Characteristics of main research directions investigated at the institute and the achievements 2010−2014

Institute	Institute of Biophysics of the CAS, v. v. i.

The mission of the Institute of Biophysics, Czech Academy of Sciences (IBP) is a basic research of the structure, function and dynamics of biological systems (biomolecules, cell components, cells and cell populations) using methods of molecular biology, biophysics, biochemistry and bioinformatics. IBP contributes to increasing the level of knowledge and education, to the development of biotechnologies and to the transfer of reseach results to practical applications, particularly in the field of diagnostics and treatment of deleterious human diseases. In the following, the main reseach directions of the Institute and their achievements are represented by the characteristics and best results of the departments.

Department of Molecular Biophysics and Pharmacology

The key theme of the research carried out at the Department in the period of 2010-2014 was to establish fundamental principles that will enable the design of safe and effective metal-containing therapeutic. The 5 major and specific (inter-related) areas of work were chosen because they are challenging, have a ground-breaking nature, and potentially a high impact.

a) *Thermodynamic stability and energetics of DNA, damaged by antitumor metallodrugs, relations to their mechanism of action.* In the attempt at sketching an atlas of DNA–antitumor metallodrug energetics, a research program was conducted, in which spectroscopic and microcalorimetric techniques were employed, to present a detailed thermodynamic view of several complexes formed between antitumor metallodrugs and DNA host duplexes. The data made it possible to explain the influence of DNA–antitumor metallodrug energetics on the ability of many DNA polymerases to bypass adducts of antitumor platinum drugs and the ability of cellular systems to repair damage induced by these adducts. Notably, it has also been shown that the migration of some antitumor platinum complexes from one strand to another in double-helical DNA controlled by energetic signatures of these agents may contribute to a better understanding of their cytotoxic and mutagenic potential.

b) *Mechanistic insights into unique antitumor effects of new polynuclear platinum complexes.* It was demonstrated that DNA adducts of polynuclear bifunctional and trifunctional platinum (II) complexes, whose formation is associated with antitumor effects of these agents, differed significantly in structure and type from those of mononuclear platinum complexes used in the clinic. The results indicate that in particular the unique properties of DNA interstrand cross-links of polynuclear platinum complexes, recognition of these cross-links and ability to condense nucleic acids may play a prevalent role in antitumor effects of this new class of metallodrugs.

c) *Metallo-supramolecular helicates with antitumor and antibiotic aktivity.* The substitution-inert cationic helicates - chiral assemblies of two or more metal atoms linked by short multidentate organic ligands - are regarded as non-peptide mimetics of α-helices. These helicates exhibit promising antitumor and antimicrobial activity. It was demonstrated that the helicates exhibited specific interactions with DNA and recognized and stabilized unusual DNA structures.

d) *Photoactivatable platinum anticancer drugs*. Selective photoactivation of platinum complexes by irradiation of cancer cells may avoid enhancement of toxic side-effects, but may increase toxicity selectively in cancer cells and extend the application of photoactivatable platinum complexes to resistant cells and to a wider range of cancer types. In addition, recent advances in laser and fiber-optic technologies make it possible to irradiate also internal organs with light of highly defined intensity and wavelength. The research aimed at molecular mechanisms underlying toxic effects in cancer cells of the photoactivatable platinum drugs provided new insights into mechanisms associated with the antitumor effects of platinum complexes photoactivated by UVA and visible light.

e) *Dual-targeting anticancer metallodrugs, mechanism of action.* A strategy was explored to examine the biological effects of substitutionally inert Pt (IV) prodrugs, combining bioactive axial ligands with Pt (IV) derivatives of antitumor Pt (II) compound (oxaliplatin). The results have demonstrated that the dual targeting strategy is a valuable route to pursue in the design of platinum agents which may be more effective in cancer types that are typically resistant to therapy by conventional cisplatin. Moreover, platinum (IV) derivatives containing valproic acid as axial ligands seem to be promising dual-targeting candidates for additional preclinical studies.

Department of Biophysical Chemistry and Molecular Oncology

The DBCMO pursues interdisciplinary research on structure and interactions of biopolymers (nucleic acids, proteins and carbohydrates) in cells, in solution and at electrodes, and it is also involved in development of novel bioanalytical methods and biosensors, primarily based on electrochemical detection. In 2010-2014, the members and research groups within DBCMO contributed significantly to the progress in the areas of chemical modification and redox labelling of nucleic acids and carbohydrates, and of protein-DNA interactions with focus on proteins involved in cancer. DBCMO studies were particularly successful in the field of electrochemistry of non-conjugate proteins.

The beginning of electrochemical analysis of proteins dates back to 1930. Since the beginning of the 1970s, electrochemistry of proteins dealt predominantly with conjugated proteins containing non-protein redox centers yielding reversible electrode processes. Until recently, it was generally accepted that adsorption of proteins at metal electrodes leads to denaturation of the proteins. Nevertheless, despite these literature data, it was shown,at the DBCMO using constant current chronopotentiometric stripping (CPS) analysis that (a) close to the potential of zero charge, proteins adsorbed at Hg-containing electrodes are not denatured; they can however denature due to a prolonged exposure to negative potentials, (b) proteins produce well-resolved electrocatalytic signal (peak H) at bare mercury electrodes regardless of the presence or absence of redox centers in their molecules, which is useful (i) for detection of proteins down to nanomolar and subnanomolar concentrations and (ii) for studying local and global changes in protein structures.

In 2010, thiol-modified Hg electrodes, suitable for immobilization of a given protein were proposed. Using dithiothreitol (DTT)-modified mercury or solid amalgam electrodes, the effect of oncogenic mutations on the DNA-binding domain of the p53 tumor suppressor were studied. The CPS responses of wild-type and mutant p53 showed excellent correlation with existing structural and stability data and provided additional insights into the differential dynamic behavior of the proteins. Further, it is possible to monitor the loss of an essential zinc ion resulting from mutation (R175H) or metal chelation. It can be envisaged that the CPS method can be applied to the analysis of virtually any protein as a sensor for conformational transitions or ligand binding to complement conventional techniques, but with the added benefit that only relatively small amounts of protein are needed and instant results are obtained. This work may lay the foundation for the wide application of electrochemistry in

protein science, including proteomics and biomedicine. In addition to cancer research, CPS was applied in studies of proteins and peptides involved in neurodegenerative diseases.

Recently, specific properties of CPS have been utilized in the analysis of DNA-protein complex nanolayers. Rapid potential changes at high negative current intensities (I_{str}) in CPS are utilized in the analysis of DNA-protein interactions at DTT-modified mercury electrodes. P53 core domain (p53CD) sequence-specific binding to DNA results in a striking decrease in the electro-catalytic signal of free p53. This decrease is related to changes in the accessibility of the electroactive amino acid residues in the p53CD-DNA complex. By adjusting *I*str and temperature, weaker non-specific binding can be eliminated or distinguished from the sequence-specific binding. This method also reflects differences in the stabilities of different sequence-specific complexes, including those containing spacers between half-sites of the DNA consensus sequence. The high resolving power of this method is based on the disintegration of the p53CD-DNA complex by the electric field effects at a negatively charged surface and fine adjustment of the millisecond time intervals for which the complex is exposed to these effects. Picomole amounts of p53 proteins and DNA were used for the analysis at full electrode coverage.

Department of Molecular Epigenetics

Exploring the evolution of allopolyploid genomes using the Nicotiana model. Angiosperm evolution is heavily impacted by polyploidy, which has occurred in the ancestry of all or most species. It is thought that revolutionary changes in genome composition occur in early generations after the production of new polyploid lineages. The *Nicotiana* genus provides an excellent model group for such studies, since the genus consists of c. 70 species, and ~40% of these are thought to be polyploids derived from six independent polyploidy events, for which the members of the diploid lineages giving rise to these polyploids are still extant. Here, we have used molecular, cytogenetic and genomic approaches to address significant questions of genome dynamics following a polyploidisation event. The major achievements can be summarised as follows:

a) The allotetraploid genome of *Nicotiana tabacum* (tobacco, <0.2 myrs old) shows evidence of sequences loss, which is particularly evident among the Ty3-*gypsy* retroelements, but also involving tandem repeats and 35S ribosomal DNA. Moreover, the paternally (*N. tomentosiformis*) derived T-genome of tobacco shows evidence of erosion, while the maternal (*N. sylvestris*) S-genome appears materially unchanged, indicating the possibility of preferential loss of paternally derived repetitive DNAs.

b) The allotetraploid genomes of ancient (5 myrs old) Nicotiana allopolyploids from section Repanda show complete loss of parental subtelomeric repeats and amplification of their novel variants. This may be considered as a hallmark of diploidization processes that also involves erosion of low copy-number nuclear DNA. The genome size divergence is manifested through the differential removal and/or accumulation of high copy-number sequences.

Department of Molecular Cytology and Cytometry

For a long time, the Department has been interested in studies of the higher-order chromatin 3D structure in various cell nuclei and its relation to functional aspect such as differentiation, DNA repair, cell transformation etc. In addition, we recently also focus to other epigenetic phenomena (acetylation, methylation of histones) and detailed mechanisms of DNA repair in relation to structural features. We use not only normal and cancer cells but some important results have been obtained also with pluriponent cells. Some scientists are involved in plant cell biology (collaboration with Masaryk University), studies of chromosomal HMG proteins

and radioprotection against ionizing radiation. In the following we present the results obtained basically in our Department.

a) *Dynamics of DNA repair proteins.* We have studied accumulation of epigenetically important proteins to UVA-damaged chromatin. We have observed that heterochromatin-related proteins such as BMI1 and HP1β have an ability to recognize DNA lesions and this nuclear event was dependent on acetylation state at locally irradiated chromatin. We have also analysed whether OCT4 protein, as pluripotency factor of ES cells, has the ability to recognize UVA-induced DNA lesions. In this case, we have observed that OCT4 accumulates visibly in the UVA-damaged chromatin, immediately after irradiation, and also that this process was ATP and acetylation dependent. Because we have discovered that accumulation of the above mentioned proteins to UV-induced DNA lesions is linked to hypoacetylation events, we have continued with analyses using inhibition of histone deacetylases (HDACs) that caused protein hyperacetylation. We found that HDAC inhibitors, including Trichostatin A (TSA) or SAHA, abrogate accumulation of HP1β and OCT4 in UVA-induced DNA lesions. In addition, we have attempted to discover whether A-type lamin function is responsible for HP1β recruitment to DNA lesions. And, using the FRET analysis in DNA lesions, we addressed the question whether HP1β interacts with other DNA damage-related proteins, including 53BP1 or BMI1. On the basis of the results of our experiments, we can summarize that when nuclear lamina is injured by UVA-irradiation, fluorescence of mCherry-tagged A-type lamins disappears. However, damage in nuclear lamina did not influence recruitment of DNA repair-related proteins, including HP1β, 53BP1 and BMI1. Only the 53BP1 status at DNA lesions was affected by A-type lamin deficiency. This has been observed on the level of mCherry-53BP1 fluorescence in DNA lesions of A-type lamin deficient mouse embryonic fibroblasts.

b) *Detection of HP1 and DSB repair proteins represents a sensitive instrument for monitoring AML.* We have discovered that maturation of chromatin during granulocyte differentiation determines the ability of neutrophils to release chromatin NETs (neutrophil extracellular traps) and thus their capability to immobile and inactivate pathogens upon an immune stimulation. In addition, the chromatin maturation was followed by a loss of DNA damage response; the DNA repair is necessary neither for fulfilling neutrophils' functions, nor for their short life. Importantly, neutrophils isolated from AML patients showed, in a variable extent, features of incomplete chromatin differentiation and condensation, which precluded their ability to form NETs. On the other hand, DNA repair remained active. Thus, it appears that the purpose of changes in chromatin structure during the neutrophil differentiation is to enable the unique function of these cells in their immune defense. Moreover, not even successful AML treatment always leads to complete maturation of granulocytes; this may have a direct impact on the ability of AML patients in remission to fight infection, which is an important cause of mortality during the AML therapy. On the basis of our results, we suggest the immunodetection of HP1 protein and DSB repair proteins to be a sensitive method for monitoring AML neutrophil maturation and functionality in clinical practice.

c) *The role of chromatin structure in DSB repair and formation of exchange aberrations.* We have described new relationship between the chromatin structure and indirect induction of DSBs by low-LET radiations and have also proposed a new model of how chromosomal translocations arise upon irradiating cells with radiations of low and high LET, respectively. We have discovered that although most of DSBs are spatially stable and do not migrate into putative repair factories, 'heterochromatic' DSBs may show higher mobility because of 'heterochromatin' decondensation required for repair of these lesions. Hence, interactions between damaged genetic loci that are far from each other in the cell nucleus are possible, but not frequent and purposeful. We have demonstrated that DSB cluster represents the sites with an increased risk of chromosomal translocations rather than repair factories. In

addition, mutual distribution of damaged loci, mutual distribution of chromatin domains, and distribution of damaged loci relative to these domains largely influence the probability of interactions and chromatin translocations between particular loci.

Department of Cytokinetics

In general, the research of DC has been concentrated mainly on elucidation of molecular and cellular mechanisms involved in various aspects of carcinogenesis and cancer cell biology, as well as on potential novel therapeutic strategies. Some of our major research results are highlighted below:

a) *Novel type of GF-β1 and IL-6 cytokines signaling.* We have demonstrated a novel type of interaction between TGF-β1 and IL-6 cytokines signaling, and we have described yet another mechanism of how defects in TGF-β signaling, may contribute to the disruption of tissue homeostasis; we have shown for a first time that GDF-15 cytokine, TGF-β family member is an abundant cytokine in seminal plasma that displays immunosuppressive characteristics.

b) *The effects of androgen deprivation therapy.* We have documented that androgen deprivation therapy, a common treatment for advanced prostate cancer, induces both the emergence of neuroendocrine-like prostate cancer cells and the senescence-associated secretory phenotype in prostate cancer epithelial cells.

c) *Signal transduction of Dishevelled and its role in Wnt signaling*. We have described several key mechanisms in the signal transduction of Dishevelled and its role in Wnt signaling; importantly, our study for the first time identified the critical role of non-canonical Wnt pathway (also referred to as planar cell polarity (PCP) pathway) in the pathogenesis of chronic lymphocytic leukemia (CLL).

d) *Novel inhibitor of FGFR3.* We have identified a novel, potentially therapeutic inhibitor of FGFR3 and described a novel phenotype, a premature senescence, induced by FGFR3 signaling in chondrocytes. We also characterized a novel type of FGFR3 interaction with TGF-β-activating kinase 1 and its role in pathological FGFR3 signaling, associated with human diseases.

e) *Cell-specific-modulation of CYP1B1 expression*. We have demostrated that inflammatory signaling may alter metabolism of carcinogens and thus contribute to their tumor initiating effects via a cell-specific-modulation of CYP1B1 expression, which involves a unique type of the regulation of CYP1B1 expression via the p38/MSK1 kinase cascade, leading increased CYP1B1 gene transcriptional elongation.

f) *Toxic ligands of the Ah receptor disrupt intercellular communication.* We have shown that toxic ligands of the Ah receptor may, through a variety of mechanisms, disrupt expression and/or function of proteins mediating intercellular communication via gap junctions, adherens junctions or desmosomes in epithelial cells. This is linked with alterations of cell proliferation, cell adhesion or cell phenotype as well as with inhibition of Wnt/β-catenin signaling. Importantly, we have documented that the Wnt/β-catenin signaling simultaneously co-regulates the AhR-dependent expression of xenobiotic metabolizing enzymes, such as CYP1 enzymes, which may have profound effects on metabolism of carcinogenic compounds.

g) *Lipid composition in colon epithelial cells is alterd by fatty acids.* We have documented, via lipidomic and functional analyses, that short-chain fatty acid butyrate and essential polyunsaturated fatty acids coordinately alter lipid composition in colon epithelial cells, and we have shown a significant association between lipid alterations and differentiation/ apoptotic responses in colon cells.

h) *Combination cancer therapy using polyunsaturated fatty acids and TRAIL*. We have identified the potential of polyunsaturated fatty acids for a combination therapy with clinically

useful cytokine TRAIL. Importantly, when evaluating the promising anti-cancer potential of a novel platinum cytostatic drug LA-12, we provided important novel mechanistic insights into its cooperative action with TRAIL in carcinoma cells.

These results have contributed, by our opinion, to a better understanding of important mechanisms that play a role during cancer development, and which can be explored in diagnosis, prevention and/or therapy of neoplastic diseases.

Department of Free Radical Pathophysiology

The attention of the Department was focused on: 1) the study of mechanisms leading to the generation of reactive oxygen and nitrogen metabolites by phagocytes, and 2) the evaluation and modulation of molecular mechanisms underlying the inflammation-derived damage of vascular endothelium and heart tissue. The following major results were reached:

a) The role of myeloperoxidase in the blood vessel inflammation. Clarifying the importance of myeloperoxidase (MPO) in the pathogenesis of vascular inflammatory processes, We have demonstrated that upon degranulation from neutrophils, MPO binds to the surface of endothelial cells in an electrostatic-dependent manner and undergoes transcytotic migration to the underlying extracellular matrix. MPO also binds to the surface of blood cells including thrombocytes and erythrocytes. Interaction of thrombocytes with MPO induces their partial activation. Furthermore, we have demonstrated that MPO binds to erythrocytes, which correlates with the clinical conditions of patients with cardiovascular diseases. MPO bound to erythrocytes adversely affects the function of blood vessels *in vivo*. The importance of MPO in the development of blood vessel inflammation and endothelial dysfunction opens pharmacological interventions focused on MPO.

b) Nitrated unsaturated fatty acids (NO_2-FAs), endogenously occurring products of nitration reactions, represent novel signalling mediators leading to secondary changes in protein function via electrophilic-based modifications. Our studies have shown that NO_2-FAs are present in the vasculature at nanomolar to low micromolar concentrations in an amount sufficient for exertion of biological actions including inhibition of neutrophil, macrophage, and fibroblast activation, proinflammatory cytokine secretion, and vascular smooth muscle cell proliferation. In the light of the above-described cell signaling roles of NO_2-FAs, one may expect an existence of a "threshold level" over which their physiological activities switch from signaling to pharmacological actions. Therefore, NO_2-FAs are currently suggested as highly promising compounds for treatment of various cardiovascular and inflammatory disorders.

c) Inhibitory effect of histamine on the oxidatiove burst of neutrophils. Platelets and activated neutrophils are in close contact at the sites of inflammation. Mediators like serotonin and histamine, released from platelets, might have a protective function against neutrophil-derived oxidative stress and oxidative damages. The increase in local concentrations of serotonin and histamine reduced the formation of reactive oxygen species by neutrophils. We have revealed that the inhibitory effects of histamine on the oxidative burst of neutrophils were caused rather by the binding of histamine to H2R than to H4R.

d) A*nti-inflammatory and antioxidative properties of medicinal plants*. We have also studied the anti-inflammatory and antioxidative properties of medicinal plants, vegetables and small fruits, which are a rich source of bioactive substances, including polysaccharides and polyphenols, and therefore they are suitable raw materials for the production of functional foods. The extracts from berries especially blocked the formation of neutrophil-derived reactive oxygen species induced with the use of receptor binding activators almost completely. On the other hand, the effect of extracts on neutrophils activated with receptor by-passing stimuli was much milder, indicating that these extracts interfere with the signalling cascade of phagocyte activation upstream to the protein kinase C activation.

Department of Molecular Dynamics of Nucleic Acids

The Department if focused on modelling of the structure and dynamics of large biomolecules, particularly, nucleic acids. The productivity of DMDNA measured by bibliometry is for a long time the best in the institute. In order to show practical significance and importance of this work for human society the following outputs of DMDNA are used as an example.

a) Origin of Life on the Earth - formamide pathway for the prebiotic synthesis of the first RNA molecules. The formamide-based synthesis of nucleic acid components offers a new alternative for the origin of informational polymers. This chemistry represents an elegant and continuous way, from simple prebiotic precursors up to short catalytic oligonucleotides. Since this multistep synthesis proceeds in a very complex reaction mixture, it is difficult to study its mechanism using purely experimental methods. In such complicated cases, computational chemistry might be instrumental to provide an atomic-level insight into the mechanistic details of the reactions. In the last few years, we have devoted a special attention to this topic, and in cooperation with experimental groups, we have addressed various stages of the formamide-based origin of life scenario. Our results strongly suggest that life on Earth could have been spontaneously created via genuine chemical processes and that there was no need to bring the life and its components from the Space. We have proposed an impact-based model for the origin of terrestrial life: we suggest that heavy impact of extraterrestrial bodies during the Late Heavy Bombardment period about 3.8 billion years ago could sparkle life (synthesis of basic components of biomolecules) in small formamide-ponds (Ferus et al., 2015). Thus, our model further elaborates the scenario proposed by Saladino and suggests that the energy, which was brought in by extraterrestrial impacts could have been used to create building blocks of life.

The paper has been published as feature article (very rare for PNAS) and further identified to be of "exceptional significance" by the editorial board of PNAS. Immediately after its publication (Dec 8, 2014) on the internet, it has been highlighted by Science mag and New Scientist and Chemistry World. Apart from intense reactions of the scientific world, the paper has ignited a cascade of press releases intended to the public: through the AP press agency, apart from leading Czech newspapers and magazines like Hospodářské noviny, Lidové noviny, Respekt, etc., our results have been discussed in many newspapers all over the world, e.g. Los Angeles Times, The Telegraph. We gave several radio interviews (see e.g. German state radio, Czech state radio, Czech BBC). In addition, major news channels on the internet reported our results (see e.g. Huffington Post, Yahoo news channel, Discovery News).

b) How the first oligonucleotides acquire their catalytic function. In our recent Chemistry – A European Journal VIP paper (very important paper, ~1% of publications in the Journal) (Stadlbauer et al., 2015), we address a later step of the origin of terrestrial life: i.e. how the first oligonucleotides could acquire their catalytic function, a feature that stays behind evolution of species on Earth. This paper has also been highlighted in the Chemistry World magazine. All computational work in the projects (which is essential) is done by the Brno group. The foreign partners are providing the necessary experiments.

Department of CD Spectroscopy of Nucleic Acids

The main goal of the Department is better understanding biophysical and conformational properties of quadruplexes, primarily of quadruplexes of the human telomere (htel) DNA. Quadruplexes are biologically important anomalous DNA structures which frequently form in gene promoters and were shown to control their expression. In telomeres quadruplexes control genome integrity and their formation is associated with ageing and carcinogenesis. Our work contributed to the polymorphic view of the htel quadruplex by the following results.

(a) We have shown that apart from the concentration and type of present cations, the quadruplex structure depends on the length of the htel molecule and its precise oligonucleotide sequence including appended nucleotides on the 5´and 3´end.

(b) We were among the first laboratories demonstrating the transition of the htel quadruplex from antiparallel to parallel arrangement under dehydrating conditions.

(c) Based on thermodynamic data we found that long htel molecules form, similarly to nucleosomes, beads on a strng-like structure where the beads are the basic 21 nucleotide long three-tetrad quadruplex units.

(d) Various quadruplex structures were suggested for this basic quadruplex unit by different methods. According to the results of optical spectroscopies the basic htel sequence adopted an antiparallel quadruplex at physiological conditions. NMR unambiguously found that the quadruplex is under the same conditions in a so-called (3+1) hybrid structure, with three parallel and one antiparallel strands. X-ray analysis of the htel sequence in crystal identified that this quadruplex has propeller-like double chain reversal loops resulting in an all-chains-parallel quadruplex structure. We have explained the cause of distinct results on the htel quadruplex structure reported by different laboratories: The reason is the dependence of the quadruplex arrangement on DNA concentration, which is by orders distinct in the individual methodological approaches.

(e) We have confirmed the concentration dependence of the htel quadruplex conformation in the framework of a joint grant with our colleagues from the Institute of Physics, Charles University, Prague by Raman spectroscopy, which enables spectroscopic measurements in a wide region of DNA concentrations.

(f) We have determined thermodynamic parameters of particular quadruplex structures and identified association of the quadruplex particles as the source of these intramolecular transformations. The association has been visualized by AFM.

The laboratory has undertaken an extensive study of the influence of the most frequent naturally occurring DNA damages on the structure and stability of the htel quadruplex. The damages to core guanines destabilized the quadruplexes and their impact depended more on the lesion position in the htel sequence than on its type. The damages to quadruplex loops were able, depending on their type and position, to change topology of quadruplex folding. The obtained results indicate that, in view of the important biological functions of telomeres, the change in their DNA quadruplex structure may have serious biological consequences.

Department of Plant Developmental Genetics

The Department is focused on studies of developmental processes, which play the key role in plant reproduction. Our aim is to characterize sex determining mechanisms and processes controlling sexual pathways. Our research is based on advanced techniques of molecular biology and genomics.

Sex chromosomes in the majority of animal species are ancient, but plant sex chromosomes have evolved relatively recently, making dioecious plants good models to study the early steps of sex chromosome evolution. We work on selected dioecious species that evolved heteromorphic sex chromosomes to reveal specific processes connected with separate evolution of male and female genomes. *Silene latifolia* (syn. *Melandrium album* or white campion) is our key dioecious plant model possessing heteromorphic sex chromosomes, X and Y. It formally resembles the mammalian type of sex determination since the gender is controlled by dominant Y chromosome-linked genes present only in male individuals. Our recent data on *S. latifolia* show that both the X and Y chromosomes harbor active genes, but that they are divergent due to the genetic degeneration of alleles in the non-recombining region of the Y. Most important achievements include (a) genetic mapping of model dioecious plants, (b) discovery of phylogenetic relationships in sex chromosome evolution, (c) identification of new sex-linked genes, and (d) deciphering a role of repetitive DNA in sex

chromosome evolution. Special attention is taken to develop original methods for targeted modification of plant genes and genomes.

To focus directly on sex chromosome structure and function, we have constructed X- and Y-specific libraries using laser microdissected and/or flow-sorted chromosomes. We have isolated new sex linked genes by screening a bacterial artificial chromosome (BAC) library. We also study the impact of repetitive DNA sequences on the evolution of sex chromosomes. Our results reveal specific mechanisms connecting retrotransposon spread with tandem repeat amplification in the genomes. Our data suggest that the absence of one family of retrotransposons on the Y chromosome is caused by small RNA-mediated silencing leading to a female-specific distribution. The results of our current research show that divergence of the sex chromosomes of *S. latifolia* is already in process and degeneration of the Y chromosome accompanied by accumulation of specific sequences has begun.

Our phylogenetic studies reveal that there are at least two independently evolved groups of dioecious species within the genus *Silene* (sections Elisanthe and Otites). We found that the sex determining system in *S. otites* was based on female heterogamety (ZW system), a sex determining system that is unique among the other *Silene* species having male heterogamety (XY system). Our observations imply that a switch from an XX/XY sex determination to a ZZ/ZW system (or vice versa) occurred in the subsection Otites. This is the first report of two different types of heterogamety within one plant genus of this mostly non-dioecious plant family

Research Report of the team in the period 2010–2014

Institute	Institute of Biophysics of the CAS, v. v. i.

Scientific team	Department of Molecular Biophysics and Pharmacology

(1) Thermodynamic stability and energetics of DNA damaged by antitumor metallodrugs. Relations to their mechanism of action

To understand the molecular basis for biological activities of metallodrugs, their interaction with DNA molecules has been investigated by several laboratories using a variety of experimental techniques. Numerous studies have produced rather impressive structural pictures that show where antitumor metallodrugs bind to DNA and that suggest the formation of specific molecular interactions in the complex. Despite the obvious value of such structural models, their information content is lacking in several important respects. Specifically, the structural picture does not provide us with insight into (i) the stability (ΔG^0) of the metallodrug-DNA complex in solution; (ii) the thermodynamic nature of the overall molecular forces that drive complex formation in solution (ΔH^0, ΔC_p, and ΔS^0); (iii) the relative contributions made by specific molecular interactions to stabilization of the complex in solution; and (iv) the temperature-dependent (melting) behavior of the complex. In short, despite their fundamental

importance, the structural studies on metallodrug-DNA complexes have not characterized the thermodynamic nature of the driving forces nor defined the relative contributions made by each molecular interaction to the DNA binding affinity and specificity of the drug. Such a characterization and ranking of molecular interactions requires thermodynamic data on metallodrug binding and the resulting metallodrug-DNA complex. In recognition of this need and in a comprehensive attempt at sketching an atlas of DNA–antitumor metallodrug energetics, we have been conducting a program in which spectroscopic and microcalorimetric techniques are being employed, to present a detailed thermodynamic view of several complexes formed between antitumor metallodrugs and DNA host duplexes. Our investigation has been paralleled and/or preceded by the structural studies performed by us or others which have provided us with a microscopic framework in which to interpret our macroscopic thermodynamic data. The major results of this study are summarized below:

(i) We employed differential scanning calorimetry to measure the thermodynamic changes associated with replication bypass past major adducts formed by conventional and novel antitumor metallodrugs. Depending on a nucleotide sequence context these DNA adducts inhibit DNA polymerization. The thermodynamic data helped understand the effect of the alterations in thermodynamic stability of DNA caused by the adducts upon DNA polymerization across these lesions. Moreover, these data can explain the influence of these alterations on the ability of many DNA polymerases to bypass adducts of antitumor platinum drugs. These results also highlight the usefulness of differential scanning calorimetry in evaluating the impact of DNA adducts of antitumor metallodrugs on the processing of these lesions by damaged-DNA processing-proteins (enzymes).

(ii) Recent clinical studies suggest that high levels of expression of proteins associated with removal of adducts of antitumor metallodrugs from DNA result in tumor resistance and, ultimately, are responsible for the low efficacy of classical metallodrug-based regimens. DNA repair systems most efficiently recognize and remove irreversible DNA adducts that are bulky in nature and/or thermodynamically destabilize double-stranded DNA due to severe conformational distortions. Our results support the view that antitumor transition metal-based complexes belong to a class of anticancer agents for which structure–pharmacological relationships might be correlated with their capability to thermodynamically affect DNA stability.

