylceramides, and several more complex GSLs, in lactosylceramide synthase-deficient cells (LC-MS/MS). We found that downregulation of B4GALT5 or B4GALT6 enhanced the DLD-1 colon cancer cell sensitivity to the cytotoxic effects of oxaliplatin, a drug frequently used in the colorectal cancer treatment, which was demonstrated by general decrease in cell viability (resazurin assay), an enhanced apoptosis/cell death (annexin V/PI assay, flow cytometry), caspase activation and cleavage of their substrates (specific antibodies, flow cytometry and Western blotting) or stimulation of other cell death-related changes at the level of selected intracellular organelles. We continue to evaluate the molecular mechanisms involved in B4GALT5/B4GALT6-mediated modulation of colon cancer cell sensitivity to oxaliplatin treatment. Our findings seem to support the functional roles of B4GALT5/B4GALT6 enzymes in the regulation of survival and chemosensitivity of colon cancer cells.

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Wnt ligand secretion regulates emergency granulopoeisis by inducing myeloid differentiation

Karolina Vanickova[1], Petr Danek[1], Mirko Milosevic[2], Miroslava K. Adamcova[1], Francesca Matteini[3], Lucie Janeckova[1], Vladimir Korinek[1], M. Carolina Florian[3], Jakub Rohlena[2], Katerina Rohlenova[2], Meritxell Alberich-Jorda[1]

[1]Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic.
[2]Institute of Biotechnology of the Czech Academy of Sciences, Prague, Czech Republic.
[3]The Bellvitge Institute for Biomedical Research, Barcelona, Spain

β-catenin-TCF/LEF-mediated transcription in hematopoietic stem and progenitor cells (HSPCs) is critical for myeloid differentiation during bacterial infections, where increased numbers of granulocytes are rapidly generated in a process known as emergency granulopoiesis (EG). The β-catenin-TCF/LEF pathway is activated by Wnt ligands, a family of secreted glycoproteins accounting for 19 members, both in human and mice. Nevertheless, whether some of these ligands are secreted during infection and regulate emergency granulopoiesis is largely unknown. Here, we employed Wls conditional KO mice, in which the secretion of all Wnt ligands is blocked upon tamoxifen administration. We observed a severe impairment of EG upon lipopolysaccharide (LPS) treatment at the level of multipotent progenitors, which were not able to execute the lymphoid-to-myeloid bias switch. scRNAseq analysis of HSCs isolated from mice challenged with LPS or PBS control revealed that Wnt10b was the only Wnt ligand upregulated in a myeloid-biased HSC population exclusive of the LPS-treated mice. Next, we confirmed that Wnt10b is released in the serum and in the bone marrow following LPS treatment *in vivo* using ELISA and whole mount microscopy. We generated Wnt10b KO mice and observed impaired myeloid differentiation in steady-state conditions. Accordingly, *in vitro* stimulation of WT HSPCs with recombinant Wnt10b led to decrease in proliferation and increased myeloid differentiation. Finally, EG response in these mice was impaired in a similar manner to the Wls mice, albeit to a lesser extent. Altogether, our data shows that proper secretion of Wnt ligands is crucial for EG and that at least part of the response is mediated by Wnt10b, which acts by blocking proliferation and inducing myeloid differentiation. This work was supported by GACR 22-18300S, GAUK 327722, and IMG institutional funding RVO68378050

CD53 and MHCII expression define a unique HSC present in extramedullary hematopoietic sites under chronic inflammation

Maria Kuzmina[1,2], Jiri Brezina[3,2], Mirko Milosevic[4], Srdjan Grusanovic[1], Karolina Vanickova[1,2], Nataliia Pavliuchenko[5], Tomas Brdicka[5], Katerina Rohlenova[4], Jakub Rohlena[4], Dominik Filipp[3], Meritxell Alberich-Jorda[1,6]

[1]Laboratory of Hemato-oncology, Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic.
[2]Faculty of Science, Charles University, Prague, Czech Republic.
[3]Laboratory of Immunobiology, Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic.
[4]Laboratory of Cellular metabolism, Institute of Biotechnology of the Czech Academy of Sciences, Vestec, Czech Republic.
[5]Laboratory of Leukocyte Signalling, Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic.
[6]Childhood Leukemia Investigation Prague, Department of Pediatric Hematology and Oncology, 2nd Faculty of Medicine, Charles University in Prague, University Hospital Motol, Prague, Czech Republic

