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**Structure and Function
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Abstracts of COMBIOM Final Scientific Meeting

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Aminoacyl-tRNA Synthetases in Cancer Diagnostics

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Aminoacyl-tRNA synthetases (aaRSs) are ancient ubiquitous “house-keeping” enzymes that are responsible for cellular protein synthesis and cell viability. Nontranslational functions of vertebrate aaRSs have also recently been discovered. Taken together, new findings suggest that aaRSs have critical mechanistic roles in a variety of cellular processes which are relevant for disease development and pathology, and these roles may be used as one possible avenue for improvement of diagnostics and open a new dimension for cancer therapy. To uncover the biomarkers related to tumorigenesis and behavior of tumor we have studied the differently expressed genes of four or six aaRSs in tissues of colon and kidney cancers by the quantitative polymerase chain reaction (Q-PCR) method. In the case of kidney cancer from 18 samples of tumor tissue, an increased expression (more than 2 fold) of seryl-tRNA synthetase (*SARS*) was observed in 10, and only two were noted for its slight decrease. In the case of human colon cancer we have observed the expression profile for leucyl-tRNA synthetase (*LARS*), histidyl-tRNA synthetase (*HARS*), *SARS* and lysyl-tRNA synthetase (*KARS*) in 16 primary cancer samples. We have found that the genes of *LARS*, *SARS* and *KARS* underwent the most changes. The *LARS* and *SARS* genes expression decreased significantly (from 2 to 60 fold) in 38% of samples (6 of 16) in comparison with a surrounding normal tissue according to the Q-PCR data. The *KARS* expression was increased significantly (from 2 to 3000 fold) in 50% (8 from 16) and was decreased in 25% of the cancer samples. These genes might be used for diagnosis of colon tumors.

COMBIOM in Research of Translation Elongation Factors

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During the COMBIOM action a number of scientific visits to Warsaw have been conducted. The scientific part of the project has been developed in several directions. To study hydrodynamic and thermodynamic properties of the purified

translation elongation factors in solution we used a method of analytical ultracentrifugation. The aggregation states, sedimentation coefficients and approximate molecular masses of translation elongation factors eEF1B α , eEF1B β and eEF1B γ were determined by the sedimentation velocity approach. The data were supported by the sedimentation equilibrium analysis, which does not depend on the shape of a protein. The same approach was applied to characterize the binary eEF1B $\alpha\gamma$ and ternary eEF1B $\alpha\beta\gamma$ complexes. The manuscript describing the properties of the elongation factor eEF1B α is submitted to an international journal. Both the purified translation elongation factor eEF1B α and eEF1B $\alpha\gamma$ complex were subjected for crystallization trials. Several screens with different crystallization conditions were performed. A crystal of the eEF1B β mutant has been obtained. Intracellular localization of the components of the eEF1B complex was examined by immunofluorescent microscopy. The data on eEF1B γ localization in lung cancer cells were included into the paper published in BMC Cancer journal. An attempt to identify protein partners of the mutant eEF1A protein in which the specifically methylated lysine residues were substituted with arginine ones, by means of mass-spectrometry was undertaken. Obtaining a crystal of the eEF1B β mutant under specifically elaborated conditions suggests a need for more financing to further pursue these studies. During the COMBIOM action the collaboration with Institute of Biochemistry and Biophysics PAN has been also developed. A research on the protein partners of the elongation factor eEF1B complex in the cancer cell line A549 has been performed. The results provide a ground for further research of the eEF1B interactome. We plan to get financial support for continuation of this research via EU and NATO research financing structures.