(iii) We have shown employing short oligodeoxyribonucleotide duplexes containing single, site-specific cross-links of antitumor platinum complexes of the second generation that in contrast to major DNA adducts of conventional cisplatin, under physiological conditions the coordination bonds between platinum and N7 of guanine residues involved in the cross-links of these second generation platinum complexes can be cleaved. This cleavage may lead to the linkage isomerization reactions between these metallodrugs and double-helical DNA. Differential scanning calorimetry of duplexes containing single, site-specific cross-links of these second generation platinum complexes has revealed that one of the driving forces that leads to the lability of DNA cross-links of these metallodrugs is a difference between the thermodynamic destabilization induced by the cross-link and by the adduct into which it could isomerize. The rearrangements may proceed in the way that cross-links originally formed in one strand of DNA can spontaneously translocate from one DNA strand to its complementary counterpart, which may evoke walking of the platinum complex on DNA molecule. Interesting generalization of these results is that the migration of some antitumor platinum complexes from one strand to another in double-helical DNA controlled by energetic signatures of these agents may contribute to a better understanding of their cytotoxic and mutagenic potential (Figure 1).

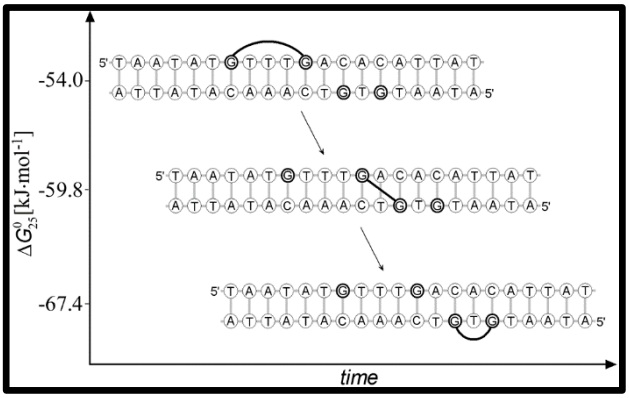

Figure 1. Scheme of the spontaneous linkage isomerization reactions between trinuclear Pt complex [{*trans*-$PtCl(NH_3)_2$}$_2$(μ-trans-Pt-$(NH_3)_2${$NH_2(CH_2)_6NH_2$}$_2$)]$^{4+}$ (BBR3464) and double-helical DNA substrate. The driving force that leads to the lability of DNA cross-links of this metallodrug is a difference between the thermodynamic destabilization induced by the cross-link and by the adduct into which it isomerizes.

The share of the Department in the creation of this result:

The authors from the Department designed research, performed research, analyzed data and wrote the papers (the first and corresponding authors were from the Department, several authors were PhD students supervised at the Department). The authors from the collaborating foreign laboratories only supplied the platinum complexes because of the lack of unit at the Institute having adequate equipment and experience in synthetic chemistry.

Selected relevant articles (authors from the Department are typeset in boldface):

Florian, J. and **Brabec, V.** (2012) Thermodynamics of translesion synthesis across a major DNA adduct of antitumor oxaliplatin: Differential scanning calorimetric study. *Chem. Eur. J.*, **18**, 1634-1639.

Brabec, V., Malina, J., Margiotta, N., Natile, G. **and Kasparkova, J.** (2012) Thermodynamic and mechanistic insights into translesion DNA synthesis catalyzed by Y-family DNA polymerase across a bulky double-base lesion of an antitumor platinum drug. *Chem. Eur. J.*, **18**, 15439-15448.

Mlcouskova, J., Malina, J., Novohradsky, V., Kasparkova, J., Komeda, S. and **Brabec, V.** (2012) Energetics, conformation, and recognition of DNA duplexes containing a major adduct of an anticancer azolato-bridged dinuclear PtII complex. *Biochim. Biophys. Acta*, **1820**, 1502-1511.

Malina, J., Kasparkova, J., Farrell, N.P. and **Brabec, V.** (2011) Walking of antitumor bifunctional trinuclear Pt^{II} complex on double-helical DNA. *Nucleic Acids Res.*, **39**, 720-728.

Malina, J., Natile, G. and **Brabec, V.** (2013) Spontaneous translocation of antitumor oxaliplatin, its enantiomeric analogue, and cisplatin from one strand to another in double-helical DNA. *Chem. Eur. J.*, **19**, 11984-11991.

(2) Mechanistic insights into unique antitumor effects of new polynuclear platinum complexes

The polynuclear platinum compounds represent a class of new antitumor metallodrugs that is structurally distinct from conventional cisplatin (*cis*-$[PtCl_2(NH_3)_2]$) and its mononuclear analogs, and whose clinical profile and mechanism of action are different from these established platinum mononuclear compounds. Importantly, cells with resistance to cisplatin showed no cross-resistance to polynuclear platinum compounds. We have demonstrated that DNA adducts of polynuclear bifunctional and trifunctional platinum(II) complexes, whose formation is associated with antitumor effects of these agents, differed significantly in structure and type from those of mononuclear platinum complexes. Especially because of markedly more distant leaving groups, long-range intra- and interstrand cross-links are formed in DNA which affect DNA conformation in a unique way, are recognized by specific proteins and repaired differently in comparison with the cross-links of cisplatin and its mononuclear analogs. More specifically,

our results indicate that the unique properties of DNA interstrand cross-links of polynuclear platinum complexes and recognition of these cross-links may play a prevalent role in antitumor effects of these metallodrugs.

Quite recently, new, biologically active trinuclear platinum(II) complexes were designed that bind to DNA through noncovalent (hydrogen bonding, electrostatic) interactions (Figure 2). We have shown that these trinuclear platinum complexes condense nucleic acids with a much higher potency than conventional DNA condensing agents. The complexes even induce aggregation of small transfer RNA molecules, and completely inhibit DNA transcription at lower concentrations than naturally occurring polyamines. Topoisomerase I mediated relaxation of supercoiled DNA is inhibited by these substitution-inert platinum complexes at concentrations which are even 250 times lower than that of polyamines. It is suggested that the mechanisms for the biological activity of these substitution-inert trinuclear platinum complexes may be associated with their ability to condense/aggregate nucleic acids with consequent inhibitory effects on crucial enzymatic activities.

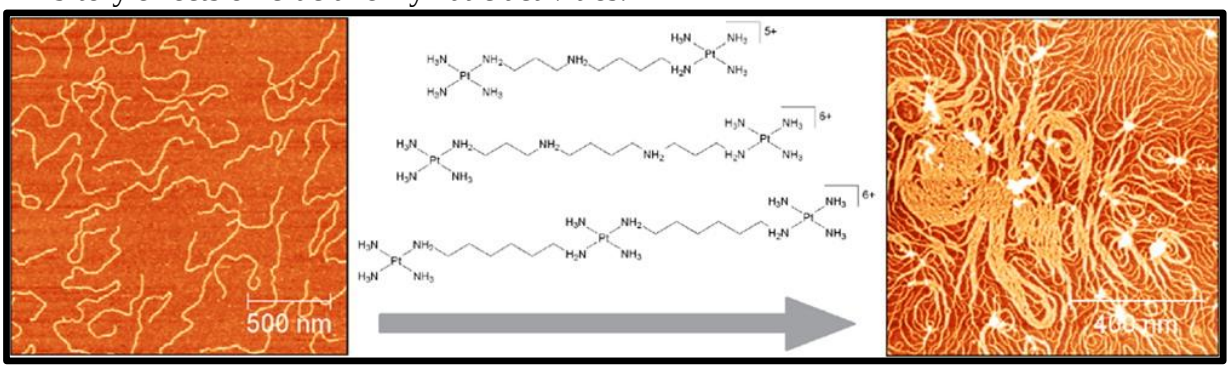

Figure 2. Atomic force microscopic image demonstrating condensation of linearized plasmid pSP73 in the presence of 25 μM triplatinNC-A (bottom structure in the figure).

The share of the Department in the creation of this result:

The authors from the Department designed research, performed research, analyzed data and wrote the papers (the first and corresponding authors were from the Department, several authors were PhD students supervised at the Department). The authors from the collaborating foreign laboratories only supplied the platinum complexes because of the lack of unit at the Institute having adequate equipment and experience in synthetic chemistry.

Selected relevant articles (authors from the Department are typeset in boldface):

Zerzankova, L., Suchankova, T., Vrana, O., Farrell, N.P., **Brabec, V.** and **Kasparkova, J.** (2010) Conformation and recognition of DNA modified by a new antitumor dinuclear Pt^{II} complex resistant to decomposition by sulfur nucleophiles. *Biochem. Pharmacol.*, **79**, 112-121.

Zerzankova, L., Kostrhunova, H., Vojtiskova, M., Novakova, O., Suchankova, T., Lin, M., Guo, Z., **Kasparkova, J.** and **Brabec, V.** (2010) Mechanistic insights into antitumor effects of new dinuclear cis Pt^{II} complexes containing aromatic linkers. *Biochem. Pharmacol.*, **80**, 344–351.

Olivova, R., Kasparkova, J., Vrana, O., Vojtiskova, M., Suchankova, T., Novakova, O., He, W., Guo, Z. and **Brabec, V.** (2011) Unique DNA binding mode of antitumor trinuclear tridentate platinum(II) compound. *Mol. Pharmaceutics*, **8**, 2368-2378.

Malina, J., Farrell, N.P. and **Brabec, V.** (2014) Substitution-inert trinuclear platinum complexes efficiently condense/aggregate nucleic acids and inhibit enzymatic activity. *Angew. Chem. Int. Ed.*, **53**, 12812-12816.

Malina, J., Farrell, N.P. and **Brabec, V.** (2014) DNA condensing effects and sequence selectivity of DNA binding of antitumor noncovalent polynuclear platinum complexes. *Inorg. Chem.*, **53**, 1662-1671.

(3) Metallo-supramolecular helicates with antitumor and antibiotic activity

The substitution-inert cationic helicates - chiral assemblies of two or more metal atoms linked by short multidentate organic ligands (Figure 3) - are regarded as non-peptide mimetics of α-helices. Thermodynamically stable monometallic units connected by organic linkers were synthesized. These helicates exhibit promising antitumor and antimicrobial activity, as well as low toxicity towards a non-mammalian model organism. It has been hypothesized that binding to DNA is responsible for this behavior. We have demonstrated that the helicates exhibit specific interactions with DNA and recognize and stabilize unusual DNA structures.

Figure 3. Structure-dependent anticancer and antimicrobial activity of metallo-helices is discovered along with novel interactions with potential drug targets.

The share of the Department in the creation of this result:

This study was performed in collaboration with the researchers from the University of Warwick and University of Birmingham. Contribution to this study by the Department author's team included experiments focused on DNA interactions, their interpretation and writing the parts of the manuscripts dealing with these studies.

Selected relevant articles (authors from the Department are typeset in boldface):

Howson, S.E., Bolhuis, A., **Brabec, V.**, Clarkson, G.J., **Malina, J.**, Rodger, A. and Scott, P. (2012) Optically pure, water-stable metallo-helical 'flexicate' assemblies with antibiotic activity. *Nature Chemistry*, **4**, 31-36.

Brabec, V., Howson, S.E., Kaner, R.A., Lord, R.M., **Malina, J.**, Phillips, R.M., Abdallah, Q.M.A., McGowan, P.C., Rodger, A. and Scott, P. (2013) Metallohelices with activity against cisplatin-resistant cancer cells; does the mechanism involve DNA binding? *Chem. Sci.*, **4**, 4407-4416.

Malina, J., Hannon, M.J. and **Brabec, V.** (2014) Recognition of DNA bulges by dinuclear iron(II) metallosupramolecular helicates. *FEBS J.*, **281**, 987-997.

(4) Photoactivatable platinum anticancer drugs

Cisplatin is a widely used antitumor agent that acts primarily by adducting DNA to form mainly intrastrand cross-links. Toxicity to the patient limits the use of this highly effective drug, indicating the need for a more selective alternative. One approach is also to use photoactivatable platinum complexes. Selective photoactivation of platinum complexes by irradiation of cancer cells may avoid enhancement of toxic side-effects, but may increase toxicity selectively in cancer cells and extend the application of photoactivatable platinum complexes to resistant cells and to a wider range of cancer types. In addition, recent advances in laser and fiber-optic technologies make it possible to irradiate also internal organs with light of highly defined intensity and wavelength.

We examined molecular mechanisms underlying toxic effects in cancer cells of the photoactivatable Pt(IV) diazido complex, *trans,trans,trans*-[Pt-$(N_3)_2(OH)_2$(pyridine)$_2$] (**1**) (Figure 4). This complex is unreactive in the dark but is cytotoxic when photoactivated by UVA and visible light. In addition, its activation with UVA or visible light irradiation to produce cytotoxic and reactive Pt(II) analogue does not require oxygen, an advantage over conventional photosensitizers currently used in photodynamic therapy (PDT). We have shown that **1** when photoactivated accumulates in tumor cells and binds strongly to nuclear DNA under conditions

in which it is toxic to tumor cells. The nature of the DNA adducts, including conformational alterations, induced by photoactivated **1** are distinctly different from those produced in DNA by conventional cisplatin or transplatin. In addition, the observation that major DNA adducts of photoactivated **1** are able to efficiently stall RNA polymerase II more efficiently than cisplatin suggests that transcription inhibition may contribute to the cytotoxicity levels observed for photoactivated **1**. Hence, DNA adducts of **1** could trigger a number of downstream cellular effects different from those triggered in cancer cells by DNA adducts of cisplatin. This might lead to the therapeutic effects that could radically improve chemotherapy by platinum complexes (Figure 4). The findings of the present work help to explain the different cytotoxic effects of photoactivated **1** and conventional cisplatin and thereby provide new insights into mechanisms associated with the antitumor effects of platinum complexes photoactivated by UVA and visible light.

Other candidate among platinum complexes for use in photoactivated cancer chemotherapy is conventional carboplatin, an analogue of "classical" cisplatin, a widely used second-generation platinum anticancer drug. The reduced toxic effects in tumor cells and a more acceptable side-effect profile in comparison with cisplatin are attributable to the lower reactivity of carboplatin with nucleophiles, since the cyclobutanedicarboxylate ligand is a poorer leaving group than the chlorides in cisplatin. Therefore, it was of interest to examine whether carboplatin can be affected by irradiation with light to the extent that its DNA binding and cytotoxic properties are altered. We have found that carboplatin is converted to species capable of enhanced DNA binding by UVA irradiation and consequently its toxicity in cancer cells is markedly enhanced.

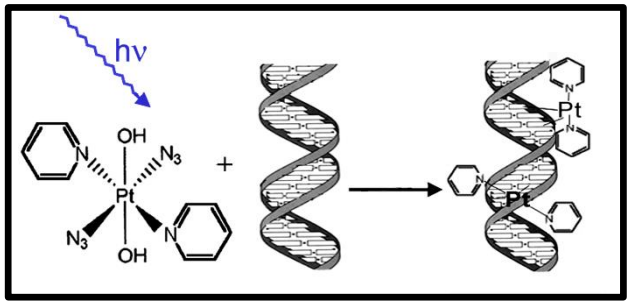

Figure 4. Photoactivated Pt(IV) diazido complex, *trans*,*trans*,*trans*-[Pt-$(N_3)_2(OH)_2(pyridine)_2$] is a more efficient DNA cross-linking agent than cisplatin; however, due to its lack of toxicity in the absence of light, this complex may be a safer antitumor agent for clinical use.

The share of the Department in the creation of this result:

The authors from the Department designed research, performed research, analyzed data and wrote the papers (the first and corresponding authors were from the Department, several authors were PhD students supervised at the Department). The authors from the collaborating laboratory at University of Warwick only supplied the platinum(IV) complex because of the lack of unit at the Institute having adequate equipment and experience in synthetic chemistry.

Selected relevant articles (authors from the Department are typeset in boldface):

Pracharova, J., Zerzankova, L., Stepankova, J., Novakova, O., Farrer, N.J., Sadler, P.J., **Brabec, V.** and **Kasparkova, J.** (2012) Interactions of DNA with a new platinum(IV) azide dipyridine complex activated by UVA and visible light: Relationship to toxicity in tumor cells. *Chem. Res. Toxicol.*, **25**, 1099-1111.

Mlcouskova, J., Stepankova, J. and **Brabec, V.** (2012) Antitumor carboplatin is more toxic in tumor cells when photoactivated: enhanced DNA binding. *J. Biol. Inorg. Chem.*, **17**, 891-898.

(5) Dual-targeting anticancer metallodrugs. Mechanism of action

The mechanisms of resistance of tumor cells to platinum drugs are multifactorial processes. Moreover, the inhibition of single pathways that sustain cisplatin resistance fails to restore

sensitivity to normal levels. These findings give impetus to design several conjugated transition metal-based complexes with dual functionality to improve antitumor activity of clinically used anticancer metallodrugs. A class of such agents involves bifunctional compounds in which histone deacetylase inhibitors (HDACis) are tethered to a Pt(II)-based DNA binding agent. HDACs are key regulators of chromatin structure and transcription. Agents, such as HDACis, that induce hyperacetylation of histone proteins complexed with DNA, could increase the accessibility of DNA within chromatin and consequently potentiate the anticancer activities of metallodrugs, such as DNA-binding platinum complexes. These facts suggest that simultaneous HDAC inhibition and DNA damage triggering apoptosis, autophagy and cell death could be a viable alternative approach in cancer therapy. As regards Pt(II)-HDACi conjugates, it was anticipated that these new conjugated agents after accumulation in tumor cells will aquate in the cytoplasm releasing simultaneously Pt(II) complex capable of coordinating to DNA base residues and HDACi which will increase the accessibility of DNA within chromatin to this Pt(II) agent so that a higher degree of DNA damage mediated by the Pt(II) agent would be achieved. Unfortunately, we showed that the anticipated enhancement of toxicity of these Pt(II)-HDACis in tumor cells (due to dual functionality of these conjugated Pt(II) complexes) has not been evident, apparently since the covalent linker group negatively impacted these processes.

We further explored a strategy to examine the biological effects of substitutionally inert Pt(IV) prodrugs, combining bioactive axial ligands with Pt(IV) derivatives of antitumor Pt(II) compound (oxaliplatin). The rationale behind this prodrug is to release, by reductive elimination inside the cancer cell, an active Pt(II) drug (oxaliplatin) which binds nuclear DNA as well as bioactive ligands that may potentiate toxic effects of the Pt(II) drugs by an independent pathway. We demonstrate that platinum prodrugs, such as Pt(IV) derivatives of oxaliplatin containing axial valproic acid (VPA, well-known HDACi) ligands, destroy cancer cells with greater efficacy than conventional oxaliplatin and display activity in both cisplatin sensitive- and resistant tumor cells. The prodrugs are capable of both markedly enhanced accumulation in tumor cells and acting in a dual threat manner, concurrently targeting histone deacetylase and genomic DNA. The results demonstrate that the dual targeting strategy is a valuable route to pursue in the design of platinum agents which may be more effective in cancer types that are typically resistant to therapy by conventional cisplatin. Moreover, platinum(IV) derivatives containing VPA axial ligands seem to be promising dual-targeting candidates for additional preclinical studies.

Theoretical calculations predicted that a novel half-sandwich organometallic iridium(III) cyclopentadienyl complexes should be potent cytostatic and cytotoxic anticancer agents, and that these complexes, in contrast to cisplatin, may belong to the class of anticancer agents whose mechanism of action can be associated not only with DNA interaction, but also with protein synthesis disruption and redox mediation, all closely related to mitochondrial effects. The cellular mechanism of action of an iridium(III) half-sandwich complex [(η^5-$C_5Me_4C_6H_4C_6H_5$)Ir(phen)Cl]PF_6 (phen = phenanthroline) (**2**) has been examined. We have shown that complex **2** preferentially kills cancer cells over nontumorigenic cells, exhibits no cross-resistance with cisplatin and retains significant activity in human tumor cell lines that are refractory or poorly responsive to cisplatin. Notably, in contrast to cisplatin it displays a high activity in human tumor cell lines that are characterized by both wild type and mutant p53 gene. The mechanism of cell killing was established through detailed cell-based assays. Complex **2** exhibits dual effects in killing cancer cells causing nuclear DNA damage and mitochondrial dysfunction involving ROS production simultaneously. Overall, our findings confirm that complex **2** and its iridium derivatives represent promising candidates for further pre-clinical studies and new additions to the growing family of nonplatinum metal-based anticancer complexes.

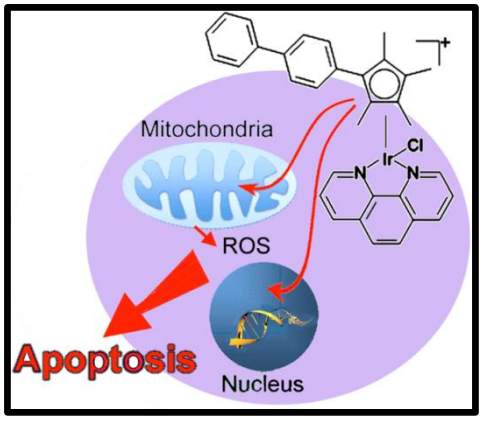

Figure 5. Schematic representation of mechanism of action and polypharmacology of anticancer half-sandwich organometallic Ir^{III} cyclopentadienyl complexes.

The share of the Department in the creation of this result:
The authors from the Department designed research, performed research, analyzed data and wrote the papers (the first and corresponding authors were from the Department, several authors were PhD students supervised at the Department). The authors from the collaborating foreign laboratories only supplied the metal complexes because of the lack of unit at the Institute having adequate equipment and experience in synthetic chemistry.

Selected relevant articles (authors from the Department are typeset in boldface):

Brabec, V., Griffith, D. M., **Kisova, A., Kostrhunova, H., Zerzankova, L.**, Marmion, C. J. and **Kasparkova, J.** (2012) Valuable insight into the anticancer activity of the platinum-histone deacetylase inhibitor conjugate, cis-[Pt(NH_3)$_2$malSAHA$_{-2H}$)]. *Mol. Pharmaceutics*, **9**, 1990-1999.

Novohradsky, V., Zerzankova, L., Stepankova, J., Vrana, O., Raveendran, R., Gibson, D., **Kasparkova, J.** and **Brabec, V.** (2014) Antitumor platinum(IV) derivatives of oxaliplatin with axial valproato ligands. *J. Inorg. Biochem.*, **140**, 72-79.

Novohradsky, V., Zerzankova, L., Stepankova, J., Kisova, A., Kostrhunova, H., Liu, Z., Sadler, P. J., **Kasparkova, J.** and **Brabec, V.** (2014) A dual-targeting, apoptosis-inducing organometallic half-sandwich iridium anticancer complex. *Metallomics*, **6**, 1491-1501.

Research Report of the team in the period 2010–2014

Institute	Institute of Biophysics of the CAS, v. v. i.

Scientific team	Department of Biophysical Chemistry and Molecular Oncology

(1) Electrochemistry of proteins

The beginning of electrochemical analysis of proteins dates back to 1930 when it was shown in J. Heyrovský Prague laboratory that proteins are able to catalyze hydrogen evolution at a mercury electrode producing a polarographic wave close to the background discharge. This wave was poorly developed and considered of little analytical use. Since the beginning of the 1970's, electrochemistry of proteins dealt predominantly with conjugated proteins containing non-protein redox centers yielding reversible electrode processes. This important field of protein electrochemistry was, however, limited to a very small fraction of proteins among the tens of thousands of proteins occurring in nature and studied by proteomics and biomedicine. Until recently it was generally accepted (e.g., F. A. Armstrong, Encyclop. Electrochem. Vol. 9, 2002), that adsorption of proteins at metal electrodes, such as gold, mercury, platinum and silver, leads to denaturation of the proteins. Using constant current chronopotentiometric stripping (CPS) analysis, we have shown that (a) close to the potential of zero charge, proteins

adsorbed at Hg-containing electrodes are not denatured; they can however denature due to a prolonged exposure to negative potentials, (b) proteins produce well-resolved electrocatalytic signal (peak H) at bare mercury electrodes regardless of the presence or absence of redox centers in their molecules, (c) Peak H differs from the previously studied electrochemical signals of proteins particularly (i) by its ability to detect proteins down to nanomolar and subnanomolar concentrations and (ii) by its high sensitivity to local and global changes in protein structures.

In 2010 we proposed thiol-modified Hg electrodes suitable for immobilization of a given protein (as with SAM-modified Au electrodes but providing a number of additional advantages) [1]. Using dithiothreitol (DTT)-modified mercury or solid amalgam electrodes [1-5] we studied effect of oncogenic mutations on the DNA-binding domain of the tumor suppressor p53 (this protein is usually kept under DTT) [3]. The CPS responses of wild-type and mutant p53 showed excellent correlation with structural and stability data and provided additional insights into the differential dynamic behavior of the proteins [3]. Further, we were able to monitor the loss of an essential zinc ion resulting from mutation (R175H) or metal chelation. We envisage that our CPS method can be applied to the analysis of virtually any protein as a sensor for conformational transitions or ligand binding to complement conventional techniques, but with the added benefit that only relatively small amounts of protein are needed and instant results are obtained. This work may lay the foundation for the wide application of electrochemistry in protein science, including proteomics and biomedicine. In addition to cancer research, CPS was applied in studies of proteins and peptides involved in neurodegenerative diseases.

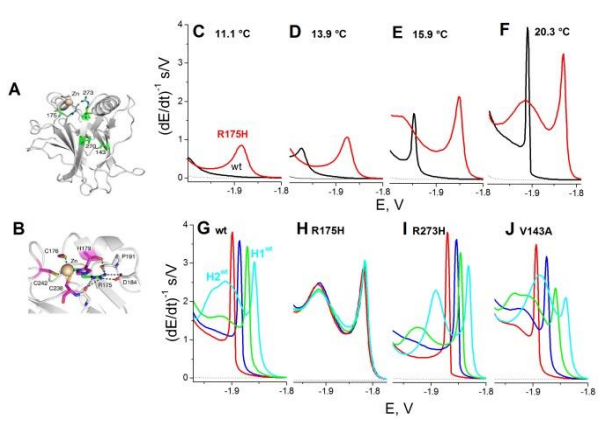

Fig. 1 Structure of DNA-binding domain of p53. **A.** Overall structure of T-p53C (PDB entry 1UOL). Sites of cancer mutations investigated in this study (V143A, R175H, F270L, and R273H) are highlighted as green stick models. **B.** Close-up view of the zinc coordination sphere, with the four zinc ligands shown in magenta. **C.-F.** CPS peak H of wild type T-p53C (black) and mutant R175H (red) at DTT-HMDE in 50 mM phosphate, pH 7 at **C.** 11.1 °C, **D.** 13.9 °C, **E.** 15.9 °C and **F.** 20.3 °C. **G.-J.** CPS peaks H of **G.** wt, **H.** R175H, **I.** R273H and **J.** V143A treated by 0 mM (red), 5 mM (blue), 10 mM (green) and 20 mM (cyan) EDTA at 0 °C for 10 min. CPS measurements were performed at 18 °C.

Foundations of electrochemistry of DNA and RNA were laid by the end of the 1950's and in 1960's at IBP (e.g., E.Palecek (1960). *Nature* **188**, 656, reviewed in [6]). Recently we have utilized specific properties of CPS in the analysis of DNA-protein complex nanolayers [5]. Rapid potential changes at high negative current intensities (I_{str}) in CPS are utilized in the analysis of DNA-protein interactions at DTT-modified mercury electrodes. P53 core domain (p53CD) sequence-specific binding to DNA results in a striking decrease in the electro-catalytic signal of free p53. This decrease is related to changes in the accessibility of the electroactive amino acid residues in the p53CD-DNA complex. By adjusting I_{str} and temperature, weaker non-specific binding can be eliminated or distinguished from the sequence-specific binding. The method also reflects differences in the stabilities of different sequence-specific complexes, including those containing spacers between half-sites of the DNA consensus sequence. The high resolving power of this method is based on the disintegration of the p53CD-DNA complex by the electric field effects at a negatively charged surface and fine adjustment of the millisecond time intervals for which the complex is exposed to these effects. Picomole amounts of p53 proteins and DNA were used for the analysis at full electrode coverage.

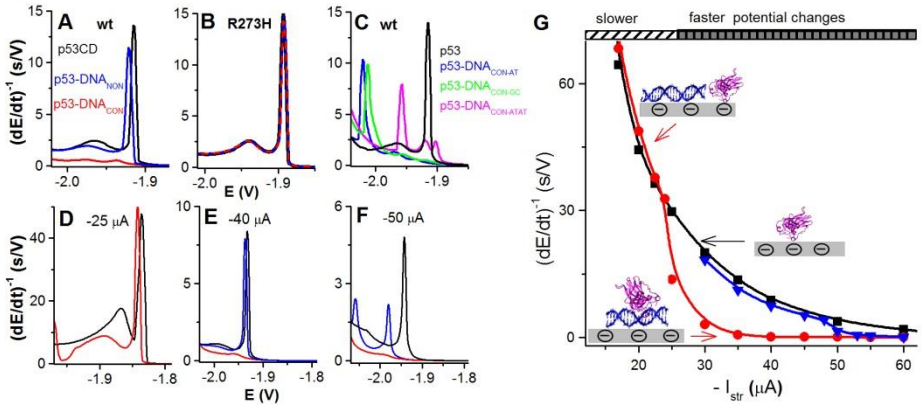

Fig. 2. A. Sequence-specific binding of wild type p53 core domain (p53CD) to dsDNA$_{CON}$ as detected by CPS at a DTT-modified hanging mercury drop electrode (DTT-HMDE). Free p53CD (black), sequence-specific p53CD-DNA$_{CON}$ complex (red) and mixture of p53CD with 40-mer dsDNA not containing the consensus sequence (blue, p53CD + dsDNA$_{NON}$), background electrolyte, 50 mM Na-phosphate, pH 7, (dashed line). **B.** Interaction of mutant p53CD R273H with dsDNA$_{CON}$ (showing no DNA binding). **C.** Peak H of p53CD (black) and p53CD complexes with spacer-containing DNAs: DNA$_{CON–GC}$ (green), DNA$_{CON–AT}$ (blue) and DNA$_{CON–ATAT}$ (magenta). Free p53CD R273H (black), p53CD R273H + dsDNA$_{CON}$ (red), p53CD R273H + dsDNA$_{NON}$ (blue); I_{str} –35 μA at 21 ºC. **D-F**. Peak H of p53CD (black), p53CD-DNA$_{CON}$ complex (red) and p53CD-DNA$_{NON}$ (blue) at I_{str} of **D**. –20 μA, **E.** –40 μA and **F.** –50 μA. **G.** Dependence of peak H1 height of free p53CD (black), p53CD-DNA$_{CON}$ complex (red) and p53CD-DNA$_{NON}$ (blue) on stripping current ($-I_{str}$).