In steady-state hematopoiesis, hematopoietic stem cells (HSCs) reside in specialized niches of the bone marrow (BM). Nevertheless, during hematological disorders or infections, HSC function in the BM is diminished, and there is a need to create new sites of hematopoiesis. This compensatory mechanism is known as extramedullary hematopoiesis (EMH). Remarkably, chronic inflammation promotes EMH, but the impact of chronic inflammation on HSCs outside the BM is poorly understood. Here, using mice suffering from a progressive chronic autoinflammatory disease described as chronic multifocal osteomyelitis (CMO), we observed increased numbers of functional HSCs in blood, spleen, and inflamed paws of CMO mice compared to WT controls. Single cell transcriptomics revealed that HSCs in CMO EMH sites have a unique expression profile, characterized by upregulated Cd53 that distinguishes HSCs from BM HSCs in WT or CMO mice. This upregulation correlates positively with MHCII-associated and immunosuppressive genes such as Cd274 (PD-L1) and Icosl. We observed that CD53+ HSCs isolated from CMO EMH sites displayed lower proliferation but enhanced myeloid colony-forming capacity in comparison to CD53- HSCs. Further, CD53+Lin-c-kit+ cells isolated from CMO EMH sites strongly induced proliferation and survival of T cells in comparison to CD53-Lin-c-kit+ cells. Additionally, CD53+Lin-c-kit+ also promoted the development of Tregs *in vitro*. Altogether, our findings revealed a unique type of EMH HSCs characterized by enhanced expression of CD53, MHCII, and immunosuppressive genes. Notably, under chronic inflammation, EMH CD53+ HSCs might act as antigen presenting cells inducing naive T cell proliferation and the development of Tregs, suggesting an immunoregulatory role at the periphery. This work was supported by GACR24-10938S and IMG institutional funding RVO68378050.

Structural and functional study of histone deacetylase 11

Zora Nováková[1], Pavla Bartošová-Sojková[2], Julia Kudláčová[1], Zsófia Kutil[1], Lucia Motlová[1], Cyril Bařinka[1]

[1]Institute of Biotechnology of the Czech Academy of Sciences, Vestec, Czech Republic.
[2]Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice, Czech Republic

Histone deacetylase 11 (HDAC11) is the youngest member of the HDAC family implicated in cell proliferation, apoptosis and differentiation. HDAC11 represents a biomarker of various cancers and thus can serve as a putative target for therapy of cancer and immune-related diseases. However, its physiological roles are not fully understood. Furthermore, our current knowledge of its 3D structure is mainly based on predictive models, as no experimental structural data have been published to date. This information gap hinders progress in the development of new tools that would specifically target the enzyme. To gain deeper insights into the function and structure of the HDAC11, we expanded our study by including a set of HDAC11 orthologs from various species. Data mining and bioinformatics allowed us to classify HDAC11 orthologs into two clades and to identify sequence motifs critical for deacetylase activity and substrate specificity as determined by biochemical assays. In addition, X-ray crystallography of an ancestral HDAC11 ortholog provided valuable structural insights into structural features governing substrate recognition by HDAC11. The results of this study enhance our understanding of HDAC11 functionality, and the available structural data provide basis for the rational design of inhibitors specifically tailored to different HDAC11 isoforms and species.

Unveiling the AGR2-NPM3 axis in PD-L1 regulation in Colorectal Cancer

Tereza Sosolikova[1], Andrea Martisova[1], Jakub Faktor[2], Iveta Klemesova[1], Tamara Kolarova[1], Jitka Holcakova[1], Roman Hrstka[1]

[1]Masaryk Memorial Cancer Institute, Brno, Czech Republic.
[2]International Centre for Cancer Vaccine Science, University of Gdansk, Gdansk, Poland

Protein Anterior Gradient 2 (AGR2) is known to be upregulated during carcinogenesis, and its increased levels are linked to clinical outcomes, prognosis, and chemoresistance. In this study, we conducted a comprehensive proteomic analysis using the SWATH-MS approach on a colorectal cancer (CRC) cell line model—SW480 and SW620 cells—with manipulated AGR2 levels. Through this, we identified

Nucleosplasmin (NPM3) as a protein co-expressed with AGR2. We validated AGR2's role in regulating NPM3 at both the mRNA (RT-qPCR) and protein (Western blot) levels. NPM3 interacts with Nucleophosmin (NPM1), a key regulator of various cellular processes and protein expression. Given that NPM3 has been shown to regulate Programmed death-ligand 1 (PD-L1), we explored the effect of AGR2 on PD-L1 expression (using RT-qPCR and flow cytometry) in alignment with CRC patient data from the cBioPortal database. Our findings shed light on the role of AGR2 in PD-L1 regulation via the AGR2-NPM3 signaling pathway, contributing to immune evasion in CRC cells.

This work was supported by MH CZ - DRO (MMCI, 00209805) and by the project SALVAGE (OP JAC; reg. no. CZ.02.01.01/00/22_008/0004644) – funded by the European Union and by the State Budget of the Czech Republic.

Isolation of Extracellular Vesicles: Comparison of Five Different Isolation Methods

Klára Mrázová, Roman Hrstka

Masaryk Memorial Cancer Institute, Brno, Czech Republic

Introduction: Extracellular vesicles (EVs) are a heterogenous group of vesicles enclosed by a lipid bilayer varying in size, biogenesis, composition, and function. Exosomes (30– 150 nm) as a subgroup of EVs are a promising candidate for liquid biopsies. Their cargo is rich in various molecules, some of which may be potential biomarkers for many diseases, including cancer. As their presence has been proven in body fluids such as blood, urine, saliva, or cerebrospinal fluid, the main focus of EV researchers is to improve methods of isolation and subsequently explore the contents of their cargo with the aim of finding a link between the cargo composition and cancerous state.