Colocalization of Mtor Kinase with Cytoskeleton – Implication to Regulation of Cytoskeleton Dynamics

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It is well known that the PI3K/mTOR/S6K signaling pathway is a main in integration of inputs coming from at least five major intracellular and extracellular cues: growth factors, stress, energy status, oxygen, and amino acids – to control major processes, including protein and lipid synthesis and autophagy. During last years the research efforts in the department of cell signaling, Institute of molecular biology and genetics NAS of Ukraine were focused on the structural

and functional organization of the mTOR/S6K-dependent signaling pathway. Recently we have identified a number of mTOR kinase splice variants and for one of them (mTOR β) the oncogenic properties have been demonstrated on cellular and animal models. In addition applying immunocytochemical analysis of different cell types with a set of anti-mTOR antibodies generated in the department we have found colocalization of mTOR with different elements of cytoskeleton that suggested the involvement of mTOR in the regulation of cytoskeleton dynamics. In the frame of COMBIOM project we have established collaboration with the Laboratory of Molecular and Cellular Neurobiology headed by Dr. Jacek Jaworski that focused on the mTOR functions, mainly on the role of mTOR in the control of proper neuronal morphology in health and disease in part by regulation of cytoskeleton rearrangements. During visits to prof. Jaworski's laboratory, the researchers from the department of cell signaling received training in immunocytochemical analysis of different cell types using confocal microscopy, neuronal cell culture and application of shRNA. The application of these approaches helped to evaluate the data of mTOR colocalization with cytoskeleton and to perform quantitative analysis. According to the data received about 30% of mTOR is colocalized with cytokeratins. Colocalization of mTOR with cytokeratins was further confirmed by immunoprecipitation. Additionally it was found that efficacy of colocalization depended on phosphorylation (activity) status of mTOR. Exchange of research material (monoclonal antibodies specific to mTOR, shRNA constructs, cDNA clones of mTOR isoforms) provided a basis for further collaboration, that was discussed, with focus on the functions of novel mTOR isoforms in cancer and neuronal cells and place of mTOR in the regulation of cytoskeleton dynamics during dendritic tree formation and cancer cells invasion.

The Development of Pseudosubstrates for MGMT Enzyme as Possible Drugs to Improve Chemotherapy Efficacy

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In frame of the COMBIOM project, the IMBG young scientist, Dr. Anna Iatsyshyna, had two research trainings in the International Institute of Molecular and Cell Biology (IIMCB), the Laboratory of Structural Biology headed by Prof. Matthias Bochtler. Prof. Lyubov Lukash (IMBG) had a visit to IIMCB for the

discussion of further directions of the scientific collaboration strengthening with Prof. Matthias Bochtler and with IIMCB Director Prof. Kuznicki Jacek. During mentioned visits several scientific seminars have been conducted in the host laboratory, where the main questions and tasks for future collaboration have been presented. Also, Prof. Bochtler had a visit to IMBG for a scientific discussion. During COMBIOM activity, 83 compounds of different chemical classes have been selected and synthesized after virtual screening experiments in the Department of Medicinal Chemistry (IMBG). These compounds can be potential MGMT inhibitors. So, we started the experiments (Department of Human Genetics, IMBG) to test an inhibitory activity of them using the fluorescent assay (the SNAP-Vista Green™ substrate, BioLabs NE) to label cell lysates. Next, we have done cloning experiments (Laboratory of Structural Biology, IIMCB) to get the rec-MGMT for the in vitro MGMT enzyme activity assay. The selected compounds may be therapeutically useful modulators of the MGMT activity. The general aim of our future investigations is to test the inhibitory activity and toxicity of these compounds. A collaborative work is planned to test the compounds, to continue this study and apply for a joint grant/s. We have prepared a draft of the Research collaboration agreement between IIMCB (Warsaw, Poland) and IMBG (Kyiv, Ukraine) to undertake joint research activities in frame of the project: “The development of the O(6)-methylguanine DNA methyltransferase inhibitors”. During the project activity we were also involved in other research collaborative topics, in particular, in pilot investigations to process approaches for the future studies on possible mechanisms of the *MGMT* gene re-activation via active promoter demethylation and to study ubiquitin ligases involved in ubiquitination and proteasomal degradation of the alkylated MGMT protein.