Selected publications of 2010-2014:

[1] V. Ostatna, H. Cernocka, E. Palecek, Protein Structure-Sensitive Electrocatalysis at Dithiothreitol-Modified Electrodes, Journal of the American Chemical Society 132 (2010) 9408-9413.

[2] P. Juskova, V. Ostatna, E. Palecek, F. Foret, Fabrication and Characterization of Solid Mercury Amalgam Electrodes for Protein Analysis, Analytical Chemistry 82 (2010) 2690-2695.

[3] E. Palecek, V. Ostatna, H. Cernocka, A.C. Joerger, A.R. Fersht, Electrocatalytic Monitoring of Metal Binding and Mutation-Induced Conformational Changes in p53 at Picomole Level, Journal of the American Chemical Society 133 (2011) 7190-7196.

[4] V. Dorcak, V. Ostatna, E. Palecek, Electrochemical reduction and oxidation signals of angiotensin peptides. Role of individual amino acid residues, Electrochemistry Communications 31 (2013) 80-83.

[5] E. Palecek, H. Cernocka, V. Ostatna, L. Navratilova, M. Brazdova, Electrochemical sensing of tumor suppressor protein p53-deoxyribonucleic acid complex stability at an electrified interface, Analytica Chimica Acta 828 (2014) 1-8.

[6] E. Palecek, M. Bartosik, Electrochemistry of Nucleic Acids, Chemical Reviews 112 (2012) 3427-3481. According to WoS ESI: "As of September/October 2014 this highly cited review received enough citations to place it in the top 1% of its academic field based on a highly cited threshold for the field and publication year".

Papers under 1, 4, 5 were done solely at IBP. Paper 2 was done in collaboration with the Institute of Instrumental Analytical Chemistry AS CR, where electrode chips were prepared. Paper 3 was prepared in the frame of the EU 6th FP; p53 protein samples were isolated and characterized in the Cambridge A. Fersht's lab while all electrochemical measurements were done at IBP.

(2) Chemical modification and electroanalysis of sugar residues in biomacromolecules

It is estimated that 70 - 80% of proteins are glycosylated. It is thus very important to analyze glycans for better understanding of their role in the cell physiology and pathology and to develop novel and robust methods applicable in diagnostics. Electrochemical (EC) analysis of glycans is gaining increasing attention, providing exceptionally low limit of detections and in some cases also a label-free format of analysis. In 2014 we submitted a review on this topic, which was recently published [1].

Label-free detection of poly- and oligosaccharides based on catalytic hydrogen evolution

Until recently label-free direct EC reduction or oxidation of polysaccharides (PS) and longer oligosaccharides (OLS) under conditions close to physiological was not possible. In 2009 it was found that some sulfated PS are able to catalyze hydrogen evolution at Hg electrodes. Using CPS, carrageen H_{PS} peaks were obtained, resembling the CPS peak H produced by proteins. However, compared to unfolded proteins, much larger PS concentrations were necessary to obtain peak H_{PS}. Later it was shown that using adsorptive transfer (*ex situ*) stripping it was possible to adsorb sulfated PS directly from seawater and analyze them in buffered solutions. Very recent data suggest [2] that other types of OLS and PS, such as chitosan, catalyze hydrogen evolution reaction (CHER) as well. In the past decades chitosan has attracted great attention as a biodegradable biomaterial with interesting properties, such as anti-microbial, anti-inflammatory and anti-cholesterolemic activities making chitosan useful in biomedicine and various fields of practical life. Chitosan mostly exists as a random linear copolymer of D-glucosamine and N-acetyl-D-glucosamine units. Recently we have shown [2] that chitosan produces voltammetric and chronopotentiometric reduction peaks at mercury and solid amalgam electrodes (Figure 1A-C) in a wide pH range and can be determined down to concentrations of 1 μg/mL and below. Chitosan peaks occurring at highly negative potentials (Fig. 1) are well separated from the background discharge. These peaks were assigned to the CHER and were much larger than those of carrageenans, suggesting that chitosan is a much better catalyst than carrageenans.

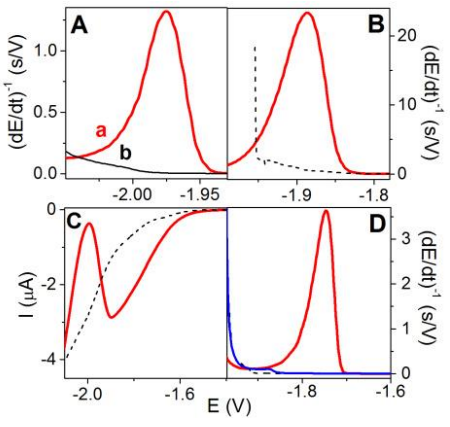

Fig. 1 A., **B.** CPS and **C.** SWV curves of chitosan at mercury electrodes. **A., C.** 10 μg/mL of chitosan at **A.**, **C.** HMDE and 15 μg/mL of chitosan at **B.** solid amalgam electrode; Accumulation time, t_A: 60 s, stripping current intensity, **A.** I_{str}: –70 μA **B.** I_{str}: –40 μA, **C.** frequency 20 Hz, **D.** CPS curves of 12 μM chitohexaose (red) and N, N', N'', N''', N'''', N'''''-hexaacetylchitohexaose (blue), t_A: 60 s (dashed), I_{str}: –40 μA. Background: 0.1 M sodium acetate, pH 5.2. [2].

It was shown that that free -NH_2 groups in glucosamine residues are responsible for the chitosan CHER. In agreement with this suggestion chemical deacetylation of the above chitin oligosaccharides resulted in voltammetric and chronopotentiometric peaks similar to those of chitosan. In weakly acid media chitosan hexamer was detectable at nM concentrations. In contrast to chitosan, PS containing acetylated glucosamine residues, such as hyaluronic acid, heparin and chondroitin sulfate, did not produce any significant reduction signal (not shown) even at much higher concentrations than that of chitosan (Fig. 1A). Finding of very strong ability of glucosamine-containing PS and OLS to

catalyze hydrogen evolution opens the door for a simple label-free EC analysis of PS and OLS, including glycans of glycoproteins. Such glycans frequently contain N-acetylated glucosamine residues (not producing CHER signals), which may become electroactive as a result of chemical or enzymatic deacetylation (Fig. 1D). Further work on this topic is in progress. These new approaches to the study of PS and OLS inspired private company Contipro Pharma a.s. (involved in the business with hyaluronic acids and other PS and OLS) with which we signed an agreement on a collaborative research.

Covalent labeling with Os(VI) complexes

Osmium tetroxide complexes, Os(VIII)L (L standing for a nitrogenous ligand), binding covalently to pyrimidine residues in single-stranded DNA and RNA (Fig. 2Aa), have shown their usefulness as probes of DNA structure *in vitro* and in cells. In contrast, six-valent Os(VI)L complexes were shown to bind ribose (but not deoxyribose) in nucleosides (Fig. 2Ab).

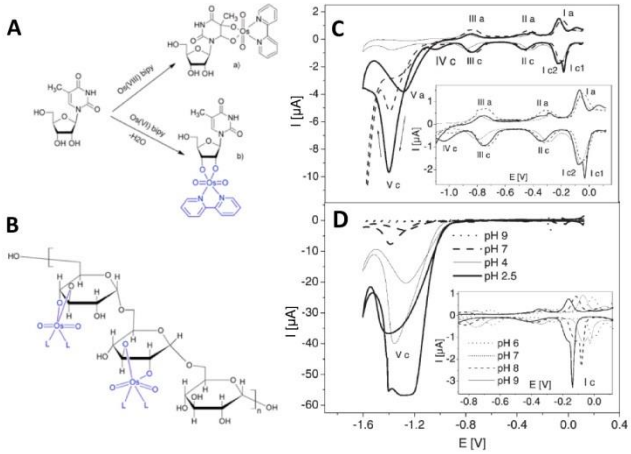

Fig. 2 A. Reaction of Os(VIII)L and Os(VI)L complexes with different parts of a nucleoside showing Os(VI)L complex specifically modifying ribose moiety. **B.** Fragment of Os(VI)L-modified dextran. Adsorptive stripping cyclic voltammograms of **C.** 10 mM (—) base-modified and (**▬**) sugar-modified thymine riboside, and (– – –) sugar-modified adenine riboside. **D.** Dependence on pH, 10 mM sugar-modified thymine riboside, HMDE, with stirring; Britton – Robinson buffer, pH 7.0; scan rate **C.** 2 V/s, **D.** 1 V/s; t_A 60 s; E_A 0 V, step potential 5mV.

We found that products of this reaction were electroactive, displaying redox couples on CV of Os(VI)L-modified ribosides (Fig. 2C) in a wide pH range (Fig. 2D) at mercury and carbon electrodes, similar but not identical to those of the base-Os(VIII)-modified ribosides. In addition to redox couples seen on CV, reaction products of some Os(VI)L complexes yielded electrocatalytic peaks (see peak Vc in Fig. 2C,D). Os(VI)L riboside reactions turned out to be useful for end-labeling of RNA and were utilized in sensing of microRNA [3-5].

We also showed that the above reactions yield a redox active product, which could be useful in EC analysis of PS and OLS [6]. Thus PS lacking any redox moiety can be transformed into electroactive species by their reaction with six-valent Os(VI)L complexes **(**Fig. 2B). Os(VI)L did not produce any electroactive adducts with DNA and proteins suggesting high specificity in the glycoprotein measurements. The reaction is very simple and does not require any special equipment. The complex can be only mixed with the carbohydrate at room temperature and the adduct is formed within hours. Using a ligand exchange process or elevated temperature (e.g. 75 °C) the reaction can proceed in about 15 min. In adsorptive transfer stripping (*ex situ*) experiments the electrode with strongly adsorbed PS adduct is washed to remove the weakly adsorbed Os(VI)L complex. The PS-modified electrode is then transferred to the electrolytic cell containing blank background electrolyte followed by voltammetric measurement. In this way the purification step is avoided and the PS adsorption can be performed from a small analyte drop (e.g., 3 - 10 μL, depending on the electrode size). Using adsorptive transfer stripping, abundance of monomeric carbohydrates (e.g. glucose) does not interfere with PS determination as the monomers can be easily washed away from the electrode [6].

Properties and EC behavior of PS-Os(VI)L adducts can be significantly influenced by the nature of the chosen ligand. For example, by using Os(VI)bpds (bpds = bathophenanthrolinedisulfonic acid) negative charges can be introduced in the PS or OLS adducts. Using Os(VI)bipy, electrocatalytic peaks can be obtained allowing determination of OLS at pM level. The reaction of Os(VI)temed producing PS and OLS adducts appears particularly interesting. These adducts can be determined using adsorptive stripping method (*in situ*) at mercury electrodes directly in the reaction mixture, without any purification step because free Os(VI)temed adsorbs very weakly on Hg electrodes. Recently we proposed modification of glycans directly in glycoproteins using Os(VI)L complexes followed by glycan voltammetric determination at carbon electrodes [7]. The electrochemical responses of two glycoproteins ribonuclease B and avidin as compared to their non-glycosylated counterparts were recorded directly in the reaction mixture. Hundreds of femtomoles of the glycoprotein were sufficient for the analysis. Our preliminary results suggest that using Hg electrodes much higher sensitivity can be obtained.

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Original papers under 2-3 and 6-7 were done solely at IBP. Papers under 4-5 were done in collaboration with the Masaryk Memorial Cancer Institute in Brno.

(3) Redox labeling of nucleic acids for electrochemical analysis of nucleotide sequences

Although nucleic acids are inherently electrochemically active due to reducibility or oxidizability of natural nucleobases, and a number of label-free electrochemical techniques have been applied in nucleic acids studies, various redox DNA labels have been developed for specific purposes (reviewed in [1,2]).

Earlier DBCMO significantly contributed in this area by introducing osmium tetroxide complexes as the very first electroactive markers for DNA (reviewed in [1]). These species form electroactive covalent adducts with pyrimidine (primarily thymine) residues in DNA and display a remarkable selectivity for unpaired or mispaired pyrimidines, making them excellent tools for probing DNA structure, detecting DNA damage and selective labeling of single-stranded DNA tails. In 2011 we utilized these features for a sensitive electrochemical detection of single-base mismatches and/or single-nucleotide insertions in DNA duplexes as a potential technique for a simple analysis of point mutations [3]. In 2014 we applied oxoosmium tail-

labelled probes to study protein-DNA interactions (see **(4)**). Analogous technique utilizing six-valent osmium complexes for the modification of sugar residues has been applied for 3'-terminal labeling of RNA, with applications in e.g., microRNA analysis (for more details see **(2)**).

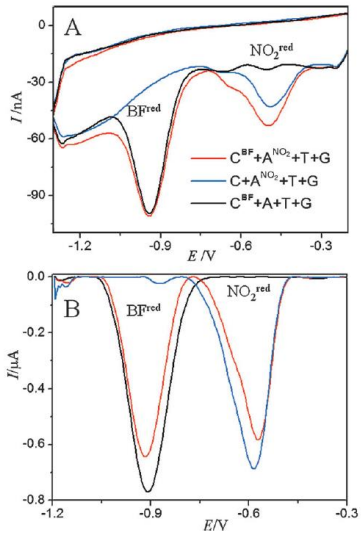

Fig. 1. Cyclic voltammograms measured at HMDE (A) and square-wave voltammograms measured at PGE (B) for DNA enzymatically synthesized with combinations of benzofurazan- (BF) and/or nitrophenyl- modified dNTPs with unmodified dNTPs, as indicated in the legend (valid for both panels). BF^{red} and NO_2^{red}: peaks due to reduction of the respective labels.

Another approach to DNA labeling has intensively been studied at DBCMO in a close collaboration with *prof. Michal Hocek group (Institute of Organic Chemistry and Biochemistry of the ASCR, Prague)* since 2006. These techniques consist of enzymatic (either sequence-specific, using template-dependent DNA polymerases, or statistical, using terminal transferases) incorporation of labelled nucleotides into DNA using nucleobase-modified deoxynucleotide triphosphates (dNTPs; designed and synthesized by the M.H. group) bearing redox tags such as ferrocene, osmium or ruthenium complexes, organic nitro- and amino derivatives and others (reviewed in [2]). The goal of this research is to develop a robust bioorthogonal system of redox coding of nucleobases generally applicable in nucleotide sequence analysis. In the period 2010-2014, the palette of useful redox labelled dNTPs has been extended by e.g., anthraquinone conjugates giving well defined redox electrochemistry (2011; [4]) and benzofurazan (2013; [5]), a new type of DNA label providing a high sensitivity of detection owing to an irreversible 6-electron reduction process (Fig. 1)

Along with the development and application of novel redox labels and labelled dNTPs, we have been studying their applicability in various techniques of construction of modified DNA. In 2011 we published a report [6] on the preparation of single-stranded tail-labelled DNA probes using terminal deoxynucleotidyl transferase (TdT), an enzyme attaching nucleotides at 3'-OH terminus of DNA without template i.e., in a statistical manner. We have shown that using nitrophenyl dNTP conjugates, reasonably long DNA tails can be prepared to attain remarkable amplification of the analytical signal, and first application of thus labelled probes in protein-DNA binding experiments were reported.

In 2012 we published [7] a study on the preparation of „reactive DNA" via incorporation of formylthiophene nucleotide conjugates to attach, in the second step, redox markers via formation hydrazones in reaction of the incorporated formyl groups in DNA and hydrazines bearing the ultimate labels (such as dinitrophenyl or nitrobenzofurazan). This approach is suitable particularly for preparation of densely modified PCR-amplified fragments which is problematic with bulky labels due to inhibition of the amplification reaction, but feasible with relatively small conjugate groups such as the formylthiophene. In this study we demonstrated usefulness of electrochemistry in microanalysis of the modification products and simple monitoring of conversion of the „reactive DNA" to the final modification product. Strengths of electrochemical techniques lie also in the possibility of conversion of an introduced label moiety (such as nitrophenyl) into a product of its electrochemical conversion (corresponding to hydroxylamine, a product of four-electron reduction of the nitro group that can be attained in a well-controlled way) offering a better resolution from another, simultaneously applied label (such as anthraquinone [4]). Via combination of different, independently detectable redox

labels (in publication [5], nitrophenyl and benzofurazan), we designed a new ratiometric approach of analysis of nucleotide sequences and evaluation of base substitutions in short DNA stretches, potentially applicable in mutation analysis. Detailed studies of the newly introduced DNA labels have resulted in extension of knowledge in the area of organic electrochemistry, particularly mechanisms of electrochemical processes of complex organic compounds [8].

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(4) Protein-DNA interactions

Protein-DNA interactions have systematically been studied at the DBCMO since the middle of 1990's when the supercoil-selective DNA binding of tumor suppressor protein p53 (a transcription factor involved in cell cycle control and particularly in defense of cells against malignant transformation) was discovered (published in Oncogene, 1997). Following this pilot study, we have significantly contributed to the knowledge about the effects of DNA conformation and/or topological state on the p53 protein-DNA recognition, roles of the p53 DNA binding domains and effects of various factors, such as redox conditions or metal ions etc. DNA superhelicity has been identified as an important factor influencing both sequence-specific and sequence non-specific p53-DNA binding. In 2010-2014 these studies were extended also to electrochemical methods and problems of stabilities of DNA-protein complexes at electrically charged surfaces.

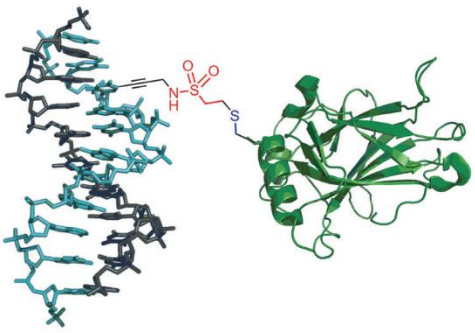
In 2010 we reported on the effects of DNA intercalating drugs (modulatoing the level of DNA supercoiling), such as chloroquin, acridine derivatives or doxorubicin, on the interactions of

wild type (wt) p53 protein with topologically constrained circular duplex DNA [1],. The p53 was found to lose gradually its preferential binding to negatively scDNA with increasing concentrations of intercalators until the DNA negative superhelix turns were relaxed, while formation of positive superhelices rendered the circular duplex DNA to be preferentially bound by the p53 again. In 2013, these studies were extended towards „hot spot“ p53 mutants (mutp53) exerting oncogenic gain-of-function activities [2]. We analyzed the interaction of seven hot spot mutp53 proteins with topologically different DNA substrates (supercoiled, linear and relaxed) containing and/or lacking mutp53-specific binding sites using a variety of electrophoresis and immunoprecipitation based techniques. All mutp53 proteins retained the ability of wildtype p53 to preferentially bind circular DNA at native negative superhelix density. Furthermore, we have shown for the first time by luciferase reporter assay that the DNA topology influences p53 regulation of BAX and MSP/MST1 promoters in cells.

In a series of studies (e.g., [3]) involving natural p53 binding sites differing in the level of internal symmetry, we have shown binding sites containing inverted repeats to bind the wt p53 protein with a higher affinity, compared to binding of the same protein to sites lacking this property. It has been concluded that the susceptibility of p53 response elements to form cruciform (stem-loop) structures is an important factor involved in preferential binding of the wt p53 to such sites under negative superhelical stress that stabilizes the alternative (cruciform) DNA conformation. Analogous abilities to recognize topologically constrained DNA and non-B DNA targets have been reported for other important cancer-related transcription factor such as IFI16 [4].

Development and application of novel methods for the protein-DNA binding studies, including those exploiting findings mentioned under results **(1-3)**, represents another important aspect of these studies. A technique utilizing constant current chronopotentiometric stripping of proteins and protein-DNA complexes at DTT-modified mercury electrodes is described under result **(1)** [5]. In 2010 we designed a label-free electrochemical test for DNA-binding activities of tumor suppressor protein p53 using immunoprecipitation (IP) at magnetic beads (MB) [6]. The technique relies on capture of the p53-DNA complexes at MB via anti-p53 antibodies, followed by selective salt-induced dissociation of linear DNA from the complex and its voltammetric detection. Compared to gel electrophoresis which is usually applied to analyze different plasmid DNA forms and their complexes with proteins, the electrochemical detection is faster and allows simpler quantitation of DNA containing free ends at submicrogram levels. In 2014 we improved this IP technique by introducing osmium tail-labelled oligonucleotide probes [7]. The probes were specifically recognized by the p53 protein according to the presence or absence of a specific binding site (p53CON) in the double-stranded segment and were successfully applied in titration as well as competition binding assays. To detect the p53-bound osmium-labelled probes, we took advantage of a catalytic peak yielded by osmium-modified DNA at the mercury-based electrodes (see **(2)**).

Other methods designed for the protein-DNA interaction studies utilize DNA labeling techniques developed in collaboration with *Hocek group*. In 2012, two types of environment-sensitive fluorophores, such as 4-aminophthalimide derivatives [8] were introduced into oligonucleotides to probe p53-DNA interactions. Significant enhancement of fluorescence was observed upon binding of the p53 DNA binding domain to the specific labelled probe. Another concept was designed in 2013 via



application of reactive, Michael acceptor (MA)-modified DNA probes for specific covalent protein-DNA conjugation [9]. The MA-modified DNAs readily react with cysteine or cysteine-containing peptides or proteins through Michael addition under physiological conditions. The MA-modified DNA was successfully used for covalent cross-linking with different constructs of p53 protein. Our study involving specific p53 mutants revealed a perfect correlation between the yield of the p53-DNA bioconjugate (Fig. 1), ability of the given construct to bind specifically DNA, and presence of a cysteine residue in a proper position at the protein-DNA interface, suggesting the new method generally applicable in studies of cysteine-containing proteins with DNA and in DNA proteomics.

Fig. 1: A bioconjugate of a reactive DNA probe and wt p53 core domain resulting from Michael addition of the protein thiol group on vinylsulfonamide moiety on the DNA.

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Research Report of the team in the period 2010–2014

Institute	Institute of Biophysics of the CAS, v. v. i.

Scientific team	Department of Molecular Epigenetics

1. Exploring the evolution of allopolyploid genomes using the Nicotiana model

Angiosperm evolution is heavily impacted by polyploidy, which has occurred in the ancestry of all, or most, species. It is thought that revolutionary changes in genome composition occur in early generations after the production of new polyploid lineages. The genus *Nicotiana* provides an excellent model group for such studies, since the genus consists of c. 70 species, and ~40% of these are thought to be polyploids derived from six independent polyploidy events, for which members of the diploid lineages giving rise to these polyploids are still extant. Here, we used molecular, cytogenetic and genomic approaches to address significant questions of genome dynamics following polyploidisation event. The major achievements can be summarised as follows:

a) The allotetraploid genome of *Nicotiana tabacum* (tobacco, <0.2 myrs old) shows evidence of sequences loss, particularly evident among the Ty3-*gypsy* retroelements, but also involving tandem repeats and 35S ribosomal DNA. Moreover the paternally (*N. tomentosiformis*) derived T-genome of tobacco shows evidence of erosion, while the

maternal (*N. sylvestris*) S-genome appears materially unchanged, indicating the possibility of preferential loss of paternally derived repetitive DNAs.

b) The allotetraploid genomes of ancient (5 myrs old) Nicotiana allopolyploids from section Repanda show complete loss of parental subtelomeric repeats and amplification of their novel variants. This may be considered as a hallmark of diploidisation processes that also involves erosion of low copy-number nuclear DNA. The genome size divergence is manifested through the differential removal and/or accumulation of high copy-number sequences.

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2. Molecular mechanisms of gene dosage control in interspecific hybrids and allopolyploids

Polyploidy, in which the entire chromosome complement multiplies (3x, 4x), is widespread in plants. When polyploidy occurs after interspecific hybridisation the process is called allopolyploidy. In allopolyploids, one manifestation of gene dosage control is nucleolar dominance, an epigenetic phenomenon in which the ribosomal rRNA genes (rDNA) of one progenitor are repressed. The results achieved mostly by studies of artificial lines or recently formed natural allopolyploids can be summarised as follows:

a) Epigenetic silencing of rRNA genes arrives immediately after formation of polyploid nucleus and associates with enhanced methylation of inactive polymerase 1 promoters

b) Genetic changes at rDNA locus (deletions and shifts in homeologous genes ratios) occur in later generations possibly after previous epigenetic modification

c) The silenced homeologs could be used in evolution to ameliorate loss and/or mutational damage of functional homeologs.

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3. The influence of cell dedifferentiation on epigenetic programming

Understanding processes leading to cell dedifferentiation and differentiation are in the centre of interest of both plant and animal physiologists. Here we studied the influence of cell dedifferentiation in *Nicotiana tabacum* (tobacco) plants, also known as callusogenesis (left margin of the Figure), on epigenetic modifications of transgenes. We also addressed the question of setting of methylation cofactors levels during plant development. The major achievements can be summarised as follows:

a) We showed that cell culture resulted in blurring of the parental epigenetic expression and methylation patterns at the silencing locus associated with increased epiallelic diversity.
b) Regenerated plants showed high interindividual but low intraindividual epigenetic variability, indicating that the callus induced epiallelic variants were transmitted to plants and became fixed.
c) Transcriptional silencing of transgenes carrying the 35S cauliflower promoter is always correlated with cytosine hypermethylation. The remaining two epigenetic marks, histone modification and small RNA production are more variable.
d) Accurate setting of methylation cofactors, S-adenosylhomocystein/S-adenosylmethionine is needed for plant development. Altered cofactor ratios result in severe developmental defects and aberrant expression of homeotic genes.

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4. Organisation and evolution of 5S and 35S rRNA genes in plants

Eukaryotic ribosomes are composed of 5S, 5.8S, 18S and 26S rRNAs plus about 70 proteins. While the 18S, 5.8S and 26S genes are always co-transcribed at 35S rDNA loci, the 5S rRNA is usually transcribed from separate loci (S-arrangement). However, linked 35S-5S rDNA (L-arrangement) also occurs in several phylogenetic groups. Here we studied chromosomal and genomic organisation of rDNA in several angiosperm and gymnosperm species. We also determined their expression pattern, epigenetic modification and chromatin condensation. The major achievements can be summarised as follows:

a) The results indicate that nearly 25% of Asteraceae (angiosperm) species may have evolved unusual linked arrangement (L-type) of 5S and 35S rRNA genes. We also showed that the L-type 5S genes harbour similar transcription level and epigenetic modification as their S-type counterparts.
b) An online resource, accessible at http://www.plantrdnadatabase.com, has been developed providing information on numbers and positions of ribosomal DNA signals and their structures for 1639 plant species (out of approximately 3000 listed accessions).
c) Phylogenetic relationships based on the comparison of 5S coding sequences suggest that the 5S genes independently inserted 35S unit at least three times in the course of gymnosperm evolution.

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5. Studies of RNA directed methylation

Post-transcriptional gene silencing of a primary target gene in plants can coincide with the production of secondary small interfering RNAs (siRNAs) of coding sequences adjacent to the target region and with *de novo* RNA-directed DNA methylation (RdDM) thereof. Here, we analyzed the susceptibility of transgenic and endogenous targets to RdDM induced by primary and secondary silencing signals. We also investigated mitotic and meiotic transmission of epialleles generated by siRNA signals in the absence of silencing trigger. The results obtained in different systems can be summarised as follows:

a) Both endogenes and transgenes are equally sensitive to transitive silencing, while differences exist in their susceptibility to undergo secondary RdDM.
b) Heritable trancriptional silencing (paramutation) of transgene targets can be induced by RdDM. Simple insertions of transgenes are less susceptible to generation of meotically heritable epialleles than loci harbouring inverted repeats of T-DNA.
c) Methylation inducing RNA species of 24-nt lengths are abundantly produced by centromeric repeats in *Fritillaria imperalis*, a species with a giant genome.

References:

Becher H, Ma L, Kelly L, Kovarik A, Leitch I, Leitch A (2014). Endogenous pararetrovirus sequences associated with 24 nt small RNAs at the centromeres of *Fritillaria imperialis* L. (Liliaceae), a species with a giant genome. *Plant Journal* 80(5):823-833.

Vermeersch L, De Winne, N, Nolf J, Bleys A, Kovarik A, Depicker A (2013) Transitive RNA silencing signals induce cytosine methylation of a transgenic but not an endogenous target. *Plant Journal* 74(5):867-879.

Crhak Khaitova L, Fojtova M, Krizova K, Lunerova J, Fulnecek J, Depicker A, Kovařík A (2011) Paramutation of tobacco transgenes by small RNA-mediated transcriptional gene silencing. *Epigenetics* 6(5): 650-660

Research Report of the team in the period 2010–2014

Institute	Institute of Biophysics of the CAS, v. v. i.

Scientific team	Department of Molecular Cytology and Cytometry

(1) **Analysis of protein acetylation and A-type lamin deficiency after DNA damage.** We have studied accumulation of epigenetically important proteins to UVA-damaged chromatin. We observed that heterochromatin-related proteins such as BMI1 and HP1β have an ability to recognize DNA lesions, and this nuclear event was dependent on acetylation state of a locally irradiated chromatin (Šustáčková et al., JCP, 2012). We have also analysed whether the OCT4 protein, as a pluripotency factor of ES cells, has the ability to recognize UVA-induced DNA lesions. In this case, we have observed that OCT4 visibly accumulates in the UVA-damaged chromatin immediately after irradiation, and that this process depends on the ATP and acetylation (Bartová et al., PLoS One, 2011). With regard to what we have discovered about accumulation of the above mentioned proteins to UV-induced DNA lesions being connected to hypoacetylation events, we have continued with our analyses by an inhibition of histone deacetylases (HDACs) that caused protein hyperacetylation. We have discovered that HDAC inhibitors, including Trichostatin A (TSA) or SAHA, prevent the accumulation of HP1β and OCT4 in UVA-induced DNA lesions. In addition, we have attempted to discover whether the function of A-type lamin is responsible for HP1β recruitment to DNA lesions. And, through the means of FRET analysis in DNA lesions, we have

addressed a question of whether HP1β interacts with other DNA damage-related proteins, including 53BP1 or BMI1. On the basis of the results of our experiments, we can summarize that when nuclear lamina is injured by UVA-irradiation, the fluorescence of mCherry-tagged A-type lamins disappears. However, the damage in nuclear lamina did not influence recruitment of DNA repair-related proteins, including HP1β, 53BP1 and BMI1. Only the 53BP1 status at DNA lesions was affected by A-type lamin deficiency. This was observed on the level of mCherry-53BP1 fluorescence at DNA lesions of A-type lamin deficient mouse embryonic fibroblasts (Sehnalová et al., BoC, 2014).