Methods: EVs were isolated from conditioned cell culture media by five different approaches: ultracentrifugation, precipitation (Total Exosome Isolation (from cell culture media)), tangential flow filtration (TFF-easy cartridge), and affinity principle (exoEasy Maxi Kit, MagCapture™ Exosome Isolation Kit PS Ver. 2). Samples of isolated EVs were further analysed by mass spectrometry. Obtained datasets were compared and examined using three different bioinformatic tools: DAVID Knowledgebase (v2023q4), Gene Ontology knowledgebase version 2024-06-17, FunRich Functional Enrichment Analysis tool version 3.1.4.

Results: MS analysis revealed ultracentrifugation as the most efficient method, as this technique provided the highest number of identified proteins. Proteins commonly identified in all datasets included proteins usually found in EVs. Analysis by different databases showed proteins to be specific for exosomes, engaged in protein binding processes, cell adhesion, angiogenesis, and others.

Conclusion: Comparison of different approaches to EV isolation showed the importance of ultracentrifugation and thus proved its position as a gold standard among isolation methods. Since ultracentrifugation has significant drawbacks, there is an urgent need for establishing a standard method for EV isolation. Apart from requirements on speed, purity, and yield, an ideal isolation technique should be applicable in clinical practice.

Synthesis and characterization of silica nanoparticles functionalized with titanocene derivatives

Petr Voňka[1], Diana Díaz-García[2], Tamara Kolářová[1], Romana Kubicová[1], Roman Hrstka[1], Santiago Gómez-Ruiz[2]

[1]Research Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Žlutý kopec 7, 656 53 Brno, Czech Republic.

[2]COMET-NANO Group, Departamento de Biología y Geología, Física y Química Inorgánica, ESCET, Universidad Rey Juan Carlos, Calle Tulipán s/n, 28933 Móstoles, Comunidad de Madrid, Spain

Introduction: Titanocene dichloride is a well-known organometallic compound that has been tested in Phase II clinical trials in patients with metastatic breast carcinoma and advanced renal cell carcinoma. However, its activity was not very good due to the low solubility and stability of the compound in physiological medium. Therefore, the incorporation of this complex in nanoparticles could be an alternative to improve its pharmacokinetics. In this work, the design of systems based on mesoporous silica nanoparticles (MSNs) was carried out, as well as biological assays in order to compare the biological activity with the isolated complex and the influence of the chlorine atoms. Materials & Methods: MSNs were synthesized by the sol-gel method when tetraethyl orthosilicate was used as silica precursor and hexadecyltrimethylammonium bromide was applied as surfactant. Titanocene derivatives were covalently incorporated on the surface of MSNs through grafting reactions trough mercapto ligand. Synthesized MSNs were characterized by analytical techniques such as FTIR spectroscopy (analysis of chemical bonds), TEM analysis (morphology of MSNs), BET analysis (textural properties), and ICP-MS analysis (content of functionalized metal). The biological effects of synthesized MSNs were tested on MDA-MB-231 and MDA-MB-468 triple-negative breast cancer cell lines by metabolic assay, CM-H2DCFDA assay, immunochemical analysis, and cell cycle analysis. Results: Treatment of MSNs functionalized with titanocene derivatives caused a decrease in the viability, mainly in MDA MB 468 cells. Further, the application of synthesized MSNs introduced ROS production with influenced cell cycle progression manifested by

significant increase of the sub-G1 population after treatment with functionalized MSNs. Subsequent analysis of proteins involved in key signalling pathways revealed increased level of LC3B, the protein associated with the induction of autophagy. Conclusion: MSNs were synthesized to determine how the appearance, or absence, of the chlorine atom influences the activity of titanocene derivatives and to compare the biological activity with respect to the isolated complex. The biological experiments showed that the chlorine atom is not necessary for titanocene dichloride activity, but its occurrence contributes to it. This information could be applied in future research focused on the development of novel and more efficient derivatives of titanocene dichloride supported on nanomaterials. Acknowledgements: This project was supported by the project National Institute for Cancer Research (Programme EXCELES, ID Project No. LX22NPO5102) - Funded by the European Union - Next Generation EU, by Ministry of Health, Czech Republic - conceptual development of research organization (MMCI, 00209805), and by the project BBMRI.CZ (no. LM2023033).