Multiple faces of endocytic scaffold ITSN: elucidation of its role in the nucleus

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Within the project new protein partners of scaffold ITSN and new connection of ITSN to cellular signalling cascades were expected to be identified. We have found that ITSN protein typically associated with plasma membrane and intracellular vesicles is transported to the nucleus. Previously, localization of other endocytic proteins was reported in the nucleus. Regulation of their nuclear import and nuclear function still is not clear. We have shown that accumulation of

ITSN in nucleus depends on the stage of cell cycle. A maximal accumulation of ITSN in nucleus was observed during G1, whereas in S and G2 the protein was not abundant in nucleus. Moreover, we showed that ITSN was not transported into nucleus during G0/G1 transition state. Overexpression of ITSN in nuclear compartment caused upregulation of early response genes, namely c-fos, c-myc, and egr1. We identified heterogeneous nuclear ribonucleoprotein K (HNRNPK) as an interacting partner of ITSN that predominantly is localized in nucleus. Despite the interaction between proteins, HNRNPK has no impact on the ITSN nuclear import. Using bioinformatics tools we identified AGFG1/Hrb protein as a putative transporter of ITSN into nucleus. AGFG1/Hrb undergoes nucleocytoplasmic shuttling and binds N-terminal part of ITSN. Previously, we demonstrated that N-terminal part of ITSN is responsible for the nuclear import of the protein. Next, we plan to investigate a role of AGFG1/Hrb in the nucleocytoplasmic shuttling of ITSN and nuclear-specific roles of ITSN. For this, an effect of the nuclear-targeted variant of ITSN on cell proliferation and survival will be assessed. Second, other putative target genes regulated by ITSN will be searched. Third, the nuclear-specific proteins partners will be identified.

Role of ITSN1 in Dendritic Development

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Intersectin 1 is an endocytic scaffold protein that is highly expressed in neurons and has a prominent role in synaptic development and transmission. Recently we have identified that intersectin 1 interacts with neuronal isoform of microtubule-associated protein STOP required for the enhancement of microtubule stability. According to current understanding, the microtubule stabilization is needed for the acquirement of proper neuron morphology. Therefore, we investigated a role of intersectin 1 in the development of dendritic tree in the cultivated primary hippocampal neurons of rat. To do this we established collaboration with the Laboratory of Molecular and Cellular Neurobiology headed by Dr. Jacek Jaworski (Warsaw). We used their expertise in the neuronal culture preparation and maintenance, shRNA design, confocal microscopy, morphometrical analysis of neurons. Firstly, we developed a construct encoding shRNA for the suppression of intersectin 1 expression in rat cells. Transfection of this construct to the rat hippocampal neurons led to moderate decrease of intersectin 1 content in cells. Then we used it to evaluate its effect on the dendritic tree formation in young neurons that were cultivated during one week before an experiment start.

We transfected the construct into neurons along with the GFP-encoding construct for visualization of neuronal morphology. An empty vector was used as a control. For quantitative expression of neuronal morphology alterations we measured total dendritic length and number of dendritic tips. Furthermore, we performed a Sholl analysis for the evaluation of complexity of dendritic tree architecture. We found that a number of dendrotic tips in shRNA-expressing cells did not significantly differ from the control. However, a total dendritic length was significantly decreased when the intersectin 1 expression was downregulated. Sholl analysis revealed that the Sholl curve for hRNA-expressing cells differed from the control one on the interval of curve decay. These results suggest that intersectin 1 is important for dendritic growth and does not have a significant impact on branching of dendrites. Intersectin 1 is a known regulator of actin cytoskeleton, therefore the effect of its downregulation on the dendritic tree formation can result from deregulation of either tubulin or actin cytoskeleton (or both). In order to evaluate the effect of intersectin 1 on the STOP-mediated microtubule stabilization, we overexpressed both these proteins in HeLa cells and checked their effect on microtubule stability in response to cold and nocodazole. For this research we established a collaboration with INSERM U829 Laboratory in Evry, France, headed by Dr. Patrick Curmi. This laboratory possesses a strong expertise in studying microtubule structure and dynamics. We found that ITSN1 overexpression did not alter the Warsaw STOP-mediated microtubule stability, indicating that interaction between intersectin 1 and STOP does not interfere with STOP association with microtubules.