Relevant publications (2010-2014):

Šustáčková G, Kozubek S, Stixová L, Legartová S, Matula P, Orlova D, Bártová E. (2011) Acetylation-dependent nuclear arrangement and recruitment of BMI1 protein to UV-damaged chromatin. J Cell Physiol. 227(5):1838-50; IF=4.0.

Bártová E, Šustáčková G, Stixová L, Kozubek S, Legartová S, Foltánková V. (2011) Recruitment of Oct4 protein to UV-damaged chromatin in embryonic stem cells. PLoS One. 2011;6(12). IF=4.3.

Sehnalová, P., Legartová, S., Cmarko, D., Kozubek, S., and Bártová, E. (2014) Recruitment of HP1 beta to UVA-induced DNA lesions is independent of radiation-induced changes in A-type lamins, Biology of the Cell 106, 111-161. IF=3.87.

(2) **Function of proteins of Cajal bodies and nucleoli during DNA repair events.**

Cajal bodies (CBs) are important nuclear structures, which contain proteins that preferentially regulate RNA-related metabolism. We have investigated the cell-type specific nuclear distribution of Cajal bodies and the level of coilin, a protein, which is inherent in Cajal bodies, in non-irradiated and irradiated human tumor cell lines and embryonic stem (ES) cells. Cajal bodies were localized in various nuclear compartments, including DAPI-poor regions, in the proximity of chromocenters, and adjacent to nucleoli. We have also studied the localization of coilin in UVA-induced DNA lesions in detail. We have discovered that coilin, in contrast to SMN (another protein, inherent in CBs), is recruited to DNA lesions induced by UVA-irradiation. Coilin recruitment to UVA-damaged chromatin was cell cycle-independent and occurred immediately (15-20 s) after local micro-irradiation by an UVA laser. We have additionally observed that γ-irradiation-induced foci (IRIF) did not co-localize with CBs, and the exposure to γ-rays did not alter total coilin levels as studied by western blots. However, localized movement of coilin-positive Cajal bodies was significantly reduced by exposing the cells to γ-rays. Together, coilin was rapidly recruited to DNA lesions, which can be explained as a primary stress response. Coilin at genomic lesions might attract other proteins involved in DNA repair-related pathways, which appear in this radiation-injured chromatin later. It is well known that radiation can simultaneously induce multiple DNA damage-response pathways, which could explain why we have observed cell cycle-independent coilin recruitment to DNA lesions (Bártová et al., Nucleus, 2014).

We have also investigated the responses of nucleoli to UVA- and γ-radiation. We have focused on the accumulation patterns of UBF1 and HP1β. The majority of analyses was performed on live cells by a time-lapse confocal microscopy (see Fig. 1). Radiation is considered to be a genotoxic agent that induces genomic DNA lesions, accompanied by changes in nuclear and nucleolar morphology. Different types of radiation can cause nucleolar segregation, relocation of nuclear proteins, and changes in protein post-translational modifications. For example, we have observed that actinomycin D, an inhibitor of RNA polymerase I, or a delayed UBF1 recruitment to nucleoplasmic DNA lesions (Stixová et al., Epigenetics and Chromatin, 2014). Our results indicate that the nucleolus and the nucleolar UBF1 protein are important components of the cellular response to genome injury.

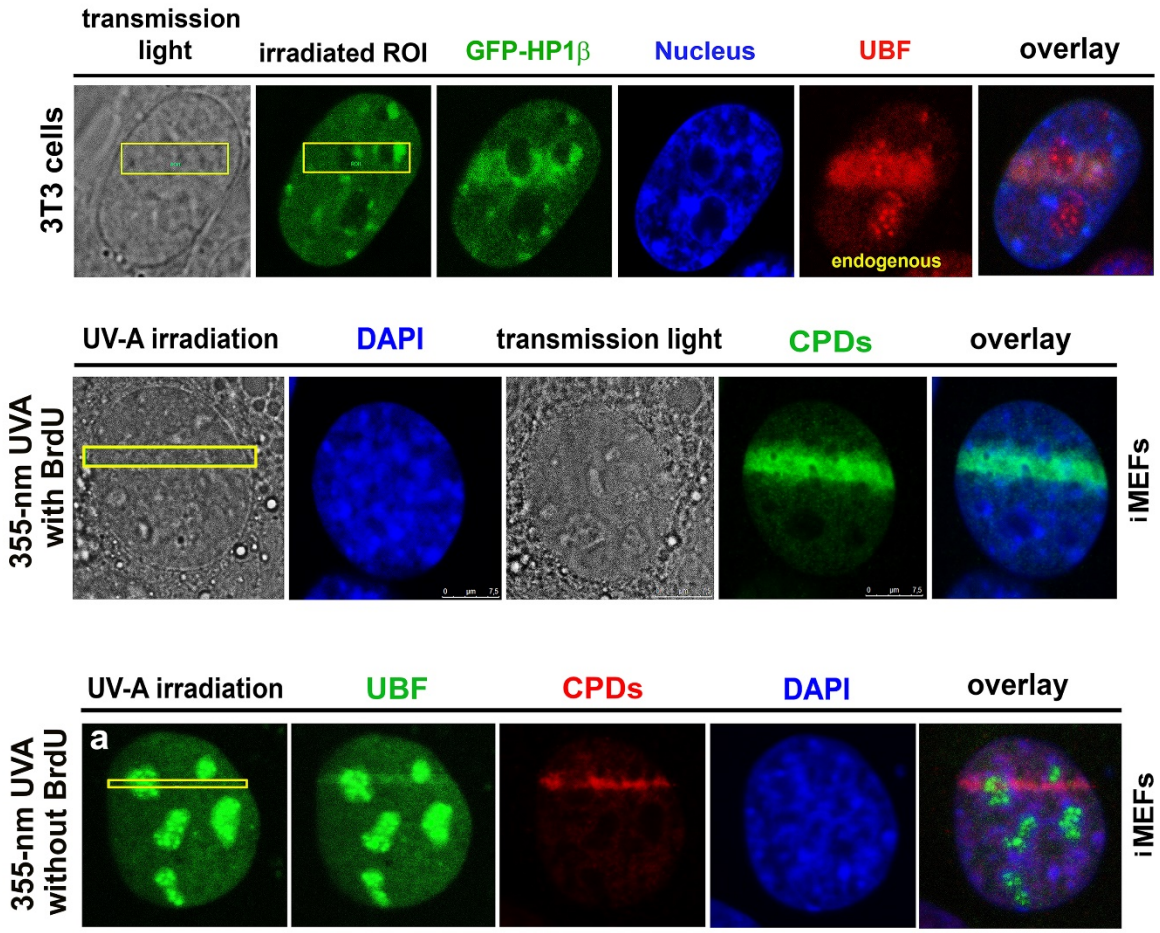


Fig. 1. Selected proteins studied at locally induced DNA lesions. (A) Recruitment of GFP-HP1β (green) and UBF1 protein (red) to UVA-irradiated region of interest (ROI, yellow frame). (B) ROIs, irradiated by a UVA-laser (of the wavelength of 355nm), were positive on cyclobutane pyrimidine dimers (CPDs) (green). (C) GFP-UBF1 (green) recruited only to CPDs-positive DNA lesions (red). DNA was visualized by DAPI staining (blue) and cell nuclei were irradiated under transmitted light (grey).

Our data show that UBF1 protein interacts with HP1β, and after UVA irradiation, both proteins co-localize with cyclobutane pyrimidine dimers (CPDs). A chromoshadow domain of HP1β, which has a dominant-negative effect on UBF1 recruitment to DNA lesions, is responsible for this radiation-induced event. Intriguingly, both UBF1 and HP1β appeared and co-localized especially at DNA lesions induced outside the nucleolus. This DNA damage response might be associated with extensive chromatin conformational changes induced by UVA radiation. Together, our results indicate that UBF1 recruitment to DNA lesions is probably dependent on CPDs. Moreover, HP1β interacts with UBF1 in DNA lesions, as shown by immunofluorescence and confirmed by FRET analysis (Förster or Fluorescence Resonance Energy Transfer) or HP1β siRNA experiments (Stixová et al., Epigenetics and Chromatin, 2014).

Relevant publications (2010-2014):

Bártová, E., Foltánková, V., Legartová, S., Sehnalová, P., Sorokin, D. V., Suchánková, J., and Kozubek, S. (2014) Coilin is rapidly recruited to UVA-induced DNA lesions and gamma-radiation affects localized movement of Cajal bodies, Nucleus-Austin 1, 269-277. IF=3.15

Stixová L., Sehnalová P., Legartová S., Suchánková J., Hrušková T., Kozubek S., Sorokin DV, Matula P., Raška I., Kovařík A., Fulneček J., Bártová E. (2014) HP1β-dependent recruitment of UBF1 to irradiated chromatin occurs simultaneously with CPDs. Epigenetics & Chromatin, 7(1):39. IF=4.46

(3) **Changes in histone post-translational modifications during cell differentiation and apoptosis and cell treatment by inhibitors of histone deacetylases.** We have studied histone acetylation patterns, particularly in ESCs undergoing differentiation, because the results of our earlier experiments demonstrated that endoderm-like differentiation of hESCs is accompanied by a reduced H3K9 acetylation (Krejčí et al. JCP, 2009). In hESCs, a high level of histone acetylation, a marker of decondensed chromatin, should correspond to a high transcription level, which is reduced in differentiated ESCs. These nuclear events also reflect changes in the nuclear pattern of heterochromatin markers, including heterochromatin protein 1 (HP1). Subtypes of HP1 are highly dynamic and homogenously dispersed in the nuclei of hESCs, whereas HP1α accumulates within clusters of centromeric heterochromatin, called chromocenters, during the differentiation of hESC (Bártová et al. Dev. Dyn. 2008). However, as a result of general clustering of acrocentric chromosomes in these pluripotent cells, mouse ESCs are completely different in this regard.

On the basis of this knowledge, we have investigated the acetylation status of histones H2A, H2B, and H4 during cellular differentiation induced in mouse and human embryonic stem cells (ESCs) and during enterocytic differentiation induced *in vitro* by HDAC inhibitor, sodium butyrate (NaBt). For such analyses, we for example used matrix-assisted laser desorption/ionization time of flight (MALDI-TOF), mass spectrometry (MS) and western analyses in order to identify specific histone markers in selected lysine residues. These analyses revealed differentiation-specific histone signatures, particularly at the level of detailed acetylation profiles. For example, endoderm-like differentiation of mouse ESCs or enterocytic differentiation induced by NaBt was accompanied by increased mono-, di-, and tri-acetylation of histone H2B and a visible increase in di- and tri-acetylation of histone H4. In enterocytes, the mono-acetylation of histone H2A also increased and the tetra-acetylation of histone H4 appeared only after induction of this differentiation pathway. During the differentiation of hESCs, we have observed an increased mono-acetylation and a decreased tri-acetylation of H2B. Mono-, di-, and tri-acetylation of H4 were reduced, manifested by a significant increase in nonacetylated H4 histones. Majority of data originate from MALDI-TOF MS (Legartová et al., Biochem. Cell Biol, 2014).

We have also studied histone signature in apoptotic cell nuclei that are characterized by a highly condensed chromatin. We have discovered that the pronounced histone hypoacetylation and the high level of H3K9 methylation in apoptotic bodies is probably essential for the dynamic exchange of HP1α protein in the apoptotic nuclei. Relatively fast recovery after photobleaching (FRAP analysis) was discovered in case of GFP-tagged HP1α in apoptotic bodies. On the other hand, diffusion properties were limited (no recovery after photobleaching was found) for the histone demethylase JMJD2b tagged by GFP or Polycomb group-related protein, GFP-BMI1 (Legartová et al., Biochime, 2012).

Relevant publications (2010-2014):

*Legartová, S., Kozubek, S., Franek, M., Zdrahál, Z., Lochmanová, G., Martinet, N., and Bártová, E. (2014) Cell differentiation along multiple pathways accompanied by changes in histone acetylation status, Biochem. Cell Biol. 92, 81-93. IF=2.35**

*Legartová S, Jugová A, Stixová L, Kozubek S, Fojtová M, Zdráhal Z, Lochmanová G, Bártová E. (2012) Epigenetic aspects of HP1 exchange kinetics in apoptotic chromatin. Biochimie, 95(2):167-79, IF=3.14**

The above mentioned results were obtained in experiments realized mainly by the members of the Department of Molecular Cytology and Cytometry, IBP AVCR, v.v.i. The underlined authors are IBP employees.

(4) Pathological changes of chromatin structure associated with leukemia. We have discovered that maturation of chromatin determines the ability of neutrophils to release chromatin NETs and loss of DNA damage response; these properties are absent in immature neutrophils of patients suffering from acute myeloid leukemia (AML). Details are shown in Figure 1.

The importance of the higher-order chromatin structure in regulating and mediating fundamental vital processes in cells has been recognized in the past decade. We were interested in how chromatin structure changes with granulocyte differentiation and how this process is influenced in AML patients.

It is known that upon an immune activation, neutrophils fire chromatin nets that serve as traps (NETs; neutrophil extracellular traps), which are capable of immobilizing and inactivating the pathogens. We have discovered that neutrophils from AML patients show, in a variable extent, features of incomplete chromatin differentiation. The protein HP1 is replaced with the serpin MNEI only partially and histone 3 dimethylated at lysine 9 (H3K9me2) can be still immunodetected in heterochromatin. This chromatin immaturity is reflected in more 'open' higher-order chromatin structure of AML neutrophils and, importantly, precludes their ability to form NETs. Thus, it appears that the purpose of changes in chromatin structure during the neutrophil differentiation is to enable the unique function of these cells in the immune defense.

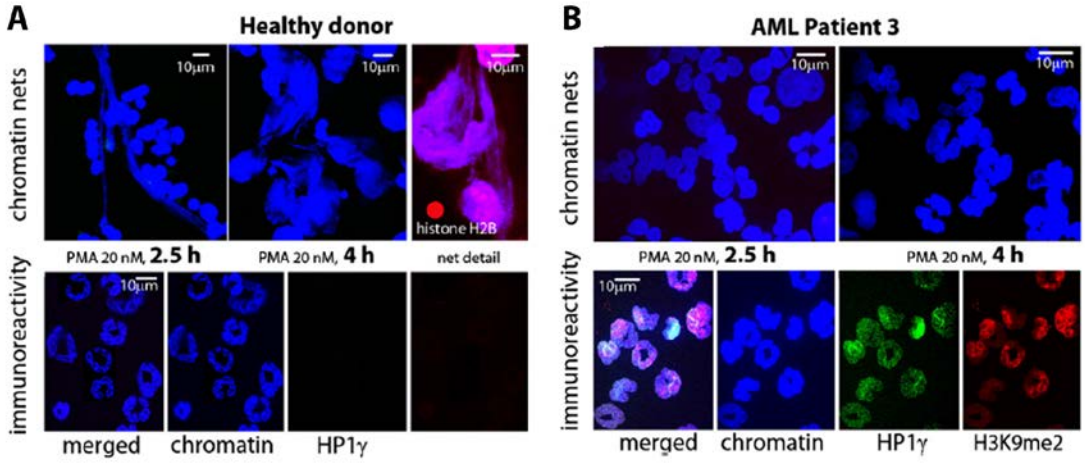


Fig. 2. Neutrophils from healthy donors **(A)** express neither HP1γ nor H3K9me2 markers of immature heterochromatin and efficiently form NETs after activation with PMA. On the contrary, neutrophiles from AML patints **(B)** express the both markers and are unable to fire NETs. (Taken from Lukasova et al., BBA – MCR 1833 (3): 767-779, 2013)

Moreover, AML treatment does not always lead to complete maturation of granulocytes, even if it restores the level of total blood cells including granulocytes to the normal values. This can have a direct impact on the ability of AML patients in remission to fight bacterial infection. Bacterial and fungal infections are an important cause of mortality during AML therapy.

We have also revealed that the repair of the DNA double strand breaks (DSB) is down-regulated in normal neutrophils but not in immature neutrophils from AML patients. The short lifespan of neutrophils and the specific role of their chromatin in forming NETs seem to be the reasons for silencing of genes participating in DSB repair in mature neutrophils. Obviously, the

conformational transition of chromatin during NET formation does not require the integrity of genome and therefore the DSBs need not to be repaired.

We propose the immunodetection of HP1 (and DSB repair proteins) to be a sensitive method to monitor AML neutrophil maturation and functionality in clinical practice.

The Institute of Biophysics of ASCR (IBP) contributed to this work fundamentally. The idea was proposed by Dr. Lukasova from IBP and also almost all experiments were performed at IBP with an exception of the flow-cytometry and *in vitro* differentiation of CD34+ cells (also followed in our study in order to reveal whether these cells can fully differentiate under *in vitro* conditions-4).

Relevant publications:

Lukášová E, Kořistek Z, Klabusay M, Ondřej V, Grigoryev S, Bačíková A, Řezáčová M, Falk M, Vávrová J, Kohútová V, and Kozubek S (2013) *Granulocyte maturation determines ability to release chromatin NETs and loss of DNA damage response; these properties are absent in immature AML granulocytes*. BIOCHIMICA ET BIOPHYSICA ACTA – MOLECULAR CELL RESEARCH 1833 (3): 767-779

(5) Biological effects of ionizing radiation and new mechanism giving rise to chromosomal translocations. We have discovered that higher-order chromatin structure influences many aspects of DNA double strand break (DSB) induction and repair and also has multiple impacts on the mechanism of how chromosomal translocations are formed. Interactions with chromatin and therefore biological effects differ for ionizing radiations of different quality. We have proposed new model on the relationship between the above-mentioned phenomena.

The 'Position-First Hypothesis' (PFH) and 'Breakage-First Hypothesis' (BFH) represent two different attempts to explain the mechanism of how chromosomal translocations could be formed. According to the PFH, translocations appear only between genetic loci that have been in close mutual proximity already before the induction of DNA double-strand breaks. On the contrary, the BFH assumes an increased chromatin movement at the sites of DSB; hence, translocations may appear also between originally distant loci. With important outputs on the formation of complex chromosomal translocations, the PFH suggests that DSBs are repaired individually, at the sites of their origin, whereas the BFH supposes DSBs to migrate into putative repair factories where several DSBs are repaired together. Not surprisingly, these hypotheses have been widely regarded as mutually exclusive.

However, we have discovered that both of these mechanisms, although greatly modified, participate in formation of translocations. Our results for low-LET γ-rays showed that DSB repair takes place, in principle, at the site of its induction. Spatial organization of the genome thus predetermines the initial nuclear positions and limits the 'movement' of individual damaged loci as well as probabilities of their mutual interactions. Up to this point, the mechanism of chromosomal translocation corresponds to the PFT. However, we have revealed that heterochromatin must decondense at the sites of DSB to allow DSB repair and – due to this extensive decondensation – some DSBs show an increased mobility. Heterochromatic DSBs move out of heterochromatin domains and, in some cases, two or (rarely) more damaged loci mutually cluster, regardless their originally larger spatial separation (see Fig. 3).

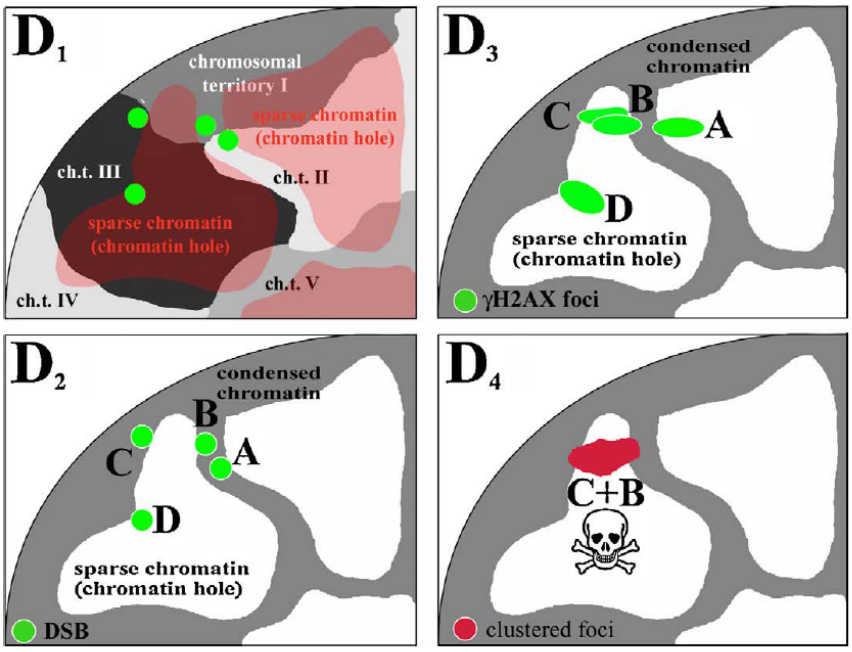


Fig. 3. Proposed model of the relationship between the higher-order chromatin structure, DSB repair and a formation of chromosomal translocations. **(D1)** Schematic location of chromosomal territories that could be subject to chromatin exchange during DSB repair. **(D2)** The higher-order chromatin structure and Brownian movement of chromatin determine the original radius of mutual DSB (gH2AX foci, green) interactions. Heterochromatin between A and B prevents their mutual interaction. **(D3)** Chromatin decondensation at sites of DSBs induced at the boundary of eu- and heterochromatin can significantly increase (foci B and C) or decrease (foci A and B) the original probability of interactions between DSBs. **(D4)** Foci B and C are at the highest risk of chromatin exchanges despite the shortest nuclear distance being between foci A and B. (Taken from Falk et al., Mutation Research – Reviews in Mutation Research 704(1–3): 88-100, 2010)

This scenario is closer to BFH; however, with a principal difference – rather than repair factories, DSB clusters represent the sites with an increased risk of giving rise to translocations. Importantly, we have shown that not only the mutual distances between DSBs, but also their distribution, relative to the local nuclear architecture (i.e. mutual organization and structure of high- and low-density chromatin domains) influence the interaction probabilities between particular genetic loci substantially. For instance, even if two damaged loci are particularly close to each other, the probability of their interaction (and chromatin translocation) is very low if they occupy the opposite sites of a heterochromatic domain and thus are forced to protrude into different low-density chromatin subdomains of the nucleus during the repair. On the other hand, we may expect this probability to be much higher for other two loci, more distant from each other, but protruding (due to their mutual location) into the same low-density chromatin domain.

Hence, the model of formation of chromosomal translocations we propose takes aspects of both PF and BF hypotheses and also incorporates new important features, such as the role of local higher-order chromatin structure. In addition to the above-described secondary DSB clusters that appear during DNA repair processes, a huge number of primary clusters arise in cells irradiated with high-LET radiations as a consequence of a localized high energy deposition and chromatin fragmentation. Hence, the manner of how the primary and secondary clusters and also the higher-order DSB clusters (secondary assembled from primary clusters) contribute to formation of chromosomal translocations largely depends on the quality of the radiation.

The idea of the new mechanism of chromosomal translocations described above was proposed by M. Falk from IBP ASCR. Also, all experiments for γ-rays and consequent data analyses and results

interpretation were exclusively performed at IBP (a contribution of 100%). Many people participated on technologically extremely difficult experiments with proton beam and ion irradiations; this explains a broad collective of authors contributing to our publication Falk et al. (2014). However, except the irradiation *per se*, the vast majority of experiments and data analyses were performed by IBP ASCR (a contribution of about 90%).

Relevant publication:

Falk M, Lukasova E and Kozubek S (2010) *Higher order chromatin structure in DSB induction, repair and misrepair*. MUTATION RESEARCH/REVIEWS IN MUTATION RESEARCH 704(1–3): 88-100

Falk M, Lukasova E, Kozubek S (2012) *Repair mechanisms of DNA double-strand breaks – Biochemical and Spatio-Temporal Aspects*. In: RADIATION DAMAGE IN BIOMOLECULAR SYSTEMS, pp. 329-359, Springer Science+Business Media, ed. García G, Fuss M, ISSN 1618-7210, ISBN 978-94-007-2563-8, e-ISBN 978-94-007-2564-5, DOI 10.1007/978-94-007-2564-5_20, Dordrecht, Heidelberg, London, New York

Falk M, Hausmann M, Lukášová E, Biswas A, Hildenbrand G, Davídková M, Krasavin E, Kleibl Z, Falková I, Ježková L, Štefančíková L, Ševčík J, Hofer M, Bačíková A, Matula P, Boreyko A, Vachelová J, Michaelidisová A, Kozubek S (2014) *Giving OMICS spatiotemporal dimensions using exciting new nanoscopy techniques to assess complex cell responses to DNA damage – PART B (Structuromics)*. CRITICAL REVIEWS IN EUKARYOTIC GENE EXPRESSION 24(3): 225-247

(6) Structure, function and regulation of telomeres.

Telomere evolution in green algae is far more dynamic and complex than thought before; human-type repeat is the most common and possibly ancestral in eukaryotes; myb-like AtTRB proteins in *Arabidopsis thaliana* play a role in telomere length regulation and telomerase recruitment to plant telomeres**.** Our results on evolution of telomeric sequences support the view that the Arabidopsis-type telomeric sequence is ancestral for green algae and has been conserved in most lineages. However, within the Chlamydomonadales, at least two independent evolutionary changes to the Chlamydomonas type have occurred, specifically in the subgroup of the Reinhardtinia clade and in the Chloromonadinia clade. Furthermore, a complex structure of telomeric repeats, including a mix of the ancestral Arabidopsis-type motifs and derived motifs identical to the human-type telomeric repeats (TTAGGG), has been discovered in the chlamydomonadalean clades Dunaliellinia and Stephanosphaeria. Our results indicate that telomere evolution in green algae, particularly in the order Chlamydomonadales, is far more dynamic and complex than thought before. The following more extensive study on eukaryotes confirmed the human-type repeat as the most common and possibly ancestral in eukaryotes, but alternative motifs replaced it along the phylogeny of diverse eukaryotic lineages, some of them independently for several times. Cell-cycle dependent regulation of telomere maintenance was described in green algae.

The research of telomere evolution in lower plants was performed dominantly at the Institute of Biophysics based on Czech Science Projects, which were done in this institution (algae cultivation, telomere analyses, telomerase activity assays). Collaboration with Masaryk University was used to quantify telomerase activity during cell cycle.

Further, we have revealed a role of the structure-function relationship in regulation of plant telomerase by a telomerase transgenic expression and reconstitution and described distinct epigenetic marks at terminal versus interstitial telomeric repeats.

The research was performed in collaboration with Masaryk University. The team at the Institute of Biophysics contributed substantially with the design of all experiments, constructs used for transgenic experiments and molecular-biology assays used in telomere analyses and protein-protein interactions.

Finally, we have described the role of myb-like AtTRB proteins in *Arabidopsis thaliana* (which show telomeric and nucleolar localization at Arabidopsis thaliana chromosomes) in telomere length regulation and telomerase recruitment to plant telomeres.

This research was performed in collaboration with Masaryk University and the team at the Institute of Biophysics contributed with molecular-biology assays as Southern hybridization, telomere and bioinformatic analyses.

Relevant publication:

Fojtová M, Peška V, Dobšáková Z, Mozgová I, Fajkus J, Sýkorová E (2011) *Molecular analysis of T-DNA insertion mutants identified putative regulatory elements in the AtTERT gene.* JOURNAL OF EXPERIMENTAL BOTANY 62: 5531-5545

Majerova E, Fojtova M, Mozgova I, Bittova M & Fajkus J (2011) *Hypomethylating drugs efficiently decrease cytosine methylation in telomeric DNA and activate telomerase without affecting telomere lengths in tobacco cells.* PLANT MOLECULAR BIOLOGY 77: 371-380

Fulnečková J, Hasíková T, Fajkus J, Lukešová A, Eliáš M, Sýkorová E (2012) *Dynamic Evolution of Telomeric Sequences in the Green Algal Order Chlamydomonadales.* GENOME BIOLOGY AND EVOLUTION 4: 248-264

Schrumpfová P, Vychodilová I, Dvořáčková M, Majerská J, Dokládal L, Schorová Š, Fajkus J (2014) *Telomere repeat binding proteins are functional components of Arabidopsis telomeres and interact with telomerase.* PLANT JOURNAL 77: 770-781

Ogrocká A, Polanská P, Majerová E, Janeba Z, Fajkus J, Fojtová M (2014) *Compromised telomere maintenance in hypomethylated Arabidopsis thaliana plants.* NUCLEIC ACIDS RESEARCH 42: 2919-2931

(7) Roles of HMGB1 protein in regulating chromatin structure and important cellular proteins and processes.

We have demonstrated that redox state of HMGB1 can modulate the ability of the protein to bind and bend DNA and that HMGB1 and HMGB2 modulate cellular activity of mammalian telomerase in a different manner.

We have demonstrated that redox state of HMGB1 can significantly modulate the ability of the protein to bind and bend DNA. We have also shown that reduced HMGB1 could displace histone H1 from DNA, while oxidized HMGB1 had a limited capacity for H1 displacement. Our results suggested a novel mechanism for modulation of histone H1 binding to DNA by HMGB1. This result contributes to our better understanding of HMGB1/H1 interplay in the functioning of chromatin.

Further, for the first time, we have reported an evidence that HMGB1 (a chromatin-associated protein in mammals, acting as a DNA chaperone in transcription, replication, recombination, and repair) could modulate cellular activity of mammalian telomerase. Knockout of the *HMGB1* gene (*HMGB1* KO) in mouse embryonic fibroblasts (MEFs) resulted in chromosomal abnormalities, enhanced co-localization of γ-H2AX foci at telomeres, and a moderate shortening of telomere lengths. *HMGB1* KO MEFs also exhibited a significantly (>5-fold) lower telomerase activity than the wild-type MEFs. Interestingly, knockout of the *HMGB2* gene elevated telomerase activity (~3-fold) in MEFs, suggesting that the two closely related proteins of the HMGB family, HMGB1 and HMGB2, had opposite effects on telomerase activity in the cell. Our results shed more light on some aspects of the HMGB proteins involvement in chromosome stability and cancer.