5-Azacytidine specific in myeloma cells promotes binding of DNMT3B to a protein complex containing of repressive histone markers

Kateřina Smešný Trtková[1,2,3], Michaela Němcová[1], Lenka Slavíčková[1], Jiří Minařík[4,5]

[1]Department of Clinical and Molecular Pathology, Faculty of Medicine and Dentistry, University Palacky Olomouc, Olomouc, Czech Republic.
[2]Department of Clinical and Molecular Pathology and Medical Genetics, Faculty of Medicine, University of Ostrava, Ostrava, Czech Republic.
[3]Life & Environment Research Center Ostrava, Faculty Hospital Ostrava, Ostrava, Czech Republic.
[4]Department of Hemato-oncology, Faculty of Medicine and Dentistry, University Palacky Olomouc, Olomouc, Czech Republic.
[5]Department of Hemato-oncology, University Hospital Olomouc, Olomouc, Czech Republic

Introduction: DNA methylation is an intensively studied posttranscriptionl epigenetic modification in which DNA methyltransferases (DNMTs) - DNMT1, DNMT3A, and DNMT3B - play a key role. DNMTs are enzymes that catalyse DNA methylation (CpG islands), thereby participating in human developmental anomalies and accompanying the arise and progression of cancer. In contrast, posttranslational modifications of histones effect the occurrence of DNMTs in protein complexes binding to specific histone sites. For example, H3K4, H3K36 are histone markers of active gene transcription, while H3K9, H3K27, H4K20 are histone markers of inactive chromatin and transcriptional repression of the respective genes.

DNMTs share a common PWWP domain through which they recognize the histone site H3K36me2 or H3K36me3. According to recent publications, H3K36me2 activates DNMT3A and affects the methylation of DNA segments located between the coding regions of the gene, while H3K36me3 functions as a repressive histone marker and mediates methylation in the coding regions of the gene through DNMT3B. The aim of our study was to determine whether our induced demethylation could influence the binding of protein complexes containing the repressive histone markers H3K9me3 and/or H3K36me3 with the methylation enzymes DNMT3A and DNMT3B.

Material & Methods: Chromatin protein lysate was isolated from three myeloma cell lines – KMS12-PE, OPM2 and U266B1 after their treatment with demethylation agents: 5-Aza-2´-deoxycytidine (5-Aza-dC; DAC) and/or 5-Azacitidine (AZA) and was subsequently precipitated with antibodies against H3K9me3 and/or H3K36me3. The binding between DNMT3A and/or DNMT3B, and protein complexes containing markers inactivating transcriptional activity H3K9me3 and/or H3K36me3 was quantified by qPCR (SyberGreen) with primers specific for both the promoter regions or the coding regions of the studied DNMT genes.

Results: In myeloma cells of the KMS12-PE line, we determined a significantly increased occurrence of the H3K9me3 protein complex binding to the DNMT3B promoter region at $p < 0.05$ (p-value 0.027) after 0.5 μmol/L AZA treatment, and at $p < 0.01$ (p-value 0.003) in cells treated with 0.2 μmol/L AZA compared with the normalized values of the respective IgG. Further, the 0.2 μmol/L AZA caused a statistically significant increase of DNMT3B in protein complex with H3K9me3 in KMS12-PE t at $p < 0.05$ (p-value 0.016) compared to the normalized value of IgG.

Conclusion: 5-Azacitidine, compared to 5-Aza-2´-deoxycitidine, significantly affects the binding of DNMT3B to a protein complex containing the repressive histone markers H3K9me3 and/or H3K36me3 with the potential to inactivate gene transcriptional activity.

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Regulation of microtubule nucleation in glioblastoma cells by ARF GTPase-activating protein GIT2 and protein kinase C

Vadym Sulimenko[1], Eduarda Dráberová[1], Vladimíra Sládková[1], Taťána Sulimenko[1], Věra Vosecká[1], Omar Skalli[2], Pavel Dráber[1]

[1]Department of Biology of Cytoskeleton, Institute of Molecular Genetics, Czech Academy of Sciences, Prague, Czech Republic.
[2]Department of Biological Sciences, University of Memphis, Memphis, USA

Glioblastoma multiforme (GBM) is the most malignant form of glioma in adults, for which no effective treatment currently exists. It is well established that microtubules (MTs) play a critical role in cell division and migration, influencing the viability and invasiveness of malignant tumors. Previously, we demonstrated that ARF GTPase-activating protein GIT2 forms complexes with γ-TuRC proteins, key regulators of MT nucleation, and associates with centrosomes. Depletion of GIT2 promoted centrosomal MT nucleation and inhibited cell migration in human U-251 MG glioblastoma cells. Here, we report results from phenotypic rescue experiments showing that ArfGAP domain of GIT2, which is targeted to centrosomes, plays a key role in the regulation of MT nucleation. Interestingly, MT nucleation was also promoted when cells were pre-treated with phorbol 12-myristate 13-acetate (PMA), which activates conventional and novel protein kinase C (PKC) isoforms. This effect was suppressed in the presence of PKC inhibitors that preferentially target conventional PKCs. Furthermore, we revealed that PKCs phosphorylate endogenous and exogenous GIT2, and identified a phosphorylation site for PKCα in the ArfGAP domain. The preparation of corresponding phosphomimetic and non-phosphorylatable mutations for phenotypic rescue experiments in cells with depleted levels of GIT2 confirmed the importance of GIT2 phosphorylation in MT nucleation.