Development of Effective Drug Combinations for Glioblastoma and Mantle Cell Lymphoma Treatment

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This research was undertaken with the aim to evaluate the anti-cancer action of complexes, which include chemotherapeutics used in clinics with newly

developed compounds, as well as with recombinant cytotoxic proteins. **Results.** To find out drug combinations that will enable the development of therapeutic regimens with improved effectiveness and less toxicity, we started collaboration with Dr. Yegor Vassetzky in frames of COMBIOM project. Bradykinin antagonists (BA) and thiazolidinone derivatives (TD) were screened for their cytotoxic effect on different glioma and mantle cell lymphoma (MCL) cells. Among all BA under investigation, BKM-570 appeared to be the most effective with IC_{50} 4 μ M and 3,3 μ M in rat glioma C6 and human glioblastoma U-251 cell lines, correspondingly. ERK1/2 and AKT1 phosphorylation was suppressed in U-251 cells after treatment with this compound, thus, growth-repression effect of BKM-570 could be mediated by the modulation of MAPK- and PI3K-signaling cascades. Temozolomide (TMZ), a first-line anti-gliomic drug used in clinics, has only temporary positive effect and severe side effects in GB patients. We showed that combination of 1 μ M BKM-570 with only 10 μ M temozolomide (TMZ), led to about 80% growth reduction of C6 and U-251 cells, compared to temozolomide used alone. Thus, BKM-570 significantly potentiates the TMZ cytotoxicity. Screening thiazolidinones revealed ID28 to be a potent suppressor of the C6 and U-251 cells growth (IC_{50} 4 μ M and 15 μ M, correspondingly). ID4523 demonstrated the highest activity in C6 cells with IC_{50} 0.13 μ M. The treatment of MCL cells by this compound and its chemical analogs showed also good results: IC_{50} values for ID 4526 (0,27 μ M) and ID 4527 (0,16 μ M) are even better than for doxorubicin, the conventional chemotherapeutic drug (0,37 μ M). It has been reported previously by us that glioma-associated protein CHI3L2 down-regulates mitogenesis and proliferation of glioma cells U-251. The use of recombinant proteins, which reduce the viability of tumor cells, as therapeutic agents together with chemotherapy and immunotherapy, is proposed to be a new approach for the complex cancer treatment. Here we reported that 100 ng/ml CHI3L2 protein inhibits cell viability of U-251 cells more effectively than TMZ in therapeutic concentrations. Furthermore, combining CHI3L2 and BKM-570 resulted in an additive cytotoxic effect in U-251 cells. To find out which molecular mechanisms could mediate the cytotoxic properties of CHI3L2, we analyzed the impact of CHI3L2 treatment on the expression of key components of cell cycle machinery, namely cyclin E and p53. While the cyclin E expression level remained unchanged, we observed a significant accumulation of p53 in the cells after addition of CHI3L2 to the cell culture medium. An increased p53 production could result in cell cycle abrogation and reduced cell viability, which we observed

after the CHI3L2 application. **Conclusions.** We showed a strong synergistic growth inhibiting effect after combination of TMZ with BKM-570. Substantial suppression of human and rat glioma, as well as MCL cells growth were obtained by TD treatment. Future collaboration with French partners will be focused on *in vivo* experiments for the evaluation of these compounds in the pre-clinical study. It was shown that CHI3L2 protein, which is overexpressed in human gliomas, inhibits glioma cells viability and enhances the cytotoxic properties of chemotherapeutic agents, thus, could be considered as a potential component for the complex chemotherapy. The reduced cell viability after CHI3L2 treatment could be associated with the accumulation of p53 protein, a key element of the cell cycle control and apoptosis.

A Genomics Approach to Study Heart Development

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The research in my lab focuses on the study of gene regulatory mechanism using genomics approach. We are currently interested in elucidating the molecular mechanism of transcriptional regulation during heart development. Using the ChIP-seq method which we have previously established, we are profiling the binding sites of several cardiac transcription factors as well as epigenetic marks in sorted heart cells from the zebrafish embryo. Our collaboration with Dr. Oksana Piven aims to implement the ChIP-seq method to profile the binding sites of b-catenin and g-catenin in attempt to elucidate the signaling function of these two Wnt pathway effectors in mouse embryonic heart development. A previous visit by Dr. Piven to our laboratory has allowed us to run a trial ChIP experiment as the initial step in optimization of a protocol suitable for mouse embryonic heart samples. Following visits are planned to further optimize this method and implement it in our actual experiments.