IBP ASCR contributed to obtaining this result dominantly. From 5 coauthors of the publication Polanska et al. (2012), the first author and the first corresponding author have an affiliation only to IBP CAS, the second corresponding author has an affiliation to IBP CAS and one other institute, and one of the 2 remaining coauthors also belongs to IBP. From 3 coauthors of the publication Polanska et al. (2014), the first author and the corresponding author are from IBP.

Relevant publication:

Polanská E, Dobšáková Z, Dvořáčková M, Fajkus J, Štros M (2012) *HMGB1 knockout in mouse embryonic fibroblasts results in reduced telomerase activity and telomere dysfunction*. CHROMOSOMA 121: 419-431

Polanská E, Pospíšilová Š, and Štros M (2014) *Binding of Histone H1 to DNA is Differentially Modulated by Redox State of HMGB1*. PLOS ONE 9: e89070

(8) The role of adenosine receptor signaling in murine hematopoiesis.

In mice, which were pharmacologically pre-treated with IB-MECA (a selective adenosine receptor A3AR agonist) and exposed to sub-lethal doses of ionizing radiation, we have observed that this drug potentiates hematopoiesis-stimulating effects of granulocyte colony-stimulating factor and meloxicam, a selective cyclooxygenase-2 inhibitor. These findings not only provide new data on regulation of hematopoiesis, but they may also have a clinical impact.

With regard to earlier obtained results on stimulatory action of an agonist of A_3ARs on radiation-suppressed hematopoiesis, we studied the hematopoiesis in A_3AR knock-out mice. We have discovered that in the peripheral blood of the A_3AR knock-out mice, defects of some parameters of mature blood cells appear. Mechanisms aimed at compensation of these defects stimulate proliferation of bone marrow cells responsible for production of functional blood cells. We have determined an interesting and currently not sufficiently explained discovery of an enhanced survival of lethally irradiated A_3AR knock-out mice in comparison with normal mice. This increased radioresistance of the A_3AR knock-out mice is the content of further hypotetical studies.

IBP CAS contributed to these findings fundamentally (>95 %), as it can be seen from the data included in the publications listed below. Except for the statistical analyses, the experiments and their interpretation have been performed at the IBP. In addition, in the evaluated period of time (2010 – 2015), the members of IBP stand as the first and/or corresponding authors of nineteen from twenty-two articles dedicated to this topic.

Relevant publication:

Hofer M, Pospíšil M, Šefc L, Dušek L, Vacek A, Holá J, Hoferová Z, Štreitová D (2010) *Activation of adenosine A3 receptors supports hematopoiesis-stimulating effects of granulocyte colony-stimulating factor in sublethally irradiated mice.* International Journal of Radiation Biology 86: 649-656

Hofer M, Pospíšil M, Dušek L, Hoferová Z, Komůrková D (2014) *Lack of adenosine A_3 receptors causes defects in mouse peripheral blood parameters.* Purinergic Signalling 10: 509-514

Hofer M, Pospíšil M, Dušek L, Hoferová Z, Komůrková D (2015) *Enhanced survival of lethally irradiated adenosine A_3 receptor knockout mice. A role for hematopoietic growth factors?* Purinergic Signalling 11: 79-85 (accepted for publication in 2014)

Research Report of the team in the period 2010–2014

Institute	Institute of Biophysics of the CAS, v. v. i.

Scientific team	Department of Cytokinetics

During the evaluation period, **our principal strategy was to support in particular younger researchers and to allow them to establish their own independent teams** tackling different aspects of the studied problematics. This approach allowed us to considerably broaden the research scope of our department and increase its relative diversity. The methodological, conceptual and personal interactions between the individual principal investigators and established research groups within our Department **helped us to effectively use our resources, introduce novel instrumentation and methodologies, and it allowed** especially the younger members of our team **to establish themselves in this competitive area of research**.

During this period, our work has concentrated mainly on elucidation of **molecular and cellular mechanisms involved in various aspects of carcinogenesis and cancer cell biology**, as well as on potential **therapeutic and/or preventive approaches applicable in**

oncology, with a potential for development of future effective anti-tumor therapeutic strategies. We particularly focused on i) **physiological protein regulators involved in developmental, microenvironmental and oncogenic signaling**, such as tumor necrosis factor (TNF) and transforming growth factor-β (TGF-β) cytokine families, fibroblast growth factors (FGF), Wnt pathway signaling and bHLH/PAS proteins (the aryl hydrocarbon receptor, AhR); ii) **specific lipid molecules**, which constitute both structural and signaling cellular components, iii) factors and the **signaling pathways involved in the control of plasticity of cancer cells, tumor heterogeneity and therapy resistance**. We further explored the **interactions** of these physiological regulatory pathways **with specific bioactive food components** (such as polyunsaturated fatty acids) or **therapeutic drugs**, which may either reduce tumor incidence, or which could be employed in cancer therapy. Finally, we studied potential role of **carcinogenic and/or endocrine-disrupting environmental chemicals** during oncogenic disease development, with a particular focus on mechanims of tumor initiation and promotion.

In general, our research activities focus on various aspects of tumor development and cancer biology, including potential practical implications, using common conceptual and experimental approaches. We have thus concentrated on the three major areas of research outlined below (***Cancer cell biology and intracellular signaling*; *Carcinogenicity and the toxic modes of action of environmental pollutants; Cancer prevention and therapy***). The principal results that we have obtained are commented individually in each of the three chapters - the results are grouped according to a major focus of the respective publications that reported our findings.

A) Cancer cell biology and intracellular signaling

Within this area, we investigated the mechanisms underlying effects of specific signaling proteins/pathways, which are linked to developmental and oncogenic signaling modules, including TGF-β family, Wnt and FGF signaling and their role in phenotype/beahviour of tumor cells. The principal results are outlined below:

1) Multifunctional cytokines from the **TGF–β family** play crucial role in modulation of tissue microenvironment through regulation of cytokinetics (proliferation, differentiation and apoptosis) in various types of normal and transformed cells. We studied functions of both TGF-β1 and one of the divergent members of TGF-β family with less known functional role, Growth/Differentiation Factor-15 (GDF-15). TGF-β cytokines belongs to the important antiinflammatory cytokines. We showed that TGF-β1 inhibits signal transduction of the proinflammatory cytokine interleukin-6 (IL-6) in a model of benign prostatic hyperplasia. Moreover, we showed that TGF-β1 inhibits the IL-6-induced expression of the cancer-associated gene MUC1. These observations **demonstrated a novel interaction between TGF-β1 and IL-6 signaling and described yet another mechanism of how defects in TGF-β signaling, frequently associated with cancer pathologies, may contribute to the disruption of tissue homeostasis**. Our previous work demonstrated that expression of GDF-15 is not essential for anti-proliferative effects of nonsteroidal anti-inflammatory drugs and significantly affects differentiation of osteoclasts. Recently, we published first demonstration that **GDF-15 displays immunosuppressive characteristics and is an abundant cytokine in seminal plasma**. We believe that these observations shed new light on the role and mechanisms of action of GDF-15 in reproduction, tumor biology and other pathological processes. These studies have been designed, and a majority of experiments and preparation of manuscripts have been carried out, at the Department of Cytokinetics (IBP AS CR). Our

collaborating partners from the Masaryk University and the Reprofit International contributed to specialized analyses (clinical samples, statistics) and data analysis. In first two papers, both first and corresponding author were from our department; paper no. 3 is a review with corresponding author being from our department.

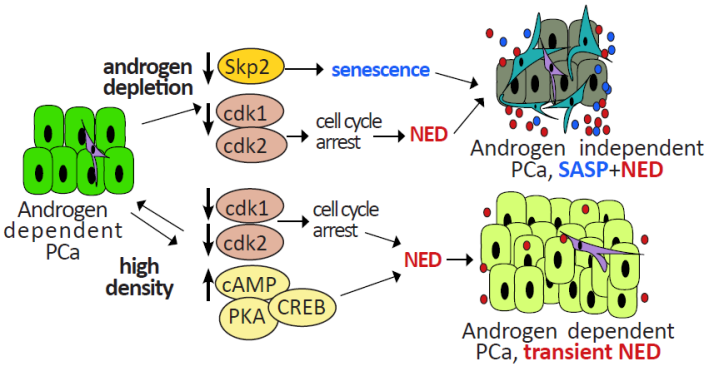
Major relevant publications:

Souček K, Slabáková E, Ovesná P, Malenovská A, Kozubík A, Hampl A (2010) *Growth/differentiation factor-15 is an abundant cytokine in human seminal plasma.* HUMAN REPRODUCTION 25:2962-2971

Staršíchová A, Lincová E, Pernicová Z, Kozubík A, Souček K (2010) *TGF-β1 suppresses IL-6-induced STAT3 activation through regulation of Jak2 expression in prostate epithelial cells.* CELLULAR SIGNALLING 22:1734-1744

Vaňhara P, Hampl A, Kozubík A, Souček K (2012) *Growth/differentiation factor-15: prostate cancer suppressor or promoter?* PROSTATE CANCER AND PROSTATIC DISEASES 15: 320-328

2) Tumor tissue microenvironment and cellular plasticity are among factors contributing to resistance to cancer chemotherapy, controlled by endocrine factors, as well as cell-to-cell communication. We showed that **androgen deprivation therapy**, a widely used treatment for advanced prostate cancer, induces both **the emergence of NE-like prostate cancer cells and the senescence-associated secretory phenotype in prostate cancer epithelial cells**. The induction of the **senescence-associated secretory phenotype is tightly connected with the regulation of the cell cycle machinery** through the downregulation of S-phase kinase-associated protein 2, whereas the emergence of neuroendocrine-like cancer cells (through the process of NED) is under separate control. Our results also imply **a novel relationship between high cell density-induced cell cycle attenuation and promotion of NED**, suggesting that high cell density may trigger intracellular signaling that can mediate reversible NED in prostate cancer cells.



Since the epithelial phenotype may change during endocrine manipulation, both neuroendocrine differentiation and epithelial-to-mesenchymal transition (EMT) could be induced by androgen ablation. EMT is a physiological process during embryogenesis, which reactivation during tumor progression is associated with therapeutic resistance, invasive metastasis and poor prognosis in cancer. Importantly, there is increasing evidence that EMT induced by different stimuli is a source of cells with characteristics of cancer stem like cells or tumor initiating cells. **We have succesfully demonstrated that, particularly in prostate epithelial cells, transcription factor SNAI2/Slug is important for EMT initiation, while the ZEB family of transcription factors in cooperation with the miR-200 family may stabilize EMT, by preventing reversion to an epithelial state**. These studies have been designed, and a majority of experiments and preparation of manuscripts have been carried out, at the Department of Cytokinetics (IBP AS CR). Our collaborating partners from the Palacký University, Masaryk University, and the Veterinary Research Institute contributed to

specialized analyses (clinical samples, immunocytochemistry), data analysis and preparation of manuscripts. In all papers, both first and corresponding author were from our department.

Major relevant publications:

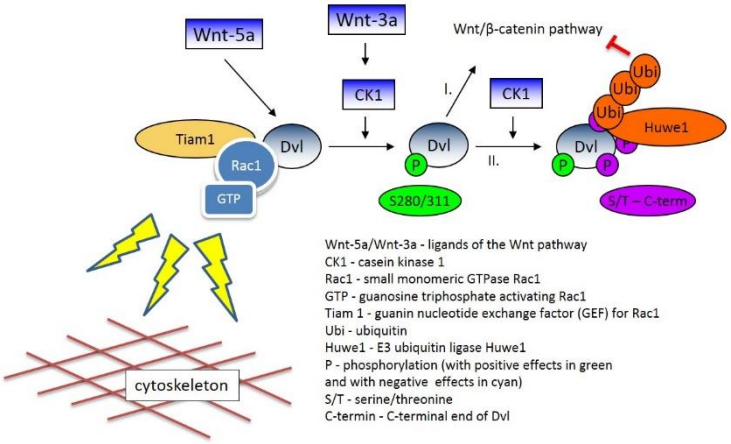
Pernicová Z, Slabáková E, Fedr R, Šimečková S, Jaroš J, Suchánková T, Bouchal J, Kharaishvili G, Král M, Kozubík A, Souček K (2014) *The role of high cell density in the promotion of neuroendocrine transdifferentiation of prostate cancer cells.* MOLECULAR CANCER 13:113

Pernicová Z, Vaňhara P, Souček K *Formation of Secretory Senescent Cells in Prostate Tumors: The Role of Androgen Receptor Activity and Cell Cycle Regulation.* In TUMOR DORMANCY, QUIESCENCE, AND SENESCENCE, Volume 1. Edited by Hayat MA: Springer Netherlands; 2013: 303-316

Pernicová Z, Slabáková E, Kharaishvili G, Bouchal J, Král M, Kunička Z, Machala M, Kozubík A, Souček K (2011) *Androgen depletion induces senescence in prostate cancer cells through down-regulation of Skp2.* NEOPLASIA 13: 526-536

Slabáková E, Pernicová Z, Slavíčková E, Staršíchová A, Kozubík A, Souček K (2011) *TGF-beta1-induced EMT of non-transformed prostate hyperplasia cells is characterized by early induction of SNAI2/Slug.* THE PROSTATE 71: 1332-1343

3) Morphogenetic proteins from the Wnt family are crucial regulators of embryonal development and homeostasis in the adult organism. **Wnt pathway deregulation often leads to the tumor formation and is implicated in the pathogenesis of many other diseases**. Despite the importance of the Wnt pathway in disease, surprisingly few details of the molecular mechanisms of the Wnt pathway are known. **Wnts bind membrane receptors from the Frizzled family, which transduce signal to phosphoprotein Dishevelled**. At the level of Dishevelled signal is analyzed and depending on the ligand/coreceptor/cell, it is further transduced downstream via one of at least four signaling pathways. However, the **molecular mechanisms directing the signal at the level of Dishevelled are largely unknown**; therefore. **we focus on understanding of the crucial events between the receptor, Dishevelled and downstream pathway components**. **We have described several important mechanisms in the signal transduction of Dishevelled** based only on **our proteomic mapping of dynamic post-translational modifications and interaction partners**. We are trying to apply our findings directly to clinically relevant problems eg. pathogenesis of cancer or leukemia. Remarkable, our study **for the first time identified the critical role of non-canonical Wnt pathway (also referred to as planar cell polarity (PCP) pathway) in the pathogenesis of chronic lymphocytic leukemia (CLL)**. Wnt/PCP pathway components are upregulated in B cells of CLL patients and their level increases with CLL progression. **Wnt/PCP pathway controls migration and chemotaxis of CLL cells, which subsequently affect the distribution of CLL cells in the patient body and disease development**. This study opens a new avenue for diagnostics and treatment of CLL. We hope that our findings will become a basis for the identification of novel therapeutic targets in



cancers caused by Wnt pathway deregulation. Papers 1 – 2 were largely designed and executed at the department, in the first case it was in collaboration with the team of local clinicians from Brno Faculty Hospital. In paper 3, both first and corresponding authorship is shared with the lab of H. Korswagen, both partners contributed equally to this work. In paper 4, we identified CEP164-Dvl interaction and described binding domains between the two proteins, and characterized CEP164 mutants.

Major relevant publications:

Kaucká M, Plevová K, Pavlová Š, Verner J, Procházková J, Janovská P, Krejčí P, Kotašková J, Ovesná P, Tichý B, Brychtová Y, Doubek M, Kozubík A, Mayer J, Pospíšilová Š, Bryja V (2013) *The planar cell polarity pathway drives pathogenesis of chronic lymphocytic leukemia by the regulation of B-lymphocyte migration*. CANCER RESEARCH 73: 1491-1501

Bernatík O, Sri Ganji R, Červenka I, Polonio T, Schulte G, Bryja V (2011) *Sequential activation and inactivation of Dishevelled in the Wnt/β-catenin pathway by casein kinases.* JOURNAL OF BIOLOGICAL CHEMISTRY 286: 10396-410

de Groot R, Sri Ganji R, Bernatík O, Lloyd-Lewis B, Seipel K, Šedová K, Zdráhal Z, Dhople VM, Dale T, Korswagen H, Bryja V (2014) *Huwe1-mediated ubiquitylation of Dvl defines a novel negative feedback loop in the Wnt signaling pathway***.** SCIENCE SIGNALING 7: ra26

Chaki M, Airik R, Ghosh AK, Giles RH, Chen R, Slaats GG, Wang H, Hurd TW, Zhou W, Cluckey A, Gee HY, Ramaswami G, Hong CJ, Hamilton BA, Cervenka I, Ganji RS, Bryja V, et al. (2012) *Exome Capture Reveals ZNF423 and CEP164 Mutations, Linking Renal Ciliopathies to DNA Damage Response Signaling*. CELL 150: 533-48

4) The **pathological signaling of fibroblast growth factor receptor kinases (FGFR)** plays an important role in human disease, including cancer. Therefore, we investigated the role of FGFR signaling in skeletal disorders and cancers caused by **activating mutations in FGFR** (skeletal dysplasia, multiple myeloma and others). We particulary focused on mechanisms regulating expression of FGF ligands *in vivo*, mechanisms of FGF/FGFR-mediated regulation of cell function, molecular mechanisms of FGFR signal transduction, basic biochemistry of FGFR kinase activation, development of biological and chemical FGFR inhibitors, and others. We also evealuted possibilities of development of novel ways to target FGFR signaling therapeutically. Our major achievements include **development of a chondrocyte-based high-throughput screening assay for identification of inhibitors of FGFR3 signaling**. Using this assay, we identified a novel, potentially therapeutic inhibitor of FGFR3. We described **a novel phenotype induced by FGFR3 signaling in chondrocytes**, i.e. the premature senescence, which is similar to oncogene-induced cellular senescence. We also characterized a **novel FGFR3 interaction with TGF-β-activating kinase 1** and its role in pathological FGFR3 signaling In addition, we contributed to **evaluation of a novel inhibitor of FGFR and JAK kinases with a potential in multiple myeloma therapy**. Finally, we described **a novel signaling interaction between the FGF and Wnt/β-catenin signaling**. The papers listed below have been largely designed and prepared at the Department of Cytokinetics (IBP AS CR). In the case of two collaborative articles listed below, we were invited by the American scientists to contribute with experiments essential to the overall impact of each particular story.

Major relevant publications:

Krejčí P, Murakami S, Procházková J, Trantírek L, Chlebová K, Ouyang Z, Aklian A, Smutný J, Bryja V, Kozubík A, Wilcox WR (2010) *NF449 is a novel inhibitor of fibroblast growth*

factor receptor 3 (FGFR3) signaling active in chondrocytes and multiple myeloma cells. JOURNAL OF BIOLOGICAL CHEMISTRY 285: 20644-53

Krejčí P, Procházková J, Smutný J, Chlebová K, Lin P, Aklian A, Bryja V, Kozubík A, Wilcox WR (2010) *FGFR3 signaling induces a reversible senescence phenotype in chondrocytes similar to oncogene-induced premature senescence. BONE 47: 102-110*

Krejčí P, Aklian A, Kaucká M, Ševčíková E, Procházková J, Mašek JK, Mikolka P, Pospíšilová T, Spoustová T, Weis M (2012*) Receptor Tyrosine Kinases Activate Canonical WNT/beta-Catenin Signaling via MAP Kinase/LRP6 Pathway and Direct beta-Catenin Phosphorylation.* PLOS ONE 7: e35826

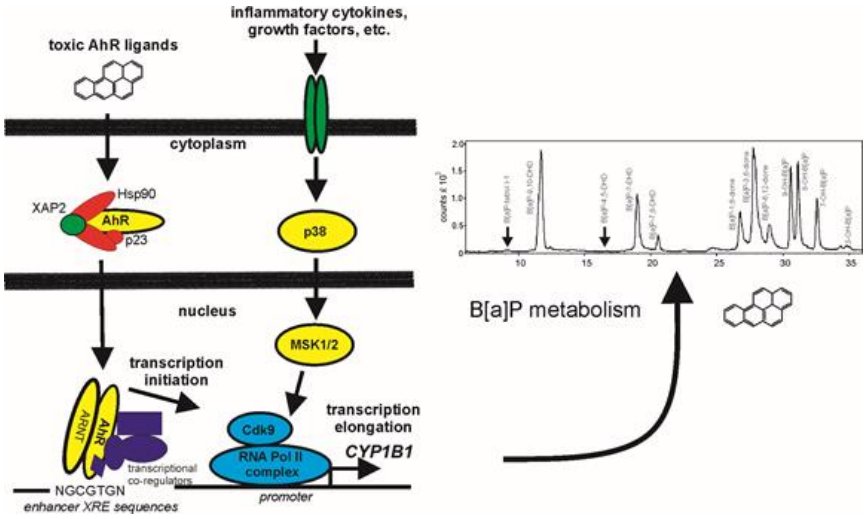
Salazar L, Kashiwada T, Krejčí P, Meyer AN, Casale M, Hallowell M, Wilcox WR, Donoghue DJ, Thompson LM (2014) *Fibroblast Growth Factor Receptor 3 Interacts with and Activates TGF beta-Activated Kinase 1 Tyrosine Phosphorylation and NFkB Signaling in Multiple Myeloma and Bladder Cancer*. PLOS ONE 9: e86470

Scuto A, Krejčí P, Popplewell L, Wu J, Wang Y, Kujawski M, Kowolik C, Xin H, Chen L, Wang Y (2011)*The novel JAK inhibitor AZD1480 blocks STAT3 and FGFR3 signaling, resulting in suppression of human myeloma cell growth and survival.* LEUKEMIA 25: 538-550

(B) Carcinogenicity and the toxic modes of action of environmental pollutants

Toxic compounds, found in the environment or food, which belong among efficient **ligands of the aryl hydrocarbon receptor (AhR)**, may negatively affect human health. The identification of molecular and cellular **mechanisms underlying the toxic effects of these organic pollutants is essential information necessary for the definition of adverse outcome pathways**, which are relevant for both individual toxicants and their complex environmental mixtures. We focused our research mainly on the possible interference of organic pollutants, in particular of the AhR agonists, with intracellular signaling and cell-to-cell communication in epithelial cells:**1)** Long-term deregulated inflammation represents one of the key factors contributing to cancer etiology. Using an in vitro model of lung alveolar type (AEII) II cells, which regulate lung inflammatory response and, simultaneously, are targeted by environmental carcinogenic factors, we documented that **tumor necrosis factor-α** (TNF-α), a cytokine which plays a key role in the initiation of inflammatory responses in the lung strongly **augments the formation of stable DNA adducts and further genotoxic effects of benzo[a]pyrene (BaP)**, a highly carcinogenic polycyclic aromatic hydrocarbon. Importantly, **BaP and TNF-alpha act synergistically to upregulate key inflammatory regulators in the cells, and we identified p38 kinase as a key mediator of this effect**. Our data demonstrated, for the first time, that a pro-inflammatory cytokine and an environmental PAH may simultaneously potentiate both DNA damage and the inflammatory response in AEII cells. The analysis of genotoxicity and metabolism of BaP revealed that **inflammatory conditions may significantly accelerate BaP metabolism and formation of ultimate mutagenic BaP metabolite, via cytochrome P450 1B1 (CYP1B1)-catalyzed metabolism** in epithelial cells. CYP1B1 is an enzyme with a unique tumor-specific expression pattern, which activates a wide range of carcinogenic compounds. Therefore, we focused on identification of the mechanisms responsible for its transcriptional regulation under inflammatory conditions. We described **a novel type of the regulation of CYP1B1 expression via the p38/MSK1 kinase cascade, which involves increased CYP1B1 gene transcriptional elongation**. Our results show, for the first time, that inflammatory cytokines may alter metabolism of carcinogens and thus contribute to their tumor initiating effects via a cell-specific-modulation of CYP1B1 expression. These studies have been designed, and a majority of experiments and preparation of manuscripts have been carried out, at the Department of Cytokinetics (IBP AS CR). Our collaborating partners from the Veterinary Research Institute, Institute of Experimental Medicine AS CR, Masaryk University and the Central European Institute of

Technology contributed to specialized analyses (ChIP assays, analyses of BaP metabolites and DNA adducts) and writing of manuscripts. In all papers, both first and corresponding author were from our department.



Major relevant publications:

Vondráček J, Umannová L, Machala M (2011) *Interactions of the aryl hydrocarbon receptor with inflammatory mediators: Beyond CYP1A regulation*. CURRENT DRUG METABOLISM 12: 89-103

Umannová L, Machala M, Topinka J, Schmuczerová J, Krčmář P, Neča J, Šujanová K, Kozubík A, Vondráček J (2011) *Benzo[a]pyrene and tumor necrosis factor-α coordinately increase genotoxic damage and the production of proinflammatory mediators in alveolar epithelial type II cells*. TOXICOLOGY LETTERS 206: 121-129

Šmerdová L, Neča J, Svobodová J, Topinka J, Schmuczerová J, Kozubík A, Machala M, Vondráček J (2013) *Inflammatory mediators accelerate metabolism of benzo[a]pyrene in rat alveolar type II cells: the role of enhanced cytochrome P450 1B1 expression*. TOXICOLOGY 314: 30-38

Šmerdová L, Svobodová J, Kabátková M, Kohoutek J, Blažek D, Machala M, Vondráček J (2014) *Up-regulation of CYP1B1 expression by inflammatory cytokines is mediated by the p38 MAP kinase signal transduction pathway*. CARCINOGENESIS 35: 2534-2543

2) It is becoming increasingly evident that the AhR alters functions of a variety of signaling proteins participating in the regulation of cell proliferation and apoptosis, cell migration and adhesion, cellular senescence, cell differentiation or intercellular communication in various tissues. Therefore, we focused on analysis of the impact of toxic, as well as endogenous, ligands of the AhR on intercellular communication mediated by adherens junctions/gap junctions, as well as two pathways involved in their control, which also play a key role in regulation of cell proliferation, differentiation and apoptosis – Wnt/β-catenin signaling and TGF-β signaling. Our work has shown **that a persistent activation of the AhR, via its highly toxic agonists, may, through a variety of mechanisms, disrupt expression and/or function of proteins mediating intercellular communication via gap junctions, adherens junctions or desmosomes** (such as β-catenin, connexin 43, E-cadherin or plakoglobin) in epithelial cell models. This is **linked with alterations of cell proliferation, cell adhesion or cell phenotype as well as with inhibition of Wnt/β-catenin signaling**. Importantly, we also documented that apart from **AhR ligands modulating the Wnt/β-catenin signaling**, this signaling pathway **simultaneously co-regulates the AhR-dependent expression of**

xenobiotic metabolizing enzymes, such as **CYP1 enzymes**, which could have profound effects on metabolism of carcinogenic compounds in cells with deregulated Wnt/beta-catenin signaling. Finally, we demonstrated that **TGF-β1 signaling plays a dominant role in the crosstalk with AhR signaling pathway in the context of prostate epithelium** and that TGF-β1 suppresses the AhR-mediated gene expression through multiple mechanisms, involving **inhibition of AhR expression and down-regulation of nuclear AhR, via a SMAD4-dependent pathway**. Given the importance of TGF-β1 and AhR signaling in regulation of prostate epithelial tissue homeostasis, development and carcinogenesis, the above findings significantly contribute to our understanding of the mechanisms underlying the crosstalk between these two signaling pathways. The studies 1 to 5 have been designed, and a majority of experiments and preparation of manuscripts have been carried out, at the Department of Cytokinetics (IBP AS CR). Our collaborating partners from the Veterinary Research Institute, and the Masaryk University contributed to specialized analyses (including immunoprecipitation, siRNA experiments) and writing of manuscripts. In all papers, both first and corresponding author were from our department. In study 6, we contributed by designing microarray experiments, preparation of RNA samples, bioinformatics analyses of global gene expression; we contributed extensively both to writing of the original manuscript and its revision (we shared first authorship).

Major relevant publications:

Procházková J, Machala M, Kozubík A, Vondráček J (2011) *Differential effects of indirubin and 2,3,7,8-tetrachlorodibenzo-p-dioxin on the aryl hydrocarbon receptor (AhR) signalling in liver progenitor cells*. TOXICOLOGY 279: 146-154

Procházková J, Kabátková M, Bryja V, Umannová L, Bernatík O, Kozubík A, Machala M, Vondráček J (2011) *The interplay of the aryl hydrocarbon receptor and β-catenin alters both AhR-dependent transcription and Wnt/β-catenin signaling in liver progenitors*. TOXICOLOGICAL SCIENCES 122: 349-360

Staršíchová A, Hrubá E, Slabáková E, Pernicová Z, Procházková J, Pěnčíková K, Šeda V, Kabátková M, Vondráček J, Kozubík A, Machala M, Souček K (2012) *TGF-β1 signaling plays a dominant role in the crosstalk between TGF-β1 and the aryl hydrocarbon receptor ligand in prostate epithelial cells*. CELLULAR SIGNALLING 24: 1665-1676

Andrysík Z, Procházková J, Kabátková M, Umannová L, Šimečková P, Kohoutek J, Kozubík A, Machala M, Vondráček J (2013) *Aryl hydrocarbon receptor-mediated disruption of contact inhibition is associated with connexin 43 downregulation and inhibition of gap junctional intercellular communication*. ARCHIVES OF TOXICOLOGY 87: 491-503

Procházková J, Kabátková M, Šmerdová L, Pacherník J, Sýkorová D, Kohoutek J, Šimečková P, Hrubá E, Kozubík A, Machala M, Vondráček J (2013) *Aryl hydrocarbon receptor negatively regulates expression of the plakoglobin gene (Jup)*. TOXICOLOGICAL SCIENCES 134: 258-270

Faust D, Vondráček J, Krčmář P, Šmerdová L, Procházková J, Hrubá E, Hulinková P, Kaina B, Dietrich C, Machala M (2013) *AhR-mediated changes in global gene expression in rat liver progenitor cells*. ARCHIVES OF TOXICOLOGY 87: 681-698

(C) Cancer prevention and therapy

Dietary compounds, physiological apoptosis regulators or chemotherapeutic drugs may interact in the strict control of the balance between the cell proliferation, differentiation, and death (apoptosis), which is impaired during cancer development. Therefore, our research in this area focused on understanding cellular and molecular mechanisms underlying their action and

especially their mutual interactions, particularly during colon cancer development, which could be employed in both cancer prevention and therapy.