Collectively, these results suggest that phosphorylation of GIT2 plays an important role in regulating MT organization and the physiology of glioblastoma cells.

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Exploring novel polycyclic hetero-aryl7-deazapurine nucleosides with potential therapeutic application

Marta Gargantilla[1], Enzo Vaiedelich[1], Michal Tichý[1], Michal Hocek[1,2]

[1]Institute of Organic Chemistry and Biochemistry of the CAS, Prague, Czech Republic.
[2]Department of Organic Chemistry, Faculty of Science, Charles University, Prague, Czech Republic

Modified purine nucleosides have been extensively studied during the last years due to the wide range of biological activities they present, especially anticancer and antiviral. In particular, our research group has focused on the research of base-modified 7-deazapurine derivatives, discovering nucleosides such as PNH173 and AB61, which showed potent and selective cytostatic activity and that are now in preclinical trials [1-3]. The presence of a 5-membered heteroaryl ring in position 7 of AB61 lead us to explore a new family of nucleoside analogues with that heteroayl ring fused directly fused to deazapurine and to study their biological activities.

To this aim, we developed two synthetic approaches to prepare the modified nucleobases. The first one is based on the substitution of chlorine in o-chloronitroarenes by the enolate present in ethyl-cyanoacetate, further reduction of the nitro group and subsequent cyclization to give the aminoaryl carboxylate. Then, the tricyclic nucleobase is obtained after cyclization with formamide. The second approach consisted in a Negishi coupling of zincated 4,6-dichloropyrimidine with the corresponding heteroaryl iodide or some arylsulfonium salt, followed by azidation and either thermal or photocyclization. Further glycosylation of those heteroaryl-fused nucleobases by Vorbruggen procedure afforded the key-intermediate benzoylated chloronucleosides. Finally, the target nucleosides were obtained by either aromatic nucleophilic substitution, Suzuki coupling, Stille coupling or Pd-catalysed methylation and further deprotection, when required [4-8].

The cytostatic activity of the synthetized nucleosides was evaluated in order to stablish some structure-activity relationships.

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Novel 2,6-disubstituted 7-deazapurine ribonucleosides: synthesis and biological activities

Ugnė Šinkevičiūtė[1,2], *Michal Tichy*[1], *Marcela Dvorakova*[1,2], *Michal Hocek*[1,2]

[1]*Institute of Organic Chemistry and Biochemistry of the CAS, Prague, Czech Republic.*
[2]*Charles University, Prague, Czech Republic*

The discovery of adenosine receptor modulation activity of 7-deazapurine nucleosides, together with the fact that many 2,6-disubstituted purine nucleosides are A3 AR agonists, inspired the design of a new class of hitherto unknown 2,6-disubstituted 7-deazapurine ribonucleosides as potential A3 AR agonists.1-3 Besides various asymmetrical 2,6-diaryl nucleosides we also aimed at 2-arylethynyl-6-aryl nucleosides. The target nucleosides were synthesized in 7 steps starting from simple D-ribose, 4-chloro-7H-pyrrolo[2,3-d]pyrimidin-2-amine, different boronic acids and/or acetylene derivatives by a sequence involving protection of the sugar, glycosylation, deamination, Suzuki and Sonogashira coupling reactions. Several 7-deazapurine nucleosides bearing different size aryl/heteroaryl substituents at position 2 and 6 were synthesized and tested for cytostatic and antiviral activities together with ability to act as an AR agonist. Synthetic details and results of these studies will be presented on the poster.

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Deciphering the relationship between cytidine metabolic pathways and cytarabine treatment of haematological tumours

Martina Horejšová[1], *Barbora Pisklákova*[1], *Aleš Kvasnička*[1], *Jakub Rozhon*[1], *Anna Ligasová*[2], *Karel Koberna*[2], *David Friedecký*[1,3]

[1]*Laboratory of Inherited Metabolic Disorders, Faculty of Medicine and Dentistry, Palacký University Olomouc, Olomouc, Czech Republic.*
[2]*Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacký University Olomouc, Olomouc, Czech Republic.*
[3]*Department of Clinical Biochemistry, University Hospital Olomouc, Olomouc, Czech Republic*

Introduction: Haematological malignancies are characterized as cancers of the blood comprising of acute and chronic myeloproliferative or lymphoproliferative diseases[1]. One possible therapy for haematological malignancies is cytarabine (ara-C), a nucleoside analogue of the naturally occurring cytidine. Ara-C is transported into tumour cells by specialised nucleoside transporters, where it undergoes a three-step phosphorylation to active ara-C triphosphate, which is subsequently incorporated into DNA to block DNA synthesis[2]. Conversely, deamination of 5-ethynyl-2‘-deoxycytidine (EdC) to its uridine counterpart is desirable in anticancer therapy due to its high toxicity to cell growth[3].

Objectives: This work is aimed at describing the metabolic pathway leading to the conversion of ara-C to the inactive product uracilarabinoside (ara-U) by cytidine deaminase (CDA).