Research training in mass-spectrometry of biopolymers: results and perspectives

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Due to the COMBIOM we had opportunity to visit Laboratory of Bioinformatics

and Protein Engineering/Core Facility, International Institute of Molecular and Cell Biology, and Mass Spectrometry Laboratory, Institute of Biochemistry and Biophysics of Polish Academy of Sciences, Warszawa, for research trainings in mass-spectrometry of oligoribonucleotides, proteins and their complexes. These laboratories have a wide range of the modern mass-spectrometric equipment and its researchers have an extensive experience in this field. As oligoribonucleotides play a key role in the antiviral cell defence and the processes of cell growth and differentiation it is very important to reveal oligoribonucleotides with unique biological activity and understand the mechanism of their action in an organism, because it could give an opportunity to create new medicines based on the native oligoribonucleotides. Therefore, the main aim of the research training is to get skills that necessary for the analysis of the oligoribonucleotides and proteins using MALDI-Tof-Tof mass spectrometric techniques: samples preparation procedure, carrying out measurements, processing and analysis of the experimental data with special software, sequencing of low molecular weight oligoribonucleotides. The analytical and investigation skills required to carry out the experiments in the field of MALDI-MS have been acquired. During this research training the EMAPII, CaM, S100 proteins and their complexes with 2',5'-A₃ and its epoxy analogue (epo-2',5'-A₃) which contain 3 nucleoside elementary links as well as RNA extracted from yeast and its fractions after FPLC were investigated by MALDI-TOF-TOF mass spectrometry. The very promising preliminary results have been obtained; the perspectives and possibilities of the future collaboration between our laboratories also have been discussed.

Structural basis of methylation and hydroxymethylation specificity of DNA binding protein

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DNA methylation and hydroxymethylation introduce relatively small changes in DNA bases, yet there are many proteins that distinguish between unmethylated, methylated and hydroxymethylated DNA. In the case of cytosine methylated DNA, solvation/desolvation effects are thought to play a major role in methylation specific binding. Using restriction endonuclease R. DpnI as an example, we have studied specific binding to adenine methylated DNA. From biochemical data, two crystal structures and hydrogen-deuterium experiments, we

conclude that the high specificity of the enzyme relies on a “double readout” of methylation by two separate domains (catalytic and winged helix). Each domain relies partially on desolvation for methylation detection, but in addition, adenine methylation in the GATC context generates DNA methyl groups in close proximity, which enforces DNA deformation. We suspect that R. DpnI specifically recognizes the deformed conformation, and hence ultimately relies on a methyl-methyl clash in the substrate to specifically bind adenine methylated DNA. As a model for hydroxymethylcytosine specific DNA binding, we have used the PvuRtsII, a bacterial enzyme, which specifically cleaves the DNA with 5-hydroxymethylcytosine bases. A crystal structure of PvuRtsII shows a previously unrecognized SRA domain in the enzyme, which can be expected to flip the modified base. Although our own and another group’s biochemical data regarding nucleotide flipping are still confusing, currently the most plausible model is that specific 5hmC binding relies on scrutiny of the flipped base, which is highly reminiscent of the way how many DNA repair proteins detect damaged bases.

Nuclear organization, oncoviruses and cancer

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Environmental factors play an important role in most human cancer. Burkitt’s lymphoma (BL), a rare B-cell lymphoma caused by specific chromosomal translocations resulting in the juxtaposition of the CMYC oncogene with an immunoglobulin gene locus, is a typical example of cancer strongly affected by environmental factors. BL is associated with the Epstein Barr virus, human immunodeficiency virus (HIV), malaria and exposure to a Euphorbiaceae plant. The molecular mechanisms of these environmental factors remain largely unknown. One of the major enigmas to solve is why all these factors specifically induce Burkitt’s lymphoma, and no other malignancies? Our data indicate that this these factors perturb the nuclear organization of B-cells inducing the prolonged colocalization of potential translocation partners, the IGH and CMYC loci. This may induce BL-specific translocations.