1) Dietary n-3 polyunsaturated fatty acids (PUFAs) and short-chain fatty acids, such as butyrate (produced by microbial fibre fermentation) exhibit beneficial effects on colon epithelial cell metabolism, signaling, and kinetics, thus preventing colon inflammation and cancer. In addition, fatty acids may influence the effects of endogenous factors regulating cytokinetics (cytokines, apoptotic inducers) and also modify the cancer cell sensitivity to chemotherapy. We **showed that anti-proliferative, differentiation and apoptotic effects of butyrate may differ at various stages of cancer development and can be further modulated by combination with n-6 arachidonic acid (AA) or especially with n-3 docosahexaenoic acid (DHA)**. This is linked with modulations of membrane lipid structure, cytoplasmic lipid droplet accumulation, modulation of fatty acid endogenous synthesis, reactive oxygen species production, and dissipation of mitochondrial membrane potential. Moreover, these effects were further altered by butyrate and PUFA combination. Our further studies **highlighted the important association of this specific cell response and cellular lipid alterations** induced by fatty acids. We documented an **effective incorporation and metabolism of supplemented PUFAs, accompanied with altered content and composition of whole fatty acid spectrum** incorporated in cellular lipids. Together, our results documented **cooperative effects of butyrate and PUFAs and show significant association between lipid alterations and differentiation/apoptotic response in colon cells**. We also documented **the ability of fatty acids to interact and cooperate with members of TNF family**, such as clinically useful **TNF-related apoptosis-inducing ligand (TRAIL)**. We described the ability of **subtoxic doses of DHA to enhance anti-proliferative and apoptotic effects of TRAIL** in colon cancer cells, associated with an extensive **engagement of mitochondrial pathway and activation of endoplasmic reticulum stress response**. We identified significant differences in sphingolipid metabolism, especially in representation of ceramides, between the cancer and normal colon cells treated with DHA and TRAIL, suggesting their potential role in the regulation of the cell response to the drug combination. These study outcomes highlight the potential of DHA for a combination therapy with TRAIL for selective elimination of colon cancer cells via simultaneous targeting of multiple steps in apoptotic pathways. These studies have been conceived, designed and largely performed at the Department of Cytokinetics (IBP AS CR). We led both writing of the original manuscript and its revision (both the first and the corresponding author are from our department). Our collaborating partners from the Veterinary Research Institute contributed by performing complex chemical lipid analyses and participated in preparation of the papers 2 -3 (corresponding author of the second study was from the VRI).

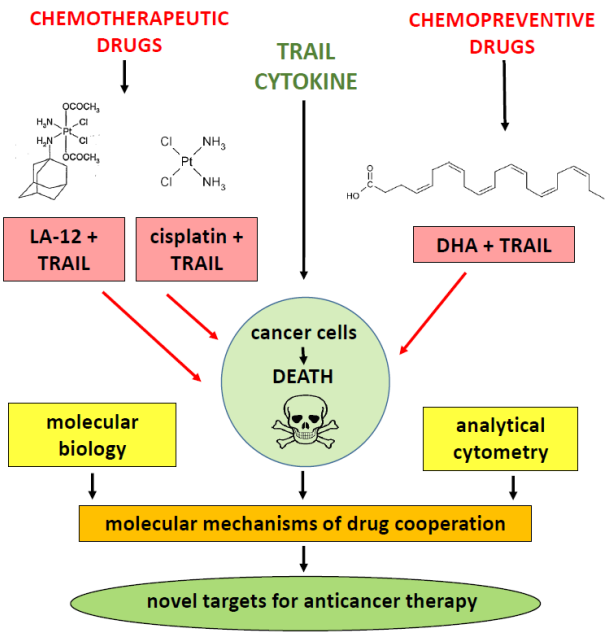
Major relevant publications:

Hofmanová J, Vaculová A, Kozubík A (2013) *Regulation of the metabolism of polyunsaturated fatty acids and butyrate in cancer cells*. CURRENT PHARMACEUTICAL BIOTECHNOLOGY 14: 274-288

Hofmanová J, Ciganek M, Slavík J, Kozubík A, Stixová L, Vaculová A, Machala M (2012) *Lipid alterations in human colon epithelial cells induced to differentiation and/or apoptosis by butyrate and polyunsaturated fatty acids*. JOURNAL OF NUTRITIONAL BIOCHEMISTRY 23: 539-548

Skender B, Hofmanová J, Slavík J, Jelínková I, Machala M, Moyer MP, Kozubík A, Hyršlová Vaculová A (2014) *DHA-mediated enhancement of TRAIL-induced apoptosis in colon cancer cells is associated with engagement of mitochondria and specific alterations in sphingolipid metabolism.* BIOCHIMICA ET BIOPHYSICA ACTA 1841:1308–1317

Hofmanová J, Straková N, Hyršlová Vaculová A, Tylichová Z, Šafaříková B, Skender B, Kozubík A (2014) *Interaction of dietary fatty acids with tumor necrosis factor family cytokines during colon inflammation and cancer.* MEDIATORS OF INFLAMMATION 2014: Article ID 848632



2) Platinum-based complexes are widely used agents in anticancer treatment. However, their application is often limited by the undesirable side effects and acquired resistance of tumor cells. These limitations evoke a search for novel effective platinum derivatives with a broader range of mechanisms of action. The LA-12, a recently introduced platinum(IV) complex containing 1-adamantylamine, offers several advantages over conventionally used platinum drugs. We demonstrated an **outstanding ability of LA-12 to induce effective elimination of colon cancer cells at significantly lower doses than oxaliplatin**. LA-12 demonstrated a higher malignant cell toxicity and especially **the ability to bypass cell cycle arrest** (thus blocking effective DNA damage repair), which makes LA-12 to be a more effective candidate for elimination of colon tumors of a variable genetic background, as compared with oxaliplatin. We also demostrated a significantly higher anti-tumor efficacy of LA-12 as compared to cisplatin when applied not only individually, but also in combination with apoptotic inducer TRAIL. This unique cytokine offers a great therapeutic potential, which is nevertheless limited by resistance of numerous cancer types, including colon or prostate carcinomas. We were the **first to show that LA-12 enhanced killing effects of TRAIL in human colon and prostate cancer cell lines via stimulation of caspase activity and overall apoptosis**. We described a novel mechanism, in which platinum drugs increase DR5 surface expression, trigger the relocalization of DR4 and DR5 receptors to lipid rafts and accelerate internalization of TRAIL, thus sensitizing cancer cells to TRAIL-induced apoptosis. Finally, **we provide the first evidence that LA-12 primes human colon cancer cells for TRAIL-induced cytotoxicity by p53-independent activation of the mitochondrial apoptotic pathway**. The cooperative action of LA-12 and TRAIL was associated with stimulation of Bax/Bak activation, drop of mitochondrial membrane potential, caspase-9 activation, and a shift of the balance among Bcl-2 family proteins in favour of the pro-apoptotic members. The selective action of LA-12 was documented by preferential priming of cancer cells, but not normal cells, to TRAIL killing effects. **Our work highlights the promising potential of LA-12 and provides new mechanistic insights into its cooperative action with physiological regulators of apoptosis, such as TRAIL**. These studies have been conceived, designed and performed at the Department of Cytokinetics (IBP AS CR). We led both writing of the original manuscript and its revision (both the first and the corresponding author on all studies were from our department). The collaborating partners from the University of Debrecen, Hungary contributed by performing confocal microscopy analyses of lipid rafts and to preparation of the manuscript. Other co-authors participated by providing cytostatic drug LA-12, specific colon cell models and/or contributed to the writing of manuscripts.

Major relevant publications:

Vondálová Blanářová O, Jelínková I, Hyršlová Vaculová A, Sova P, Hofmanová J, Kozubík A (2013) *Higher anti-tumor efficacy of platinum(IV) complex LA-12 is associated with its ability to bypass M-phase entry block induced in oxaliplatin-treated human colon cancer cells.* CELL PROLIFERATION 46:665-76

Vondálová Blanářová O, Jelínková I, Szoor A, Skender B, Souček K, Horváth V, Vaculová A, Anděra L, Sova P, Szollosi J, Hofmanová J, Vereb G, Kozubík A (2011) *Cisplatin and a potent platinum(IV) complex-mediated enhancement of TRAIL-induced cancer cells killing is associated with modulation of upstream events in the extrinsic apoptotic pathway.* CARCINOGENESIS 32:42-51

Jelínková I, Šafaříková B, Vondálová Blanářová O, Skender B, Hofmanová J, Sova P, Moyer MP, Kozubík A, Kolář Z, Ehrmann J, Hyršlová Vaculová A (2014) *Platinum(IV) complex LA-12 exerts higher ability than cisplatin to enhance TRAIL-induced cancer cell apoptosis via stimulation of mitochondrial pathway.* BIOCHEMICAL PHARMACOLOGY 92:415-24

Research Report of the team in the period 2010–2014

Institute	Institute of Biophysics of the CAS, v. v. i.

Scientific team	Department of Free Radical Pathophysiology

The attention of Department of Free Radical Pathophysiology was focused on the study of mechanisms leading to the generation of reactive oxygen and nitrogen metabolites by phagocytes, and the evaluation and modulation of molecular mechanisms underlying the inflammation-derived damage of vascular endothelium and heart tissue. The following major results were reached:

(1) Role of myeloperoxidase in regulation of endothelial and immune functions

Myeloperoxidase (MPO) is an abundant hemoprotein expressed by neutrophils. The release of MPO from neutrophils is a hallmark of vascular inflammation and contributes to the pathogenesis of vascular inflammatory processes. MPO is capable of enzymatically catalyzing the generation of reactive oxygen species and the consumption of nitric oxide. Thus MPO has both potent microbicidal and cell damaging pro-inflammatory properties.

We analysed interactions of MPO with proteins of extracellular matrix (ECM) and whether this binding modulates its enzymatic functions. We showed that the oxidizing and

chlorinating potential of MPO was preserved upon binding to collagen IV and fibronectin; even the potentiation of MPO activity in the presence of collagen IV and fibronectin was observed. Collectively, the data reveal that MPO binds to ECM proteins on the basis of electrostatic interactions, and MPO chlorinating and oxidizing activity are potentiated upon association with these proteins.

Further we provided evidence for MPO to facilitate recruitment of polymorphonuclear cells (PMNs) by its positive surface charge. In vitro, MPO evoked highly directed PMNs motility, which was solely dependent on electrostatic interactions with the leukocyte's surface. In vivo, PMNs recruitment was shown to be MPO-dependent in a model of hepatic ischemia and reperfusion, upon intraportal delivery of MPO and in the cremaster muscle exposed to local inflammation or to intraarterial MPO application. Our data revealed the importance of MPO for recruitment of PMNs that comprises a cascade of concerted events allowing for capture, adhesion and extravasation of the leukocyte. This electrostatic MPO effect not only displays a so far unrecognized, catalysis-independent function of the enzyme, but also highlights a principal mechanism of PMN attraction driven by physical forces.

Interestingly, MPO also binds to the surface of blood cells including thrombocytes and erythrocytes. Thrombocytes interaction with MPO induces their partial activation. Furthermore, we have shown that MPO binds to erythrocytes, which correlates with the clinical conditions of patients with cardiovascular diseases. Our results also demonstrate that MPO modulates the balance of pro- and anti-inflammatory lipid mediators during acute inflammation and, in this way, may control acute inflammatory diseases.

In summary, we provide complex evidence that MPO interacts with negative surface of both blood cells and vascular endothelium that significantly modulates physiological functions of these cells, particularly increases interaction among these cells that can significantly contribute to the development of chronic inflammatory processes. Proposed mechanism of MPO action in the vasculature is shown in Fig. 1.

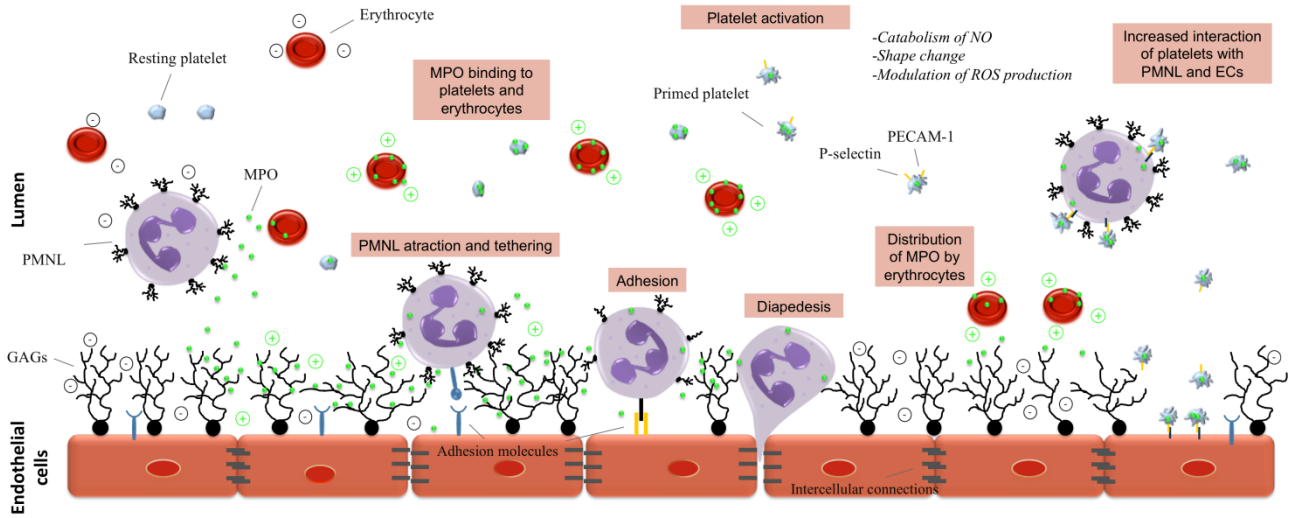


Fig. 1: Proposed mechanism of MPO action in the vasculature.

Relevant publications:

1. Adam M, Gajdova S, Kolarova H, Kubala L, Lau D, Geisler A, Ravekes T, Rudolph V, Tsao PS, Blankenberg S, Baldus S, Klinke A. (2014) *Red blood cells serve as intravascular carriers of myeloperoxidase*. J MOL CELL CARDIOL. 74:353-63.
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HJ, Ehmke H, Schumacher U, Meinertz T, Sperandio M, Baldus S. (2011) *Myeloperoxidase attracts neutrophils by physical forces*. BLOOD. 117(4):1350-8.
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4. Kubala L, Schmelzer KR, Klinke A, Kolarova H, Baldus S, Hammock BD, Eiserich JP. (2010) *Modulation of arachidonic and linoleic acid metabolites in myeloperoxidase-deficient mice during acute inflammation*. FREE RADIC BIOL MED. 48(10):1311-20.
5. Kubala L, Kolářová H, Víteček J, Kremserová S, Klinke A, Lau D, Chapman AL, Baldus S, Eiserich JP. (2013) *The potentiation of myeloperoxidase activity by the glycosaminoglycan-dependent binding of myeloperoxidase to proteins of the extracellular matrix*. BIOCHIM BIOPHYS ACTA.1830(10):4524-36.

(2) Role of L-arginine and methylarginines in regulation of innate immune and endothelial homeostasis

L-arginine (L-Arg) is the amino acid, which can be synthesized "de novo" in metabolic pathways from proline, glutamine, or glutamate. The regulation of L-Arg endogenous synthesis is very important under pathophysiological conditions when its synthesis may be insufficent to supply metabolic demands of organism and thus dietary intake remains the primary determinant of plasma L-Arg levels. L-Arg homeostasis is importantly involved in the regulation of airway function as well as cardiovascular and immune systems. Deregulation of its metabolism may contribute to chronic immune responses, sepsis, endothelial dysfunction, hypertension and asthma.

One of the most important metabolite with regulatory functions rising from catabolism of L-Arg is nitric oxide (NO). NO is synthesized by the enzyme nitric oxide synthase (NOS) and plays important role in many diverse processes, including vasodilatation, immune responses, neurotransmission and adhesion of platelets and leucocytes. Importantly, the availability of L-Arg to NOS and other L-Arg catabolizing enzymes could be regulated by several distinct mechanisms, including presence of endogenously occuring methylarginines (NOS inhibitors).

Since there existed only incomplete information about the mechanism by which L-Arg together with the action of methylarginines contributes to immune and endothelial dysfunction, we were focused on the molecular mechanisms leading to deregulation of physiological responses of innate immune and endothelial cells. Importantly we found out that the imbalance between the concentrations of L-Arg and methylarginines plays a crucial role in development of chronic and acute inflammatory response in immune, as well as endothelial cells.

L-arginine alone was shown to modulate the response of macrophages toward lipopolysaccharide via the activation of G-protein-coupled receptors and potentiation of superoxide anion production derived from uncoupled inducible NOS. According to our data, we concluded that L-arginine availability plays a key role in the initiation of intracellular signaling pathways that trigger the lipopolysaccharide-induced inflammatory responses in murine macrophages. Although macrophages are partially stimulated in the absence of extracellular L-arginine, the presence of this amino acid significantly accelerates the sensitivity of macrophages to bacterial endotoxin (LPS).

Besides that we discovered that out of the three endogenously occurring methylarginines, asymmetric dymethylarginine (ADMA), identified as one of the potential therapeutic targets in treatment of serious human diseases, is able to negatively affect the balance in LPS-induced macrophage-derived production of reactive mediators. Its effect was mediated via regulation

of intracellular signaling pathways, which could lead to increased oxidative stress. These results were further investigated in in vivo model, where ADMA was infused in mice and humans. ADMA was found to profoundly impair NO synthesis in neutrophils, resulting in increased neutrophil adhesion to endothelial cells, superoxide generation, and release of leukocyte-derived hemoprotein myeloperoxidase (MPO). These data reveal an ADMA-induced cycle of neutrophil activation, enhanced MPO release, and subsequent impairment of enzyme activity, responsible for ADMA degradation within the body. Additionally, ADMA massively contributed to acute inflammatory phenotype in endothelial cells and induced the endothelial dysfunction. These findings not only highlight so far unrecognized cytokine-like properties of ADMA but also identify MPO as a regulatory switch for ADMA bioavailability under inflammatory conditions.

Relevant publications:

1. Pekarova M, Lojek A, Martiskova H, Vasicek O, Bino L, Klinke A, Lau D, Kuchta R, Kadlec J, Vrba R, Kubala L. (2011) *New role for L-arginine in regulation of inducible nitric-oxide-synthase-derived superoxide anion production in raw 264.7 macrophages.* SCIENTIFICWORLDJOURNAL 11: 2443-2457.
2. Pekarova M, Kubala L, Martiskova H, Papezikova I, Kralova S, Baldus S, Klinke A, Kuchta R, Kadlec J, Kuchtova Z, Kolarova H, Lojek A. (2013) *The unique role of dietary L-arginine in the acceleration of peritoneal macrophage sensitivity to bacterial endotoxin.* IMMUNOLOGICAL RESEARCH 56:73-84.
3. Pekarova M, Kubala L, Martiskova H, Bino L, Twarogova M, Klinke A, Rudolph TK, Kuchtova Z, Kolarova H, Ambrozova G, Kuchta R, Kadlec J, Lojek A. (2013) *Asymmetric dimethylarginine regulates the lipopolysaccharide-induced nitric oxide production in macrophages by suppressing the activation of NF-kappaB and iNOS expression.* EUROPEAN JOURNAL OF PHARMACOLOGY 713: 68-77.
4. Pekarová M, Lojek A, Hrbáč J, Kuchta R, Kadlec J, Kubala L. (2014) *Novel insights into the electrochemical detection of nitric oxide in biological systems.* FOLIA BIOLOGICA 60: 8-12.
5. von Leitner EC, Klinke A, Atzler D, Slocum JL, Lund N, Kielstein JT, Maas R, Schmidt-Haupt R, Pekarova M, Hellwinkel O, Tsikas D, D'Alecy LG, Lau D, Willems S, Kubala L, Ehmke H, Meinertz T, Blankenberg S, Schwedhelm E, Gadegbeku CA, Böger RH, Baldus S, Sydow K. (2011) *Pathogenic cycle between the endogenous nitric oxide synthase inhibitor asymmetrical dimethylarginine and the leukocyte-derived hemoprotein myeloperoxidase.* CIRCULATION 124: 2735-2745.

(3) The importance of nitrated fatty acids as endogenous cardioprotective mediators

Inflammation is a manifestation of immune system function that is triggered by microbial invasion and/or tissue injury. It is directed toward isolating and destroying invading microorganisms and injured cells and preparing the tissue for eventual repair and regeneration. Inflammatory processes are critically dependent on functional and structural changes in the circulation including impaired vasomotor function, the recruitment of leukocytes, diminished endothelial barrier function, angiogenesis, and enhanced thrombosis. Moreover, a variety of diseases are linked to chronic inflammatory processes, including cardiovascular diseases, diabetes, or neurodegenerative diseases.

Two major effector systems are frequently implicated in the immune and endothelial cell alternations associated with inflammation and cardiovascular diseases: enhanced production of reactive oxygen species (ROS) and diminished bioavailability of nitric oxide (NO).

Importantly, the oxidative milieu generated during inflammatory processes consists of a broad spectrum of oxidizing, nitrosating, and nitrating species and their products. This is also the case of nitrated unsaturated fatty acids (NO_2-FAs) that were shown to be endogenously occurring products of oxidant-induced nitration reactions. NO_2-FAs have been identified in plasma of healthy and hypercholesterolemic patients as well as in urine, cell membranes, and tissues.

Nowadays, it is believed that similarly to polyunsatured fatty acids (PUFAs) and their metabolites, NO_2-FAs represent a novel group of pluripotent signaling molecules, endogenously generated as an adaptive response of organism to oxidative stress. The effects of NO_2-FAs are diverse; to begin with, these species serve as a potential chemical reserve of NO, or can covalently modify nucleophilic protein targets to alter the structure and function of enzymes, receptors and transcription factors.

NO_2-FAs are suggested as highly promising compounds for treatment of various cardiovascular and inflammatory disorders. Thus, solid knowledge of molecular mechanisms responsible for NO_2-FAs action on cells and effects of NO_2-FAs in various animal models is needed. Our projects were designed to bring new knowledge about interactions of NO_2-FAs (as well as PUFAs) with phagocyte-derived inflammatory responses, associated with physiological as well as various pathophysiological states, particularly with endothelial dysfunction within different pathological states (e.g. atherosclerosis and pulmonary artery hypertension). Importantly, we demonstrate that subcutaneously administered 9- and 10-nitro-octadecenoic acid (nitro-oleic acid, OA-NO_2) potently reduced atherosclerotic lesion formation in apolipoprotein E-deficient mice. OA-NO_2 attenuated lesion formation by suppressing tissue oxidant generation, inhibiting adhesion molecule expression, and decreasing vessel wall infiltration of inflammatory cells. In addition, OA-NO_2 reduced foam cell formation by attenuating oxidized low-density lipoprotein-induced phosphorylation of signal transducer and activator of transcription-1, a transcription factor linked to foam cell formation in atherosclerotic plaques.

These results reveal the antiatherogenic actions of electrophilic NO_2-FAs in a murine model of atherosclerosis. Proposed mechanism of NO_2-FAs action in cardiovascular system is shown in Fig. 2.

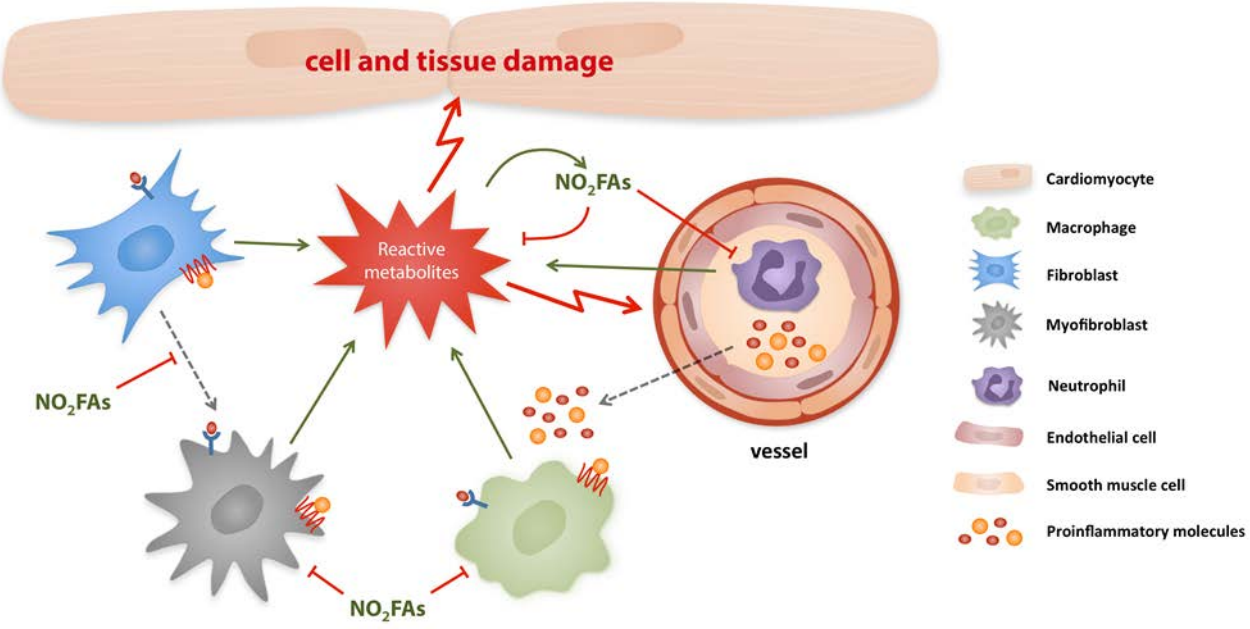

Fig 2: Proposed mechanism of nitro-fatty acids (NO_2-FAs) action in cardiovascular system.

Another study also revealed the protective effect of OA-NO_2 in pulmonary arterial hypertension (PAH), progressive vasculopathy with poor prognosis, which is characterized by adverse remodeling of pulmonary arteries. Although the origin of the disease and its underlying pathophysiology remain incompletely understood, inflammation has been identified as a central mediator of disease progression. Importantly, we discovered that OA-NO_2 decreased the right ventricular hypertrophy and fibrosis induced in mice with PAH. The infiltration of macrophages and the generation of ROS, elevated in lung tissue of mice on hypoxia were also diminished by OA-NO_2 treatment. Moreover, OA-NO_2 decreased superoxide production of activated macrophages and pulmonary smooth muscle cells. Vascular structural remodeling was also limited by OA-NO_2. Our results show that the oleic acid nitroalkene derivative OA-NO_2 attenuates hypoxia-induced pulmonary hypertension in mice. Thus, OA-NO_2 represents a potential therapeutic agent for the treatment of PAH.

Relevant publications:

1. Ambrozova G, Pekarova M, Lojek A. (2010) *Effect of polyunsaturated fatty acids on the reactive oxygen and nitrogen species production by raw 264.7 macrophages.* EUROPEAN JOURNAL OF NUTRITION 49: 133-139.
2. Ambrozova G, Pekarova M, Lojek A. (2011) *The effect of lipid peroxidation products on reactive oxygen species formation and nitric oxide production in lipopolysaccharide-stimulated RAW 264.7 macrophages.* TOXICOLOGY IN VITRO 25: 145-152.
3. Klinke A, Möller A, Pekarova M, Ravekes T, Friedrichs K, Berlin M, Scheu KM, Kubala L, Kolarova H, Ambrozova G, Schermuly RT, Woodcock SR, Freeman BA, Rosenkranz S, Baldus S, Rudolph V, Rudolph TK. (2014) *Protective effects of 10-nitro-oleic acid in a hypoxia-induced murine model of pulmonary hypertension.* AMERICAN JOURNAL OF RESPIRATORY CELL AND MOLECULAR BIOLOGY 51: 155-162.
4. Rudolph TK, Rudolph V, Edreira MM, Cole MP, Bonacci G, Schopfer FJ, Woodcock SR, Franek A, Pekarova M, Khoo NK, Hasty AH, Baldus S, Freeman BA. (2010) *Nitro-fatty acids reduce atherosclerosis in apolipoprotein E-deficient mice.* ARTERIOSCLEROSIS, THROMBOSIS AND VASCULAR BIOLOGY 30: 938-945.

(4) Efects of serotonin and histamine on the functional activity of human leukocytes

Platelets and activated PMNL are in tight contact at sites of inflammation. We have hypothetised that serotonin released from platelets might have a protective function against PMNL-derived oxidative stress and oxidative damages. We have investigated whether 5-HT mediates this modulation via 5-HT receptors (5-HTR) or whether this is due instead to 5-HT antioxidative properties. We have found that 5-HTR2 agonist DOI hydrochloride does not have any antioxidative properties, despite its ability to inhibit the CL response of activated human total leukocytes. On the other hand, DOI hydrochloride was unable to inhibit the CL response of activated human PMNL. It seems that the reduction of the oxidative burst of professional phagocytes was evoked by the activation of 5-HTR not on the neutrophil surface but on the surface of different leukocytes, which produced anti-inflammatory cytokines with NADPH oxidase activity modulating properties.

The diverse physiological functions of histamine are mediated through distinct histamine receptors. The effects of H1-antihistamines of the 1st generation (antazoline, bromadryl, brompheniramine, dithiaden, cyclizine, chlorcyclizine, chlorpheniramine, clemastine) and the 2nd generation (acrivastine, ketotifen, and loratadine) on the respiratory burst of phagocytes

were studied. Reactive oxygen species generation in neutrophils isolated from rat blood was measured using luminol-enhanced chemiluminescence. Changes in nitrite formation and iNOS protein expression by RAW 264.7 macrophages were analysed using Griess reaction and Western blotting. The antioxidative properties of drugs in cell-free systems were detected spectrophotometrically, luminometrically, fluorimetrically, and amperometrically. The majority of the H1-antihistamines tested (bromadryl, brompheniramine, chlorcyclizine, chlorpheniramine, clemastine, dithiaden, and ketotifen) exhibited a significant inhibitory effect on the chemiluminescence activity of phagocytes. H1-antihistamines did not show significant scavenging properties against superoxide anion and hydroxyl radical, thus this could not contribute to the inhibition of chemiluminescence. H1-antihistamines had a different ability to modulate nitric oxide production by LPS-stimulated macrophages. Bromadryl, clemastine, and dithiaden were the most effective since they inhibited iNOS expression, which was followed by a significant reduction in nitrite levels. H1-antihistamines had no scavenging activity against nitric oxide. It can be concluded that the effects observed in the H1-antihistamines tested are not mediated exclusively via H1-receptor pathway or by direct antioxidative properties. Based on our results, antihistamines not interfering with the microbicidal mechanisms of leukocytes (antazoline, acrivastine and cyclizine) could be used preferentially in infections. Other antihistamines should be used, under pathological conditions accompanied by the overproduction of reactive oxygen species.