Methods: Tumor cell lines, diploid cell lines, immortalized diploid cell lines, and PDX models of mouse tumor and plasma samples were used to determine CDA activity. Cell lysates and plasma were incubated with ara-C, EdC, cytidine (Cr), 5-bromo-2‘-deoxycytidine (BrdC), 5-chloro-2‘-deoxycytidine (CldC), 5-iodo-2‘-deoxycytidine (IdC) and 5-fluorocytidine (FC) under different conditions to monitor the conversion to their uridine products. Analytes were extracted using methanol and analyzed by HILIC-MS/MS. Analysis was performed using Exion LC (Sciex) and Luna NH_2 column (3 µm, 100 mm x 2 mm, Phenomenex) in conjunction with a QTRAP 6500+ mass spectrometer (Sciex) in scheduled MRM mode4. The results obtained were processed and the CDA activity was calculated from the substrate loss or product addition.

Results: High CDA activity was observed within the tumor cell lines after ara-C treatment, while low to zero enzyme activity was observed in the remaining cases. Different substrate specificities of CDA for ara-C, EdC, Cr, BrdC, CldC, IdC and FC substrates were also observed. Deamination of ara-C, used for its antineoplastic activity in chemotherapeutic treatment, may lead to reduced therapeutic effects[5].

Conclusion: This study focused on the deamination of nucleoside analogues by the CDA enzyme has potential in the context of their use in the treatment of haematological malignancies and in personalised medicine[6].

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Discovery of a Stattic-derived compound K2071 with STAT3 inhibitory, antimitotic, and senotherapeutic properties

David Rysanek[1], Patrik Oleksak[2], Marketa Vancurova[1], Pavla Vasicova[1], Alexandra Urbancokova[1], Josef Novak[1], Dominika Maurencova[1], Pavel Kashmel[1], Jana Houserova[1], Romana Mikyskova[3], Ondrej Novotny[3], Milan Reinis[3], Pavel Juda[4], Miroslav Hons[4], Jirina Kroupova[1], David Sedlak[5], Tetyana Sulimenko[6], Pavel Draber[6], Marketa Chlubnova[2], Eugenie Nepovimova[2], Kamil Kuca[2], Miroslav Lisa[2], Rudolf Andrys[2], Tereza Kobrlova[7], Ondrej Soukup[7], Jiri Janousek[7], Lukas Prchal[7], Jiri Bartek[1,8,9], Kamil Musilek[2], Zdenek Hodny[1]

[1]Laboratory of Genome Integrity, Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic.
[2]Faculty of Science, Department of Chemistry, University of Hradec Kralove, Hradec Kralove, Czech Republic.
[3]Laboratory of Immunological and Tumour Models, Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic.
[4]BIOCEV, First Faculty of Medicine, Charles University,, Prague, Czech Republic.
[5]CZ-OPENSCREEN, Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic.
[6]Laboratory of Biology of Cytoskeleton, Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic.
[7]Biomedical Research Centre, University Hospital Hradec Kralove, Hradec Kralove, Czech Republic.
[8]Danish Cancer Institute, Copenhagen, Denmark.
[9]Division of Genome Biology, Department of Medical Biochemistry and Biophysics, Science for Life Laboratory, Karolinska Institutet, Stockholm, Sweden

6-Nitrobenzo[b]thiophene 1,1-dioxide (Stattic) is a potent signal transducer and activator of the transcription 3 (STAT3) inhibitor developed originally for anticancer therapy. However, Stattic harbors several STAT3 inhibition-independent biological effects. To improve the properties of Stattic, we prepared a series of analogues derived from 6-aminobenzo[b]thiophene 1,1-dioxide, a compound directly obtained from the reduction of Stattic, that includes a methoxybenzylamino derivative (K2071) with optimized physicochemical characteristics, including the ability to cross the blood–brain barrier. Besides inhibiting the interleukin-6-stimulated activity of STAT3 mediated by tyrosine 705 phosphorylation, K2071 also showed cytotoxicity against a set of human glioblastoma-derived cell lines. In contrast to the core compound, a part of K2071 cytotoxicity reflected a STAT3 inhibition-independent block of mitotic progression in the prophase, affecting mitotic spindle formation, indicating that K2071 also acts as a mitotic poison. Compared to Stattic, K2071 was significantly less thiol-reactive. In addition, K2071 affected cell migration, suppressed cell proliferation in tumor spheroids, exerted cytotoxicity for glioblastoma temozolomide-induced senescent cells, and inhibited the secretion of the proinflammatory cytokine monocyte chemoattractant protein 1 (MCP-1) in senescent cells. Importantly, K2071 was well tolerated in mice, lacking manifestations of acute toxicity. The structure–activity relationship analysis of the K2071 molecule revealed the necessity of the para-substituted methoxyphenyl motif for antimitotic but not overall cytotoxic activity of its derivatives. Altogether, these results indicate that compound K2071 is a novel Stattic-derived STAT3 inhibitor and a mitotic poison with anticancer and senotherapeutic properties that is effective on glioblastoma cells and may be further developed as an agent for glioblastoma therapy.