Further, we studied the role of H2R and H4R histamine receptors in the effects of histamine on the production of reactive oxygen species by phagocytes in whole blood. Changes in reactive oxygen species (ROS) production by whole blood phagocytes after treatment with histamine, H4R agonists (4-methylhistamine, VUF8430), H2R agonist (dimaprit) and their combinations with H4R antagonist (JNJ10191584) and H2R antagonist (ranitidine) were determined using the chemiluminescence (CL) assay. To exclude the direct scavenging effects of the studied compounds on the CL response, the antioxidant properties of all compounds were measured using several methods (TRAP, ORAC, and luminol-HRP-H2O2 based CL). Histamine, 4-methylhistamine, VUF8430 and dimaprit inhibited the spontaneous and OZP-activated whole blood CL in a dose-dependent manner. On the other hand, only VUF8430 was able to inhibit PMA-activated whole blood CL Ranitidine, but not JNJ10191584, completely reduced the effects of histamine, 4-methylhistamine and dimaprit The direct scavenging ability of tested compounds was negligible. Our results demonstrate that the inhibitory effects of histamine on ROS production in whole blood phagocytes were caused by H2R. Our results also suggest that H4R agonists in concentrations higher than 10^{-6}M may also influence ROS production via binding to H2R.

Relevant publications:

1. Vasicek Ondrej, Lojek Antonin, Jancinova Viera, Nosal Radomir, Ciz Milan (2014): *Role of histamine receptors in the effects of histamine on the production of reactive oxygen species by whole blood phagocytes.* LIFE SCIENCES 100 (1), 67-72.
2. Ciz M, Lojek A. (2013): *Modulation of neutrophil oxidative burst via histamine receptors.* BRITISH JOURNAL OF PHARMACOLOGY 170 (1), 17-22.
3. Lojek Antonin, Ciz Milan, Pekarova Michaela, Ambrozova Gabriela, Vasicek Ondrej, Moravcova Jana, Kubala Lukas, Drabikova Katarina, Jancinova Viera, Perecko Tomas, Pecivova Jana, Macickova Tatiana, Nosal Radomir (2011): *Modulation of metabolic activity of phagocytes by antihistamines.* INTERDISCIPLINARY TOXICOLOGY 4 (1), 15-19.

4. Pracharova Lucie, Okenkova Katerina, Lojek Antonin, Ciz Milan (2010): *Serotonin and its 5-HT2 receptor agonist DOI hydrochloride inhibit the oxidative burst in total leukocytes but not in isolated neutrophils*. LIFE SCIENCES 86 (13-14), 518-523.

(5) Anti-inflammatory and antioxidative properties of medicinal plants, vegetables and small fruits

We studied the anti-inflammatory and antioxidative properties of medicinal plants, vegetables and small fruits which are a rich source of bioactive substances, including polysaccharides and polyphenols, and are therefore suitable raw materials for the production of functional foods. It is a very important area because the destructive potential of neutrophils requires a tight control of their production of reactive oxygen species. These oxidants can be highly toxic for neighbouring host tissues.

As concerns the antioxidative activity of plants, several major and original achievements were published. First, the use of solid-phase extraction as the fast, simple and reliable method for purification of anthocyanins from complex extracts was shown. Second, we compared various methods (ORAC, TRAP, HORAC and inhibition of lipid peroxidation) for detection of antioxidant properties of plant materials. ORAC has been found to be the most sensitive method to measure chain-breaking antioxidant activity. Although we have found a good correlation between TRAP, ORAC and HORAC, using more than one antioxidant assay was recommended for more detailed understanding the principles of antioxidant properties of samples. Third, the antioxidant properties of various medicinal plants, vegetables and small fruits were described.

The effect of extracts from medicinal plants and fruits on the production of reactive oxygen species (ROS) by neutrophils was also investigated. In most cases, the extracts inhibited ROS production depending on the type of used activator. All extracts studied blocked almost completely the formation of neutrophil-derived reactive oxygen species induced with the use of receptor binding activators. On the other hand, the effect of extracts on neutrophils activated with receptor by-passing stimuli was much milder. This latter result suggests that extracts (apart from their antioxidative activity) interfere with the signaling cascade of phagocyte activation upstream of the protein kinase C activation.

Relevant publications:

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2. Ciz M, Denev P, Kratchanova M, Vasicek O, Ambrozova G, Lojek A. (2012): *Flavonoids inhibit the respiratory burst of neutrophils in mammals*. OXID MED CELL LONGEV. 2012;2012:181295. Epub 2012 Apr 23.
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4. Denev P, Kratchanova M, Ciz M, Lojek A, Vasicek O, Nedelcheva P, Blazheva D, Toshkova R, Gardeva E, Yossifova L, Hyrsl P, Vojtek L. (2014): *Biological activities of selected polyphenol-rich fruits related to immunity and gastrointestinal health*. FOOD CHEM. Aug 15;157:37-44.
5. Lojek A, Denev P, Ciz M, Vasicek O, Kratchanova M (2014): *The effects of biologically active substances in medicinal plants on the metabolic activity of neutrophils*. PHYTOCHEMISTRY REVIEWS 13(2): 499-510.

The share of the team in the creation of main results:

The experiments were performed either completely by members of evaluated department or in the frame of the efficient international collaboration where the department members played the significant role. Names of authors from the department are underlined. The authors of the department contributed with performing determination of leukocyte activation, determination of nitric oxide production, evaluation of reactive oxygen species generation, antioxidant parameters of biological materials, analyses of myeloperoxidase activity, all experiments necessary to prepare samples for complex lipidomic analysis both in vivo and in vitro using myeloperoxidase deficient mouse strain provided by international partners. The authors of the department were also involved in the data processing, interpretation and in the preparation of the manuscripts.

Research Report of the team in the period 2010–2014

Institute	Institute of Biophysics of the CAS, v. v. i.

Scientific team	Department of Molecular Dynamics of Nucleic Acids

(1) Formamide pathway for the prebiotic synthesis of the first RNA molecules on the primeval Earth. The formamide-based synthesis of nucleic acid components offers a new alternative for the origin of informational polymers. This chemistry represents an elegant and continuous way from simple prebiotic precursors up to short catalytic oligonucleotides. Since this multistep synthesis proceeds in a very complex reaction mixture, it is difficult to study its mechanism using purely experimental methods. In such complicated cases computational chemistry might be instrumental to provide an atomic-level insight into the mechanistic details of the reactions. In the last few years we have devoted a special attention to this topic and in cooperation with experimental groups we have addressed various stages of the formamide-based origin of life scenario. Our results strongly suggest that life could have been spontaneously created on the Earth via genuine chemical processes and there is no need to bring the life and its components from the Space.

Recently, in a feature article (Ferus et al., 2015) published in PNAS (since 2007 PNAS has published less than 60 feature articles from all areas of science) we propose an impact-

based model for the origin of terrestrial life: we suggest that heavy impact of extraterrestrial bodies during the Late Heavy Bombardment period about 3.8 billion years ago could sparkle life (synthesis of basic components of biomolecules) in small formamide-ponds. I.e. our model further elaborates the scenario proposed by Saladino et al. and suggests that the energy brought about by extraterrestrial impacts could be used to create life's building blocks. The paper has been identified to be of "exceptional significance" by the editorial board of PNAS and immediately after its publication (Dec 8, 2014) on web it has been highlighted by Science mag, New Scientist and Chemistry World. Beyond intense reactions of the scientific world, the paper has ignited a cascade of press releases intended to the public: via the press agency AP our results have been noted in many newspapers all over the world, e.g. Los Angeles Times, The Telegraph, in addition to leading Czech newspapers and magazines like Hospodarske noviny, Lidove noviny, Respect, etc.. We gave several radio interviews (see e.g. German state radio, Czech state radio, Czech edition of BBC). In addition, major news channels on the internet reported our results (see e.g. Huffington Post, Yahoo news channel, Discovery News).

In our recent Chemistry – A European Journal VIP paper (very important paper, ~1% of publications in the Journal) (Stadlbauer et al., 2015) we address a later step of the origin of terrestrial life: i.e. how the first oligonucleotides could acquire their catalytic function, a feature that stays behind evolution of species on Earth. This paper has also been highlighted in the Chemistry World magazine.

All computational work in the projects (which is essential) is done by the Brno group. The foreign partners are providing the necessary experiments.

Ferus M, Nesvorny D, Sponer J, Kubelik P, Michalcikova R, Shestivska V, Sponer J E, Civis S (2015) *High-Energy Chemistry of Formamide: A Unified Mechanism of Nucleobase Formation.* Proceedings of the National Academy of Sciences of the United States of America 112: 657-662 (accepted Dec 2014).

Stadlbauer P, Sponer J, Costanzo G, Di Mauro E, Pino S, Sponer J E (2015) *Tetraloop-Like Geometries Could Form the Basis of the Catalytic Activity of the Most Ancient Ribooligonucleotides.* Chemistry – A European Journal 21: 3596-3604 (accepted Dec 2014).

Ferus M, Civis S, Mladek A, Sponer J, Juha L, Sponer J E (2012) *On the Road from Formamide Ices to Nucleobases: Ir-Spectroscopic Observation of a Direct Reaction between Cyano Radicals and Formamide in a High-Energy Impact Event.* Journal of the American Chemical Society 134: 20788-20796

Sponer J E, Mladek A, Sponer J, Fuentes-Cabrera M (2012) *Formamide-Based Prebiotic Synthesis of Nucleobases: A Kinetically Accessible Reaction Route.* Journal of Physical Chemistry A 116: 720-726

(2) The first successful application of accurate modern quantum chemical (QM) methods to complete nucleic acids building blocks. In computational studies, nucleic acids molecules are usually modelled using approaches based on molecular mechanics (MM) description. These methods allow, for example, extensive explicit solvent MD simulations. However, the MM potentials have substantial intrinsic accuracy limits which are unlikely to be resolved due the physical limitations of the MM model. Therefore, there is an urgent need to search for alternative theoretical methods that would complement the MM approaches.

In the last few years, we have done a major step in this direction, utilizing unprecedented developments of computer hardware and theoretical quantum chemical (QM) methods, namely, advance of accurate dispersion-corrected density functional theory (DFT) methods, such as the DFT-D3. We have pioneered application of large-scale QM calculations to complete nucleic acids building blocks, by performing the first such calculations world-wide.

Such computations must fulfil two key requirements. By complete building blocks we mean DNA and RNA segments that would be capable to fold in real experimental conditions. The other key requirement is that the QM potential energy surface description is decisively more accurate compared to the MM description (for example, semiempirical and other very fast QM methods do not fulfil this condition). This is for the first time when state-of-the-art QM methods were applied to nucleic acids models with several hundreds of atoms. Until now, accurate QM studies were done only for nucleic acids model systems having dozens of atoms (typically base pairs). The large-scale QM studies represent a significant step forward in computational studies of nucleic acids. This methodological approach can complement simulation studies based on classical MM. The first paper has been published by us in 2013 (Sponer et al., 2013). Our particular study done for relative stabilities of different topologies of guanine quadruplex stems achieved results that are in a considerably better agreement with experimental data than our earlier work based on classical MM (Cang et al., 2011a, b). The QM data has been applied as a correction to relative free energies derived using MM simulations. Overall, our calculations provided important insights into the basic principles governing the topological variability of guanine quadruplexes based on the number of their G-quartets. The principal limitations of the MM description have been convincingly demonstrated for the first time by a direct comparison with benchmark QM computations. Subsequently, we performed such large-scale QM calculations also for an RNA system, 11-nucleotide Sarcin-Ricin loop (Kruse et al, 2014). Besides demonstrating the undisputable advantages of such large-scale QM computations, we have also clearly identified their present methodological limitations, namely, the use of continuum solvent (it cab can lead to non-native interactions in the studied structures) and lack of sampling. We have initiated efforts to overcome these limitations and a first our methodological work has been accepted for publication by the end of 2014, namely, we introduced a methodology of controllable QM optimizations of nucleic acids building blocks using restraints (Kruse et al., 2015). The basic work (introduction of the large scale QM studies of NA building blocks) has been done dominantly in our lab with some initial methodological help by Stefan Grimme (Bonn, Germany). The earlier MD simulations and free energy computation studies were done primarily by laboratory of T.E. Cheatham (Salt Lake City, USA) with our contribution.

Cang X, Sponer J, Cheatham T E, III (2011) *Explaining the varied glycosidic conformational, G-tract length and sequence preferences for anti-parallel G-quadruplexes.* Nucleic Acids Research 39: 4499-4512

Cang X, Sponer J, Cheatham T E, III (2011) *Insight into G-DNA Structural Polymorphism and Folding from Sequence and Loop Connectivity through Free Energy Analysis.* Journal of the American Chemical Society 133: 14270-14279

Sponer J, Mladek A, Spackova N, Cang X, Cheatham T E, III, Grimme S (2013) *Relative Stability of Different DNA Guanine Quadruplex Stem Topologies Derived Using Large-Scale Quantum-Chemical Computations.* Journal of the American Chemical Society 135: 9785-9796

Kruse H, Sponer J (2015) *Towards biochemically relevant QM computations on nucleic acids: controlled electronic structure geometry optimization of nucleic acid structural motifs using penalty restraint functions.* Physical Chemistry Chemical Physics 17: 1399-1410

Kruse H, Havrila M, Sponer J (2014) *QM Computations on Complete Nucleic Acids Building Blocks: Analysis of the Sarcin-Ricin RNA Motif Using DFT-D3, HF-3c, PM6-D3H, and MM Approaches.* Journal of Chemical Theory and Computation 10: 2615-2629

(3) Substantial improvement of the main molecular mechanics force field for nucleic acids. Together with colleagues from the collaborating lab at Palacky university, Olomouc,

we gradually derived, tested and released several upgrades (one of them critical) to improve the classical force fields for molecular simulations of RNA and DNA (Banas et al. 2010, Zgarbova et al., 2011, 2013, Krepl et al., 2012). These upgrades have been officially included in the latest version of the AMBER program package (the world-wide leading code for modelling of nucleic acids) as the recommended (default) force field variants for nucleic acids (RNA version since 2011 and DNA version since 2014). The quality of the force fields critically affects the quality of simulations and force field refinements can be done only by the top groups in the field. As part of our work, we have published several authoritative reviews summarizing the advances and limitations of the MD simulations of nucleic acids, with special emphasize given to the force fields, for example Sponer et al. 2014.

The work is a collaborative effort of the Department of Physical Chemistry, Palacky University, Olomouc and our laboratory, with minor contributions from foreign labs. Jiri Sponer is overall supervising the nucleic acids research in both Czech laboratories.

Banas P, Hollas D, Zgarbova M, Jurecka P, Orozco M, Cheatham T E, Sponer J, Otyepka M (2010) *Performance of Molecular Mechanics Force Fields for RNA Simulations: Stability of UUCG and GNRA Hairpins.* Journal of Chemical Theory and Computation 6: 3836-3849

Zgarbova M, Otyepka M, Sponer J, Mladek A, Banas P, Cheatham T E, Jurecka P (2011) *Refinement of the Cornell et al. Nucleic Acids Force Field Based on Reference Quantum Chemical Calculations of Glycosidic Torsion Profiles.* Journal of Chemical Theory and Computation 7: 2886-2902

Krepl M, Zgarbová M, Stadlbauer P, Otyepka M, Banáš P, Koča J, Cheatham T E, Jurečka P, Šponer J (2012) *Reference Simulations of Noncanonical Nucleic Acids with Different χ Variants of the AMBER Force Field: Quadruplex DNA, Quadruplex RNA, and Z-DNA.* Journal of Chemical Theory and Computation 8: 2506-2520

Zgarbova M, Javier-Luque F, Sponer J, Cheatham T E, III, Otyepka M, Jurecka P (2013) *Toward Improved Description of DNA Backbone: Revisiting Epsilon and Zeta Torsion Force Field Parameters.* Journal of Chemical Theory and Computation 9: 2339-2354

Šponer J, Banáš P, Jurečka P, Zgarbová M, Kührová P, Havrila M, Krepl M, Stadlbauer P, Otyepka M (2014) *Molecular Dynamics Simulations of Nucleic Acids. From Tetranucleotides to the Ribosome.* Journal of Physical Chemistry Letters 5: 1771-1782

(4) Studies of topological variability and folding mechanisms/pathways of quadruplex nucleic acids (G-DNA). We have done series of studies dealing with topological preferences and potential folding pathways of monomolecular guanine quadruplexes. In contrast to the widespread views in current experimental literature often suggesting quite straightforward folding pathways with few intermeditates, our studies suggest that quadruplex folding is an extremely complicated multi-pathway process over exceptionally rugged free energy landscape with multiple deep competing basins of attraction. Wide range of potential intermediates has been visualised by us. We suggest that the simple models suggested based on experiments are due to limited resolution of the experimental techniques for quadruplex folding processes. In other words we predict that folding of G-DNA molecules from the unfolded state to the final structure is dramatically more complicated than suggested by the current very simple models based on available experiments lacking atomistic resolution (Stadlbauer et al., 2013, 2014). In contrast to these models we argue that, at the level of single molecules, the G-DNA folding is an extremely multi-pathway process involving myriads of on- and off-pathway structures, strikingly different from funnel-like folding of small fast-folding proteins and RNAs. The work is already being recognised by the leading experimental groups and we hope that further research will ultimately confirm the basic

correctness of our prediction. We consider this as a prediction of important features of nucleic acids that is not accessible to direct experimental measurements. In addition, the prediction is strikingly contrasting the simplified models currently preferred by many experimental groups. These results thus demonstrate the indispensability of complementing experiments by advanced computations.

The work is done dominantly in Brno lab (all the first and corresponding authors).

Stadlbauer P, Krepl M, Cheatham T E, III, Koca J, Sponer J (2013) *Structural dynamics of possible late-stage intermediates in folding of quadruplex DNA studied by molecular simulations.* Nucleic Acids Research 41: 7128-7143

Stadlbauer P, Trantirek L, Cheatham T E, III, Koca J, Sponer J (2014) *Triplex intermediates in folding of human telomeric quadruplexes probed by microsecond-scale molecular dynamics simulations.* Biochimie 105C: 22-35

(5) First microsecond-scale simulations of the protein – RNA complexes. We have recently opened a new theme in our research, studies of RNA-protein (RNP) complexes using microsecond-scale MD simulations. Among other results, in the year 2014, we have completed a large-scale simulation study for a set of six diverse RNP systems; the work has been subsequently published in early 2015 (Krepl at al, 2015). Such extended and systematic work is unprecedented in contemporary literature and visualises the advantages as well as the limits of contemporary simulations of RNP systems. Another our work has been carried out on CRISPR/Csy4-RNA endoribonukleaze complex (accepted 2014, Estarellas et al.) and has, besides discussing methodological issues, suggested an atomistic model for a possible catalytic scenario of this system.

The work is done dominantly in Brno lab (all the first and corresponding authors).

Estarellas C, Otyepka M, Koca J, Banas P, Krepl M, Sponer J (2015) *Molecular dynamic simulations of protein/RNA complexes: CRISPR/Csy4 endoribonuclease.* Biochimica Et Biophysica Acta-General Subjects 1850: 1072-1090

Krepl M, Havrila M, Stadlbauer P, Banas P, Otyepka M, Pasulka, J, Stefl, R, Sponer, J. (2015) Can We Execute Stable Microsecond-Scale Atomistic Simulations of Protein-RNA Complexes? JOURNAL OF CHEMICAL THEORY AND COMPUTATION 11: 1220-1243

Research Report of the team in the period 2010–2014

Institute	Institute of Biophysics of the CAS, v. v. i.

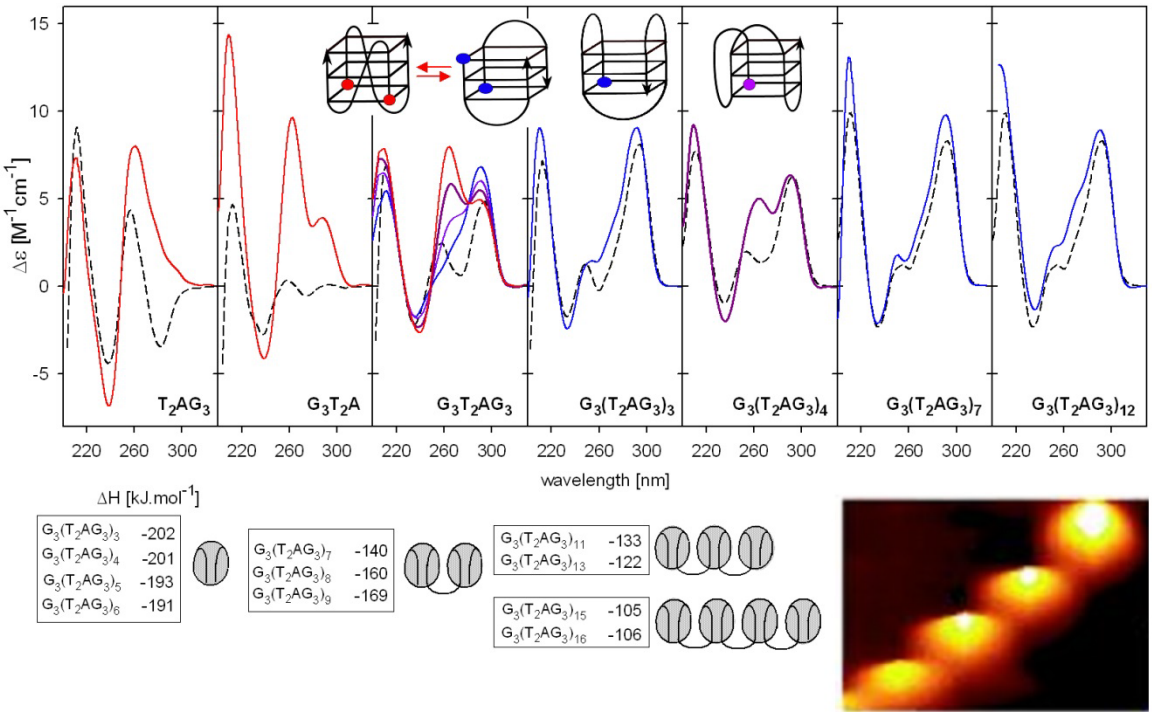
Scientific team	Department of CD Spectroscopy of Nucleic Acids

(1) Conformational properties of the human telomere quadruplex

The main result is the contribution of our laboratory to understanding biophysical and conformational properties of quadruplexes, primarily of quadruplexes of the human telomere (htel) DNA. Quadruplexes are biologically important anomalous DNA structures which frequently form in gene promoters and were shown to control their expression. In telomeres quadruplexes control genome integrity and their formation is associated with ageing and carcinogenesis.

Our results contributed to the polymorphic view of the htel quadruplex (1). (i) We have shown that apart from the concentration and type of present cations, the quadruplex structure depends on the length of the htel molecule (Figure 1) and its precise oligonucleotide sequence including appended nucleotides on the 5´and 3´end. (ii) We were among the first laboratories demonstrating the transition of the htel quadruplex from antiparallel to parallel arrangement

under dehydrating conditions. (iii) Based on thermodynamic data we found that long htel molecules form, similarly to nucleosomes, beads on a string-like structure where the beads are the basic 21 nucleotide long three-tetrad quadruplex units (Figure 1). (iv) Various quadruplex structures were suggested for this basic quadruplex unit by different methods. According to the results of optical spectroscopies the basic htel sequence adopted an antiparallel quadruplex at physiological conditions. NMR unambiguously found that the quadruplex is under the same conditions in a so-called (3+1) hybrid structure, with three parallel and one antiparallel strands. X-ray analysis of the htel sequence in crystal identified that this quadruplex has propeller-like double chain reversal loops resulting in an all-chains-parallel quadruplex structure. We have explained the cause of distinct results on the htel quadruplex structure reported by different laboratories: The reason is the dependence of the quadruplex arrangement on DNA concentration (Figure 2), which is by orders distinct in the individual methodological approaches. (v) We have confirmed the concentration dependence of the htel quadruplex conformation in the framework of a joint grant with our colleagues from the Institute of Physics, Charles University, Prague by Raman spectroscopy, which enables spectroscopic measurements in a wide region of DNA concentrations (2). (vi) We have determined thermodynamic parameters of particular quadruplex structures and identified association of the quadruplex particles as the source of these intramolecular transformations (3). The association has been visualized by AFM (3).



<u>Figure 1:</u> CD spectra and enthalpy values of human telomere DNA molecules differing in length. (upper panel) CD spectra and thus structures of human telomere DNA fragments depend on their length; (bottom panel) on the basis of sharp decreases in enthalpy values with sequences containing an integral multiple of four G3 blocks (which indicate division of the original unit into more parts) we suggested that long telomere molecules are formed by a set of small quadruplexes like beads on a string.

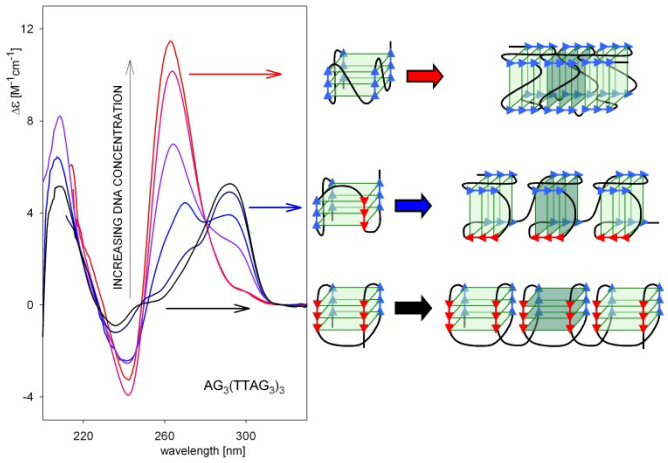


Figure 2: Polymorphous structure of the human telomere quadruplex. The quadruplex structure depends on DNA concentration: at low concentrations used with spectroscopic measurements (black spectrum) the core quadruplex sequence as well as long molecules adopt an antiparallel quadruplex structure, under concentrations used with NMR measurements (blue spectrum) the DNA forms the so-called (3+1) quadruplex reported by NMR, and at the highest DNA concentrations (red spectra) a parallel quadruplex observed by X-ray in crystal is formed.

Relevant publications:

1. Vorlickova M, Kejnovska I, Sagi J, Renciuk D, Bednarova K, Motlova J, Kypr J (2012): *Circular dichroism and guanine quadruplexes.* METHODS 57: 64-75
2. Palacky J, Vorlickova M, Kejnovska I, Mojzes P (2013) *Polymorphism of human telomeric quadruplex structure controlled by DNA concentration: a Raman study.* NUCLEIC ACIDS RES. 41: 1005–1016
3. Kejnovska I, Vorlickova M, Brazdova M, Sagi J (2014) *Stability of human telomere quadruplexes at high DNA concentrations.* BIOPOLYMERS 101: 428-438

Publications 1 and 3 were realized in our laboratory and we are their principal authors. The publication 2 was undertaken in cooperation and prepared in the laboratory of the co-authors. However, the idea and the reason of the paper are ours. Members of our laboratory are underlined.

(2) Effects of non-standard or damaged bases on DNA structures

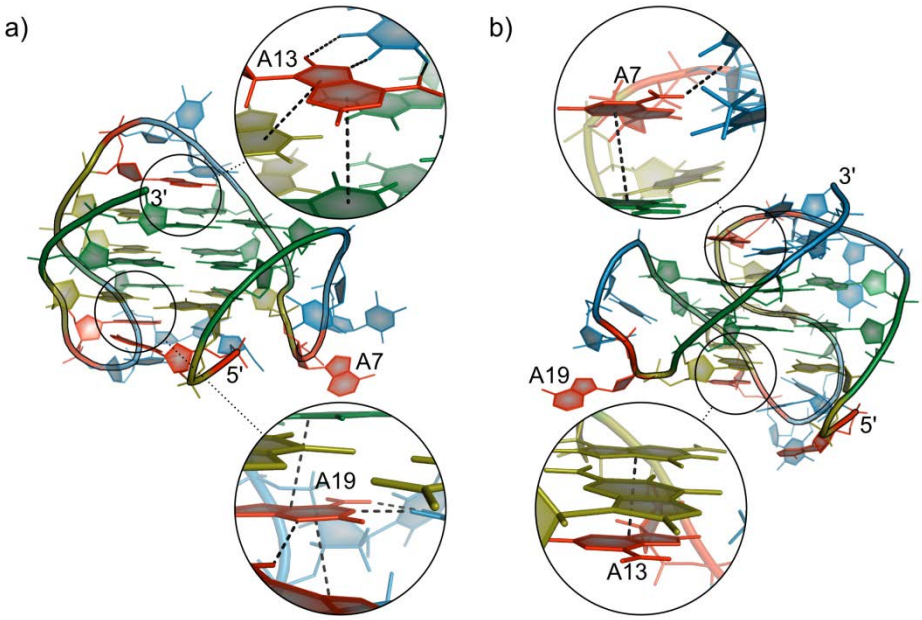
The 5-hydroxymethylcytosine (5-hmC) has been recently identified as an anomalous base existing in eukaryotic genomes, especially in the brain, in parts associated with higher brain functions. Its physiological function is still unclear, but it is supposed to serve as an intermediate in DNA de novo demethylation associated with cell dedifferentiation. The role of the anomalous base has been intensively studied at present.
We studied the effect of epigenetic modifications, 5-methycytosine (5-mC) and 5-hmC, on the B-DNA structure of the Dickerson´s dodecamer CGCGAATTCGCG. Using X-ray diffraction, we solved the structures of the dodecamer analogs containing 5-hmC or 5-mC at positions 3 or 9. The results were published (1) and the crystal structures were deposited in the NA databank.