Introduction of the digital MLPA method in adult patients with acute B-precursor lymphoblastic leukemia

Zuzana Vrzalova[1,2], Hana Halamova[3], Jiri Stika[1], Stepan Hrabovsky[3], Vaclava Polivkova[4], Katerina Machova Polakova[4], Sarka Pospisilova[1,3], Cyril Salek[4], Michael Doubek[3,1]

[1]Masaryk University, Brno, Czech Republic.
[2]University Hospital Brno, Brno, Czech Republic.
[3]University Hospital Brno, Brno, Czech Republic.
[4]Institute of Hematology and Blood Transfusion, Praha, Czech Republic

Background:

The present work by the Czech Leukemia Study Group for Life is focused on an analysis of B-cell

precursor acute lymphoblastic leukemia (BCP-ALL) patients that were enrolled in either Blina-CELL or Pona-CELL study or were treated with the standard ALL CELL 2012 Junior protocol (modified GMALL

07/2003). The analysis was performed with digital MLPA (Multiplex Ligation-dependent Probe Amplification), which was designed to detect copy number alterations (CNAs) of DNA segments by next-generation sequencing (NGS). This method can identify whole chromosome losses/gains, whole gene deletions/duplications, intrachromosomal amplifications of chromosome 21, and intragenic

deletions /duplications, which are associated with BCP-ALL.

Aims:

The study aims to identify CNAs using digital MLPA in 80 adult patients with BCP-ALL from the Czech Republic. Further, the results obtained with digital MLPA were then compared with the classical MLPA analysis (P335 kit) as well as with the results of cytogenetic examination.

Methods:

Digital MLPA analysis was performed using MLPA Probemix D007-A1 Acute Lymphoblastic Leukemia kit

(MRC Holland, D007-A1) kit and Coffalyser software (MRC Holland). According to the profile, patients were divided into 3 groups: IKZF1plus, IKZF1del, and others (aberrations excluding IKZF1 and negative results). Kaplan-Meiers survival curves were used for statistical evaluations.

Results:

CNAs using digital MLPA were found in 79% of patients. The most frequent aberration (47.5%) was a

deletion of the IKZF1 gene on chromosome 7, which is a negative prognostic factor in ALL. The second

most frequent alteration (42.5%) was a deletion of the CDKN2A gene, which is predictive of an increased relapse risk and was often accompanied with deletions of the MTAP and MLLT3 genes on chromosome 9. PAX5 gene deletions were detected in 20% and duplications of the PAR1 region in 16.3% of cases. Compared to the literature, a higher frequency of the CD200/BTLA deletions on chromosome 3 (13.8%) and partial or complete deletions of the ETV6 gene on chromosome 12 (11.4%) were observed. Low hypodiploidy due to the losses of chromosomes 8, 10, 14, 18, 21 and X/Y and complex karyotypes were found in several patients and were confirmed with cytogenetic methods. The IKZF1plus profile associated with a very poor prognosis was identified in 33% of patients. Concurrent deletions of the CDKN2A/B and PAX5 genes (18%) or CDKN2A/B genes (12%) were the most frequent alterations accompanying the IKZF1 gene deletion. Co-occurrence of the IKZF1 and PAX5 gene deletions or the IKZF1 and PAR1 region deletions were only sporadically detected.

Conclusion:

In summary, digital MLPA allowed detection of a greater number of targets with higher specificity and

sensitivity. Detection of intragenic aberrations increased by 20% and large CNAs could be detected

reliably even in subclonal populations. Thus, digital MLPA is a suitable method for CNA detection,

IKZF1plus profiling and prediction of disease prognosis. It also complements molecular genetic testing and may play an important role in cases where cytogenetic testing is not possible.

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Targeting myeloid cells and modulation of their function by fully synthetic antibody mimetics

Dominik Musil[1,2], Kristýna Blažková[3], Tereza Ormsby[1], Martin Hadzima[1], Libor Kostka[4], Tomáš Etrych[4], Vladimír Šubr[4], Pavel Šácha[1], Jan Konvalinka[1]

[1]IOCB Prague, Prague, Czech Republic.
[2]Department of Genetics and Microbiology, Faculty of Science, Charles University, Czech Republic, Prague, Czech Republic.
[3]Stanford University, Stanford, USA.
[4]Institute of Macromolecular Chemistry of the Czech Academy of Sciences, Prague, Czech Republic

In certain circumstances, monocytes, especially macrophages, significantly contribute to the onset of autoimmune diseases and tumor progression. As a result, numerous therapeutic strategies are currently being investigated to target and modulate the activity of these myeloid cells. One of the promising areas of investigation is a specific targeting of the high affinity IgG FcγRI receptor, CD64, primarily expressed on monocytes and macrophages.