Various base lesions continuously form in cellular nucleic acids and the unrepaired lesions are promutagenic and procarcinogenic. We studied the effects of various naturally occurring lesions to human telomere (htel) DNA quadruplex, which is a critical formation in the genome associated with carcinogenesis. Using CD and UV absorption spectroscopy and PAG

electrophoresis, we probed conformational properties of the htel DNA sequence $G_3(TTAG_3)_3$ or $AG_3(TTAG_3)_3$ and of their related sequences, in which each guanine base was replaced by an adenine base (2), an abasic site (3) or 8-oxo guanine (4). None of these single base substitutions (or more bases in the case of A for G substitutions) hindered the formation of antiparallel quadruplexes in NaCl solutions, but their thermal stabilities were substantially decreased. Unlike the parent wild type htel, no structural transitions were observed for the substituted sequences when sodium ions were replaced by potassium ions. The effect of substitutions differed depending on the position of the substituted base but principally not on the lesion type. As potassium is a physiological salt, these findings may have biological relevance.

Apart from the lesions to quadruplex core guanines, we have also studied the effect of three frequently occurring natural lesions that can form in the TTA loops on the structure of the htel quadruplex of $AG_3(TTAG_3)_3$. We studied the abasic site and 8-oxoadenine replacing adenine and 5-hydroxymethyluracil substituting for thymine. These lesions hindered neither the formation of the quadruplexes in Na^+ or K^+ solution nor their transition to a parallel quadruplex form. However, the three lesions impacted the stability and quadruplex folding in markedly different ways. The effect of the substitution depended on its position in the htel sequence and mainly on its type.

Abasic lesions are the most frequent damages occurring in cellular DNA. By means of CD and NMR spectroscopies we have found (5) that the loss of adenine in the first (ap7), second, or third (ap19) loop of the htel DNA quadruplex does not hinder its formation but changes its structure. The ap7 and ap19 sequences formed a hybrid-1 and hybrid-2 quadruplex respectively, with AP site located in a propeller-like loop (Figure 3). In view of the important functions of the telomeres in ageing and carcinogenesis, the change in their quadruplex structure may have serious biological consequences.



<u>Figure 3:</u> Quadruplex structures of human telomere DNA sequences lacking adenine in the first (ap7) and third (ap19) loop. (a) ap7 and (b) ap19 adopt the so-called hybrid-1 and hybrid-2 quadruplex structures respectively, in which the adenines A7 and A19 are located out of the quadruplex core so that the negative enthalpic effect of adenine depurination is minimized.

Relevant publications:

1. Renciuk D, Blacque O, Vorlickova M, Spingler B (2013) *Crystal structures of B-DNA dodecamer containing the epigenetic modifications 5-hydroxymethylcytosine or 5-methylcytosine.* NUCLEIC ACIDS RES. 41: 9891-9900
2. Sagi J, Renciuk D, Tomasko M, Vorlickova M (2010) *Quadruplexes of human telomere DNA analogs designed to contain G:A:G:A, G:G:A:A, and A:A:A:A tetrads.* BIOPOLYMERS 93: 880-886
3. Skolakova P, Bednarova K, Vorlickova M, Sagi J (2010) *Quadruplexes of human telomere $dG_3(TTAG_3)_3$ sequences containing guanine abasic sites*. BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 399: 203-208
4) Vorlickova M, Tomasko M, Sagi A, Bednarova K, Sagi J. (2012) *8-Oxoguanine in a quadruplex of the human telomere DNA sequence.* FEBS JOURNAL 279: 29–39
5) Babinsky M, Fiala R, Kejnovska I, Bednarova K, Marek R, Sagi J, Sklenar V, Vorlickova M (2014) *Loss of loop adenines alters human telomere $d[AG_3(TTAG_3)_3]$ quadruplex folding.* NUCLEIC ACIDS RES. 42: 14031–14041

The results of paper 1 were obtained in the course a stay of D. Renciuk in the laboratory of Dr. B. Spingler in the Inst. of Inorganic Chemistry of the University in Zurich. The stay was funded by our joint SCIEX grant.

Publications 2-4 were realized in our laboratory, J. Sagi is our long-time co-worker. Paper 5 belongs to the above set of our papers. It was produced in cooperation with our colleagues from the National Centre for Biomolecular Research, who are experts in determining DNA structures by NMR spectroscopy. The idea of the work was ours.
Members of our laboratory are underlined.

(3) Invited review articles

Our laboratory has been renowned in the world by its expertise in CD spectroscopy of nucleic acids. Because of our successful review published in Nucleic Acids Res. in 2009 (it has already more than 300 citations), the head of the laboratory, MV, was invited to Oxford to deliver a plenary lecture at the 13th International Conference on Chiroptical Spectroscopy (2011). Selected contributions presented on the conference were published in a special issue of Chirality (2012) (1) (Figure 4). The review has so far had 23 citations.

With respect to the contribution of our laboratory to quadruplex research we were invited by Professor S. Neidle to contribute a review on quadruplex findings obtained by CD spectroscopy (2) into a special issue of Methods devoted to studies of this biologically important structure by various methods. CD spectroscopy is the most frequently used method in this field so that we consider the invitation as a substantial success. The review published in 2012 has so far received 42 citations.

Also, our laboratory was invited by Professor Woody to contribute a chapter in the Book on conformational properties of nucleic acids revealed by CD spectroscopy (3).

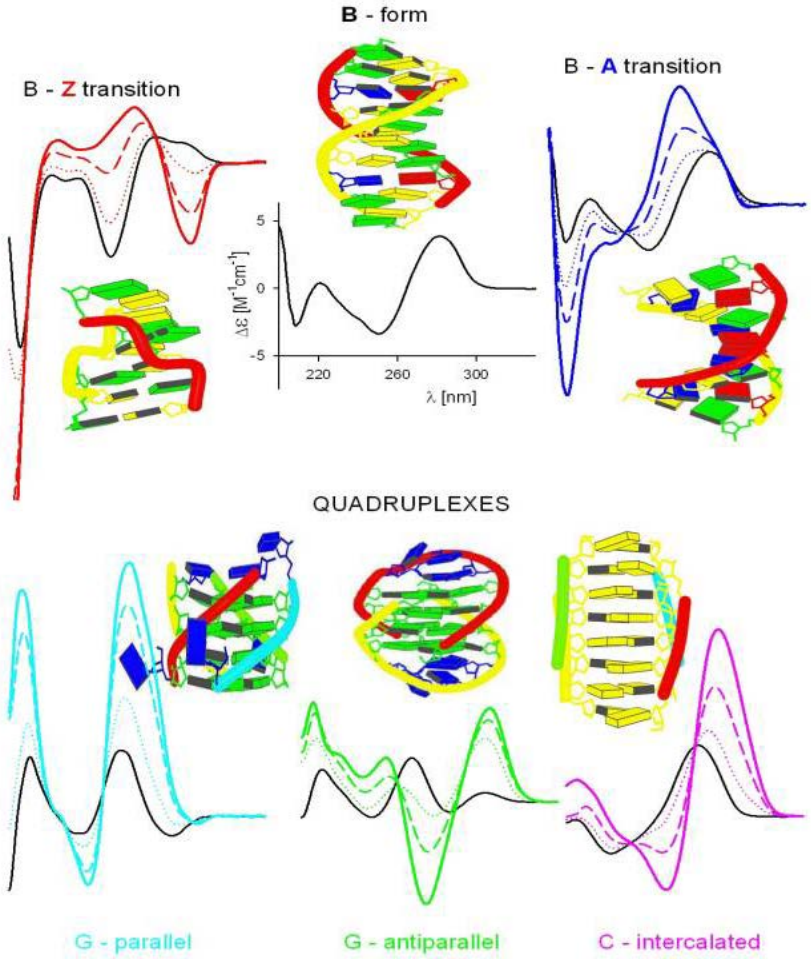


Figure 4: CD spectroscopy provides characteristic spectra of particular DNA arrangements and sensitively reflects their changes.

Relevant publications:

1. Vorlickova M, Kejnovska I, Bednarova K, Renciuk D, Kypr J (2012) *Circular dichroism spectroscopy of DNA: from duplexes to quadruplexes.* CHIRALITY 24: 691-698

2. Vorlickova M, Kejnovska I, Sagi J, Renciuk D, Bednarova K, Motlova J, Kypr J (2012) *Circular dichroism and guanine quadruplexes.* METHODS 57: 64-75

3. Kypr J, Kejnovska I, Bednarova K, Vorlickova M (2012) *Circular dichroism spectroscopy of nucleic acids.* In**:** Comprehensive Chiroptical Spectroscopy: Application in Stereochemical Analysis and Synthetic Compounds, Natural Products, and Biomolecules. 1st edition, N. Berova, P.L. Polavarapu, K. Nakanishi, R.D. Woody, Eds. John Wiley & Sons, Inc., Vol. 2: 575-586

Research Report of the team in the period 2010–2014

Institute	Institute of Biophysics of the CAS, v. v. i.

Scientific team	Department of Plant Developmental Genetics

(1) Evolution of sex determination

Members of the genus *Silene* compose an important model system for the study of the early phases of sex chromosome evolution: several dioecious species in this genus have evolutionarily recent sex chromosomes (10–20 MYA). Most *Silene* species are either gynodioecious or hermaphroditic and can therefore be used in comparative studies that search for autosomal ancestors of the sex chromosomes. To date, the attention of researchers has been concentrated mainly on the study of dioecious species possessing large heteromorphic sex chromosomes with an XX/XY sex-determining systems in *S. latifolia*, *S. dioica*, and *S. diclinis* (section Elisanthe). Phylogenetic studies conducted on these species showed that they are closely related and share a common origin of the sex chromosomes. In addition to the section Elisanthe, there is also another group of dioecious *Silene* species consisting of *S. otites* and *S. colpophylla* (subsection Otites). So far, the studied species of this group are characterized by the absence of heteromorphic sex chromosomes. This fact, together with the

supposed nondioecious origin of the genus *Silene*, suggests that the sex chromosomes in *S. otites* and its closely related species are evolutionarily younger than those in *S. latifolia*.

It was also suggested that sex determination evolved independently in the group of species around *S. latifolia* and in the group of species around *S. otites*. Our results show that *S. colpophylla* is, similarly to *S. latifolia*, a male heterogametic species, but its sex chromosomes have evolved from a different pair of autosomes than in *S. latifolia*. The results of our recent study show that the sex-determining system in *S. otites*, a species related to *S. colpophylla*, is based on female heterogamety, a sex determination system that is unique among the *Silene* species studied to date. Our phylogenetic data support the placing of *S. otites* and *S. colpophylla* in the subsection Otites and the analysis of ancestral states suggests that the most recent common ancestor of *S. otites* and *S. colpophylla* was most probably dioecious. Our data further show that female organ suppression (a common feature of subdioecious and dioecious species) evolved independently in dioecious and subdioecious species of the genus *Silene*. Our observations imply that a switch from XX/XY sex determination to a ZZ/ZW system (or vice versa) occurred in the subsection Otites. This is the first report of two different types of heterogamety within one plant genus of this mostly nondioecious plant family.

Sexual dimorphism (the systematic difference in form or other trait(s) not present in sexual organs between individuals of different sex in the same species) is a widely studied phenomenon in animal models and in humans. Much less is known about sexual dimorphism in vascular dioecious plants. Among vascular plants displaying sexual dimorphism, *Silene latifolia* is (together with *Fragaria virginiana*) the most studied species. However, the only known genes involved in sexual dimorphism are those involved in the control of flower development. During flower development, sexual dimorphism starts to occur very early. At the morphological level, the central zone of the floral meristem is significantly smaller in males than in females. This is caused by cell division arrest in male tissues. The difference between male and female flower bud morphology is preceded by differences at the gene expression level. Almost all of the sexually dimorphic traits in *S. latifolia* described so far become apparent only after the initiation of flowering. We present the first molecular evidence that sexually dimorphic gene expression is present in *S. latifolia* even at the rosette stage, a long time before the initiation of flowering, and describe three ESTs (two male specific and one female specific) with sex-specific gene expression.

Relevant publications:

Slancarova V, Zdanska J, Janousek B, Talianova M, Zschach C, Zluvova J, Siroky J, Kovacova V, Blavet H, Danihelka J, Oxelman B, Widmer A, Vyskot B (2013) *Evolution of sex determination systems with heterogametic males and females in Silene.* EVOLUTION 67: 3669-3677

Janousek B, Mrackova M (2010) *Sex chromosomes and sex determination pathway dynamics in plant and animal models*. BIOLOGICAL JOURNAL OF THE LINNEAN SOCIETY 100: 737-752

Kaefer J, Talianová M, Bogot T, Michu E, Gueguen L, Widmer A, Zluvova J, Glemin S, Marais GAB (2013) *Patterns of molecular evolution in dioecious and non-dioecious Silene*. JOURNAL OF EVOLUTIONARY BIOLOGY 26: 335-346

Zluvova J, Zak J, Janousek B, Vyskot B (2010) *Dioecious Silene latifolia plants show sexual dimorphism in the vegetative stage*. BMC PLANT BIOLOGY 10: e208

Janousek B, Hobza R, Vyskot B (2013) *Chromosomes and sex differentiation*. In: PLANT GENOME DIVERSITY, pp. 167-186, Springer Verlag, ed. Leitch I, Doležel J, Greilhuber J ISBN 978-3-7091-1159-8, Wien

(2) Structure of sex chromosomes

Silene latifolia (formerly *Melandrium album*) or white campion belongs to the Caryophyllaceae family. *S. latifolia* is a perennial herb, a weed now common nearly all over the world. Its genome is quite large (5.85 pg DNA per 2C in males), which is a disadvantage for genomic and mapping studies, but at the same time the large size of the chromosomes makes cytogenetic studies possible. Most *Silene* species possess 12 pairs of chromosomes. The karyotype of *S. latifolia* is 2n = 24, XY (or XX in females). The same number of chromosomes irrespective of whether they are autosomes or sex chromosomes indicates that the pair of sex chromosomes originated from an ordinary pair of autosomes. The Y chromosome is the largest in the genome, approximately 1.4 times larger than the X chromosome, which is larger than any of the autosomes. The sex determination system in *S. latifolia* is formally similar to the human type: the Y chromosome harbors dominant genes(s), which control the male gender. But in fact these systems are rather different. While in humans the Y-linked genes(s) switch on the testosterone pathway leading to male sexuality and blocking the female gender, the system in *S. latifolia* includes at least three sets of Y-linked genes: male fertility, male promotion, and female suppression genes.

Dioecious *S. latifolia* and gynodioecious *S. vulgaris* have the same haploid chromosome number, but differ substantially in genome size. The *S. latifolia* haploid genome is 2646 Mbp in females, with the X chromosome being about 400 Mbp in length, whereas the haploid genome size of *S. vulgaris* is 1103Mbp and autosomes are about 100 Mbp long. In our study we analyzed a part of the *S. latifolia* pseudoautosomal region (PAR) located on the p-arm of the X chromosome and on the q-arm of the Y chromosome, and of the corresponding *S. vulgaris* autosome, in order to study collinearity and divergence between these chromosome parts and to assess whether the *S. latifolia* PAR has characteristics in common with animal PARs. Furthermore, we investigated whether the *S. latifolia* size increase relative to *S. vulgaris* reflects the increase in size of the entire X chromosome or more closely resembles the increase seen in *S. latifolia* autosomes. We identified six new sex-linked genes in the *S. latifolia* PAR, together with numerous transposable elements. The same genes were found on the *S. vulgaris* autosomal segment, with no enlargement of the predicted coding sequences in *S. latifolia*. Intergenic regions were on average 1.6 times longer in *S. latifolia* than in *S. vulgaris*, mainly as a consequence of the insertion of transposable elements. The GC content did not differ significantly between the PAR region in *S. latifolia* and the corresponding autosomal region in *S. vulgaris*. Our results demonstrate the usefulness of the BAC library developed here for the analysis of plant sex chromosomes and indicate that the PAR in the evolutionarily young *S. latifolia* sex chromosomes has diverged from the corresponding autosomal region in the gynodioecious *S. vulgaris* mainly with respect to the insertion of transposable elements. Gene order between the PAR and autosomal region investigated is conserved, and the PAR does not have the high GC content observed in evolutionarily much older mammalian sex chromosomes.

Genome size evolution is a complex process influenced by polyploidization, satellite DNA accumulation, and expansion of retroelements. How this process could be affected by different reproductive strategies is still poorly understood. We analyzed differences in the number and distribution of major repetitive DNA elements in two closely related species, *S. latifolia* and *S. vulgaris*. Both species are diploid and possess the same chromosome number, but differ in their genome size and mode of reproduction. We discovered that the genome of *S. latifolia* is larger mainly due to the expansion of Ogre retrotransposons. Surprisingly, the centromeric STAR-C and TR1 tandem repeats were found to be more abundant in *S. vulgaris*, the species with the smaller genome. We further examined the distribution of major repetitive sequences in related species in the Caryophyllaceae family. The results of FISH (fluorescence

in situ hybridization) on mitotic chromosomes with the Retand element indicate that large rearrangements occurred during the evolution of the Caryophyllaceae family. Our data demonstrate that the evolution of genome size in the genus *Silene* is accompanied by the expansion of different repetitive elements with specific patterns in the dioecious species possessing the sex chromosomes.

The evolution of sex chromosomes is often accompanied by gene or chromosome rearrangements. Recently, a gene *AP3* was characterized in the dioecious plant species *S. latifolia*. It was suggested that this gene had been transferred from an autosome to the Y chromosome. We provide evidence for the existence of an X linked copy of the *AP3* gene. We further show that the Y copy is probably located in a chromosomal region where recombination restriction occurred during the first steps of sex chromosome evolution. A comparison of X and Y copies did not reveal any clear signs of degenerative processes in exon regions. Instead, both X and Y copies show evidence for relaxed selection compared to the autosomal orthologues in *S. vulgaris* and *S. conica*. We further found that promoter sequences differ significantly. Comparison of the genic region of *AP3* between the X and Y alleles and the corresponding autosomal copies in the gynodioecious species *S. vulgaris* revealed a massive accumulation of retrotransposons within one intron of the Y copy of *AP3*. Analysis of the genomic distribution of these repetitive elements does not indicate that these elements played an important role in the size increase characteristic of the Y chromosome. However, *in silico* expression analysis shows biased expression of individual domains of the identified retroelements in male plants. Taken together, we characterized the structure and evolution of *AP3*, a sex linked gene with copies on the X and Y chromosomes in the dioecious plant *S. latifolia*. These copies showed complementary expression patterns and relaxed evolution at protein level compared to autosomal orthologues, which suggests subfunctionalization. One intron of the Y-linked allele was invaded by retrotransposons that display sex-specific expression patterns that are similar to the expression pattern of the corresponding allele, which suggests that these transposable elements may have influenced evolution of expression patterns of the Y copy. These data could help researchers decipher the role of transposable elements in degenerative processes during sex chromosome evolution.

Relevant publications:

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(3) Mobility of transposable elements

Plant nuclear genomes vary widely in size. A large proportion of plant genomes are composed of repetitive DNA sequences, among which tandem repeats and transposable elements (TEs) have the most significant impact on genome size. Transposable elements can form up to 80% of the genome. The chromosomal distribution of most TEs is rather random and only a few of them have shown preferences in their chromosomal locations. We carried out a global survey of all major types of transposable elements in *Silene latifolia*, a model species with sex chromosomes that are in the early stages of their evolution. A shotgun genomic library was screened with genomic DNA to isolate and characterize the most abundant elements. We found that the most common types of elements were the subtelomeric tandem repeat X-43.1 and Gypsy retrotransposons, followed by Copia retrotransposons and LINE non-LTR elements. SINE elements and DNA transposons were less abundant. We also amplified transposable elements with degenerate primers and used them to screen the library. The localization of elements by FISH revealed that most of the Copia elements were accumulated on the Y chromosome. Surprisingly, one type of Gypsy element, which was similar to Ogre elements known from legumes, was almost absent on the Y chromosome but otherwise uniformly distributed on all chromosomes.

We studied several mechanisms that could explain the absence of Ogre elements in the Y chromosome. First, Ogre elements could have expanded in the genome before the origin of sex chromosomes and remained inactive during the evolution of sex chromosomes. Secondly, there may be a mechanism for Ogre removal from the Y chromosome. Thirdly, because they colonize only recombining parts of the genome, the spread of Ogre elements could be connected in some way with the recombination process. Fourthly, interaction with some female-specific cellular proteins could be crucial for Ogre retrotransposition. Lastly, Ogre could be active only in female individuals because of either female-specific activation or male-specific silencing. We characterized three Ogre families ubiquitous in the *S. latifolia* genome. One family is nearly absent on the Y chromosome despite all the families having similar structures and spreading mechanisms. We showed that Ogre retrotransposons evolved before sex chromosomes appeared but were mobilized after formation of the Y chromosome. Our data suggest that the absence of one Ogre family on the Y chromosome may be caused by 24-nucleotide (24-nt) small RNA-mediated silencing leading to female-specific spreading. Our findings highlight epigenetic silencing mechanisms as potentially crucial factors in sex specific spreading of some TEs. Another retroelement also missing in the Y chromosome is Athila CL3. Combining X chromosome microdissection and BAC library screening we isolated new X chromosome-linked sequences. Out of 8 identified BAC clones, only one showed an X-preferential signal after FISH experiments. Further analysis revealed the existence of the Athila retroelement which is enriched in the X chromosome and nearly absent in the Y chromosome. Based on our previous data, the Athila retroelement belongs to the CL3 group of most repetitive sequences in the *S. latifolia* genome. Structural, transcriptomics and phylogenetic analyses revealed that Athila CL3 represents an old clade in the Athila lineage. We propose a mechanism responsible for Athila CL3 distribution in the *S. latifolia* genome.

Retrotransposons with long terminal repeats (LTR) form a significant proportion of eukaryotic genomes, especially in plants. They have *gag* and *pol* genes and several regulatory regions necessary for transcription and reverse transcription. We searched for potential quadruplex forming sequences (PQSs) and potential triplex-forming sequences (PTSs) in 18 377 full-length LTR retrotransposons collected from 21 plant species. We found that PQSs were often located in LTRs, both upstream and downstream of promoters from which the whole retrotransposon is transcribed. Upstream-located guanine PQSs were dominant in the minus DNA strand, whereas downstream located guanine PQSs prevailed in the plus strand,

indicating their role both at transcriptional and posttranscriptional levels. Our circular dichroism spectroscopy measurements confirmed that these PQSs readily adopted guanine quadruplex structures - some of them were paralell-stranded, while others were anti-parallel-stranded. The PQS often formed doublets at a mutual distance of up to 400 bp. PTSs were most abundant in 3´UTR (but were also present in 5´UTR). We study the potential role of quadruplexes and triplexes as the regulators of various processes participating in LTR retrotransposon life cycle and as potential recombination sites during post-insertional retrotransposon-based genome rearrangements.

Rumex acetosa (or sorrel) is a dioecious plant with the XY1Y2 sex chromosome system. Both Y chromosomes are heterochromatic and are thought to be degenerated. We performed low-pass 454 sequencing and similarity-based clustering of male and female genomic 454 reads to identify and characterize major groups of *R. acetosa* repetitive DNA. We found that Copia and Gypsy retrotransposons dominated, followed by DNA transposons and non-long terminal repeat retrotransposons. CRM and Tat/Ogre retrotransposons dominated the Gypsy superfamily, whereas Maximus/Sireviruses were most abundant among Copia retrotransposons. Only one Gypsy subfamily had accumulated on Y1 and Y2 chromosomes, whereas many retrotransposons were ubiquitous on autosomes and the X chromosome, but absent on Y1 and Y2 chromosomes, and others were depleted from the X chromosome. One group of CRM Gypsy was specifically localized to centromeres. We also found that majority of previously described satellites (RAYSI, RAYSII, RAYSIII, and RAE180) are accumulated on the Y chromosomes where we identified Y chromosome-specific variant of RAE180. We discovered two novel satellites - RA160 satellite dominating on the X chromosome and RA690 localized mostly on the Y1 chromosome. The expression pattern obtained from Illumina RNA sequencing showed that the expression of transposable elements is similar in leaves of both sexes and that satellites are also expressed. Contrasting patterns of transposable elements (TEs) and satellite localization on sex chromosomes in *R. acetosa*, where not only accumulation but also depletion of repetitive DNA was observed, suggest that a plethora of evolutionary processes can shape sex chromosomes.

Relevant publications:

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(4) Horizontal gene transfer

Horizontal gene transfer (HGT) is defined as a heritable change caused by transfer of genetic material between two species by non-sexual means. To remain widely open to HGT is a risky strategy of evolution that probably operates only in unicellular organisms. A horizontally transferred gene may be either harmful or beneficial and there is no certainty that the acquired gene increases the fitness of the individual as it has not been tested in parents. HGT is a common and well-studied event in the microbial world and serves mainly for increasing the survival of organisms in changing conditions of the environment. In prokaryotic organisms, a rapid gain of function is facilitated by a transfer of complete operons. HGT represents a driving force in the evolution of unicellular species where fixation of transferred material occurs straightforwardly in view of the fact that there is no separate germline as in multicellular organisms with differentiated tissues. Gene transfer to multicellular organisms appears to be much rarer and more complicated than in the case of HGT between prokaryotes. In multicellular organisms, the complexity of eukaryotic organisms and their nuclei makes them more resistant to transfer of foreign DNA than in prokaryotes and unicellular eukaryotes. The number of reports of HGT in eukaryotic genomes is slowly accumulating. The detection of horizontally transferred sequences is, however, more complicated given that some of them are not expressed and cannot be found in RNAseq studies. Also in high throughput sequencing projects using DNA samples, sequences that could be obtained by HGT from bacteria are often filtered off as a possible contamination of the sample. In spite of these difficulties, rapid accumulation of a vast amount of sequencing data is uncovering the first evidence for HGT between prokaryotic and eukaryotic genomes and cases with actively expressed genes. Several examples of HGT between viruses and eukaryotes and between bacteria and eukaryotes have been found in economically important agricultural plant species and in intensively studied model species such as the nematode *Caenorhabditis elegans*, the moss *Physcomitrella patens* and bdelloid rotifers.

Few cases of spontaneously horizontally transferred bacterial genes into plant genomes have been described to date. The occurrence of horizontally transferred genes from the T-DNA of *Agrobacterium rhizogenes* into the plant genome has been reported in the genus *Nicotiana* and in the species *Linaria vulgaris*. We compared patterns of evolution in one of these genes (a gene encoding mikimopine synthase, *mis*) following three different events of horizontal gene transfer (HGT). As this gene plays an important role in *Agrobacterium*, and there are known cases showing that genes from pathogens can acquire plant protection function, we hypothesized that in at least some of the studied species we will find signs of selective pressures influencing *mis* sequence. The mikimopine synthase (*mis*) gene evolved in a different manner in the branch leading to *Nicotiana tabacum* and *N. tomentosiformis*, in the branch leading to *N. glauca* and in the genus *Linaria*. Our analyses of the genus *Linaria* suggest that the *mis* gene began to degenerate soon after the HGT. In contrast, in the case of *N. glauca*, the *mis* gene evolved under significant selective pressures. This suggests a possible role of mikimopine synthase in current *N. glauca* and its ancestor(s). In *N. tabacum* and *N. tomentosiformis*, the *mis* gene has a common frameshift mutation that disrupted its open reading frame. Interestingly, our results suggest that in spite of the frameshift, the *mis* gene could evolve under selective pressures. This sequence may still have some regulatory role at the RNA level as suggested by coverage of this sequence by small RNAs in *N. tabacum*.

Relatively frequent is the horizontal transfer of DNA from organelles (plastids and mitochondria) into the plant nuclear genome. Chloroplast DNA (cpDNA) sequences are often found in plant nuclear genomes, but patterns of their chromosomal distribution are not fully understood. The distribution of cpDNA on the sex chromosomes can only be studied in

dioecious plant species possessing heteromorphic sex chromosomes. We reconstructed the whole chloroplast genome of *Rumex acetosa* (sorrel, XY1Y2 system) from next generation sequencing data. We systematically mapped the chromosomal localization of various regions of cpDNA in *R. acetosa* and *Silene latifolia* (white campion, XY system) using fluorescence *in situ* hybridization. We found that cpDNA was accumulated on the Y chromosomes of both studied species. In *R. acetosa*, the entire Y chromosome gathered all parts of cpDNA equally. On the contrary, in *S. latifolia*, the majority of the cpDNA, corresponding to the single copy regions, was localized in the centromere of the Y chromosome, while the inverted repeat region was present also in other loci. We found a stronger accumulation of cpDNA on the more degenerated Y1 and Y2 chromosomes of *R. acetosa* than in evolutionary younger *S. latifolia* Y chromosome. Our data stressed the prominent role of the Y chromosome centromere in cpDNA accumulation.

We further analyzed the size, relative age and chromosomal localization of nuclear sequences of plastid and mitochondrial origin (NUPTs-nuclear plastid DNA and NUMTs-nuclear mitochondrial DNA) in six completely sequenced plant species (*Arabidopsis thaliana, Oryza sativa, Zea mays, Glycine max, Vitis vinifera*, and *Sorghum bicolor*). We found that the largest insertions showed lower divergence from organelle DNA than shorter insertions in all species, indicating their recent origin. The largest NUPT and NUMT insertions were localized in the vicinity of the centromeres in the small genomes of *Arabidopsis* and rice. They were also present in other chromosomal regions in the large genomes of soybean and maize. Localization of NUPTs and NUMTs correlated positively with distribution of transposable elements (TEs) in *Arabidopsis* and sorghum, negatively in grapevine and soybean, and did not correlate in rice or maize. We propose a model where new plastid and mitochondrial DNA sequences are inserted close to centromeres and are later fragmented by TE insertions and reshuffled away from the centromere or removed by ectopic recombination. The mode and tempo of TE dynamism determines the turnover of NUPTs and NUMTs resulting in their species-specific chromosomal distributions.

Relevant publications:

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These projects and publications were realised in our laboratory and the members of our laboratory played the key roles as the authors or co-authors. Their names are underlined. Each part of this text is ended by a creative review written by the members of our lab.