Recently, we have developed synthetic antibody mimetics called iBodies targeting CD64. These N (2 hydroxypropyl)methacrylamide (HPMA) copolymers are stable, non-immunogenic, highly versatile polymer-drug conjugates which allows conjugation of various functional moieties and targeting ligands, fine-tuning of their pharmacokinetics and pharmacodynamics. Additionally, iBodies can passively target tumor tissues through the enhanced permeability and retention effect.

Anti-CD64 iBodies exhibit a significant improvement in binding potency compared to an anti-CD64 ligand, showing neither overall off-target specificity nor related cytotoxicity *in vitro*. Using confocal microscopy and flow cytometry-based approaches, we confirmed a specific binding and internalization into human monocytes and monocytes-derived macrophages. The anti CD64 cytotoxic iBodies, simultaneously decorated with a specific ligand targeting CD64 and a cytotoxic moiety connected to the HPMA copolymer by a cleavable linker, showed selective elimination of myeloid CD64-expressing cells *in vitro*, including so called pro-tumorigenic M2 macrophages, even with subnanomolar effectiveness in apoptosis-dependent mechanism.

In conclusion, anti-CD64 iBodies represent a novel therapeutical strategy urgently needed for specific drug development and delivery in cancer immunotherapy.

PPM1D activity promotes cellular transformation by preventing senescence and cell death

Miroslav Stoyanov[1], Andra Martinikova[1], Katerina Matejkova[2], Zuzana Zemanova[2], Petra Kleiblova[2], Zdenek Kleibl[2], Libor Macurek[1]

[1]Institute of Molecular Genetics, Prague, Czech Republic.
[2]First Faculty of Medicine, Charles University, Prague, Czech Republic

Cell cycle checkpoints, oncogene-induced senescence and programmed cell death represent intrinsic barriers to tumorigenesis. Protein phosphatase magnesium-dependent 1 (PPM1D) is a negative regulator of the tumour suppressor p53 and has been implicated in termination of the DNA damage response. Here, we addressed the consequences of increased PPM1D activity resulting from the gain-of-function truncating mutations in exon 6 of the PPM1D. We show that while control cells permanently exit the cell cycle and reside in senescence in the presence of DNA damage caused by ionising radiation or replication stress induced by the active RAS oncogene, RPE1-hTERT and BJ-hTERT cells carrying the truncated PPM1D continue proliferation in the presence of DNA damage, form micronuclei and accumulate genomic rearrangements revealed by karyotyping. Further, we show that increased PPM1D activity promotes cell growth in the soft agar and formation of tumours in xenograft models. Finally, expression profiling of the transformed clones revealed dysregulation of several oncogenic and tumour suppressor pathways. Our data support the oncogenic potential of PPM1D in the context of exposure to ionising radiation and oncogene-induced replication stress.

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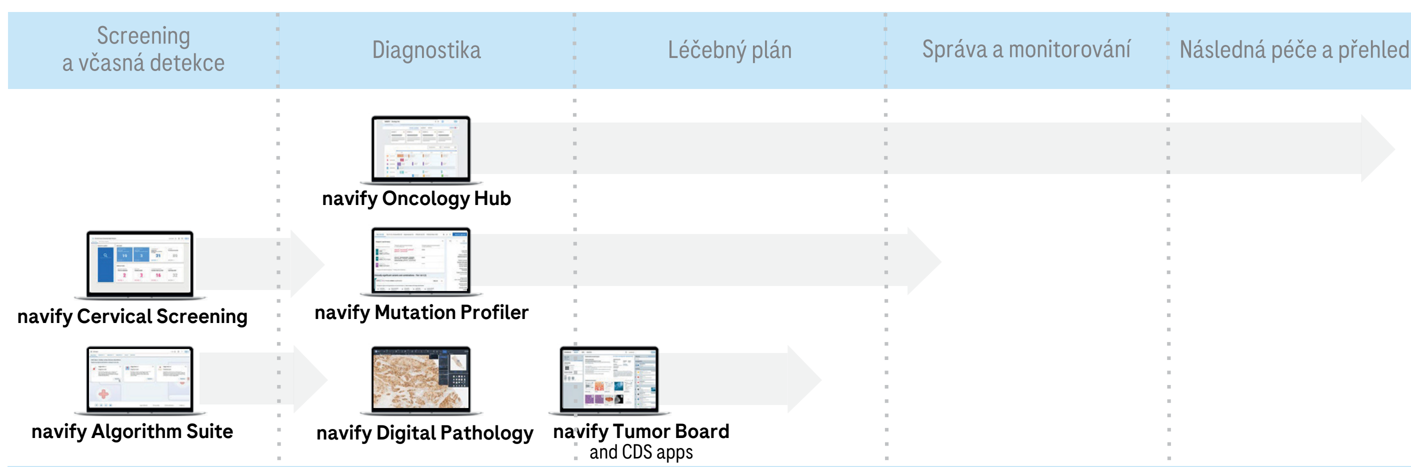

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Roche s.r.o., Diagnostics Division
Na Valentince 3336/4, 150 00 Praha 5 – Smíchov
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