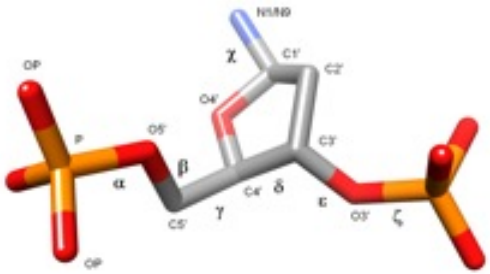
IBP
Institute of Biophysics

INSTITUTE OF BIOPHYSICS

OF THE ACADEMY OF SCIENCES OF THE CZECH REPUBLIC, V.V.I.



RESEARCH REPORT 2010

Contents

Introduction

The budget of the AS CR for the year 2010 remained similar to the budget for the previous year, with only minor adjustments to the end of the year. The budget of the Institute of Biophysics, AS CR, v.v.i. (IBP) was, therefore, also preserved, as were the budgets of individual departments. The reasons for this are as follows: (i) according to the internal evaluation there are no substantial disproportions among the departments; (ii) a comparison of the IBP with other research organizations in the CR and abroad conducted by the management of the Institute showed high-level scientific output within the framework of the CR to which all departments contribute; (iii) the academic evaluation has not yet been completed.

According to the internal evaluation, the department of J. Šponer ranked highest, with two departments (those of V. Brabec and M. Fojta) being placed in the following positions. These departments produce the highest numbers of citations, as well as the highest cumulative impact factors (IF). The teams of A. Kozubik, M. Vorlíčková, A. Lojek, S. Kozubek, A. Kovařík and B. Vyskot achieved results extremely similar to one other. The team of S. Kozubek had the highest number of citations of these departments; the department of M. Vorlíčková was the best when recalculated to the unit of funding. All departments showed improved performance this year.

The further strategy for the development of the Institute should reflect the results of the academic evaluation of individual departments. After the first phase of evaluation, the results correspond to our internal estimates from the period 2005–2009 and the following rating is suggested: the departments of V. Brabec, J. Šponer and B. Vyskot – mark 1, the departments of M. Fojta, A. Kovařík, A. Kozubik, S. Kozubek and A. Lojek – mark 2, and the department of M. Vorlíčková – mark 2.5. In response to these results, the Director and Council of the Institute have demanded an improvement to the marks for the team of M. Fojta (from 2 to 1.5) and the team of M. Vorlíčková (from 2.5 to 2). According to average marks, the Institute ranks among the best in the Academy (6th position out of 50 institutes).

Stanislav Kozubek

MOLECULAR BIOPHYSICS AND PHARMACOLOGY

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Energetics, conformation, and recognition of DNA duplexes modified by monodentate Ru^{II} complexes containing terphenyl arenes

The thermodynamic properties, conformation, and recognition of DNA duplexes site-specifically modified by monofunctional adducts of Ru^{II} complexes of the type $[Ru^{II}(\eta^6\text{-arene})(Cl)(en)]^+$, in which arene = *para*-, *meta*-, or *ortho*-terphenyl (complexes 1, 2, and 3, respectively) and en=1,2-diaminoethane were examined. It has been shown (J. Med. Chem. 2008, 51, 5310) that 1 exhibits promising cytotoxic effects in human tumor cells, whereas 2 and 3 are much less cytotoxic; concomitantly with the high cytotoxicity of 1, its DNA binding mode involves combined intercalative and monofunctional (coordination) binding modes, whereas less cytotoxic compounds 2 and 3 bind to DNA only through a monofunctional coordination to DNA bases. An analysis of conformational distortions induced in DNA by adducts of 1 and 2 revealed more extensive and

stronger distortion and concomitantly greater thermodynamic destabilization of DNA by the adducts of nonintercalating 2. Moreover, affinity of replication protein A to the DNA duplex containing adduct of 1 was pronouncedly lower than to the adduct of 2. On the other hand, another damaged-DNA-binding protein, xeroderma pigmentosum protein A, did not recognize the DNA adduct of 1 or 2. Importantly, the adducts of 1 induced a considerably lower level of repair synthesis than the adducts of 2, which suggests enhanced persistence of the adducts of the more potent and intercalating 1 in comparison with the adducts of the less potent and nonintercalating 2. Also interestingly, the adducts of 1 inhibited DNA polymerization more efficiently than the adducts of 2, and they could also be bypassed by DNA polymerases with greater difficulty. Results of the present work along with those previously published support the view that monodentate Ru^{II} arene complexes belong to a class of anticancer agents for which structure-pharmacological relationships might be correlated with their DNA-binding modes.

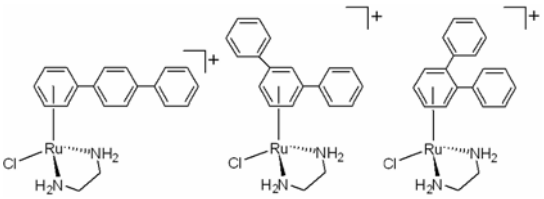

Figure 1: Structures of Ru^{II} arene complexes.

Mechanistic insights into antitumor effects of new dinuclear cis Pt^{II} complexes containing aromatic linkers

The primary objective was to understand more deeply the molecular mechanism underlying different antitumor effects of dinuclear Pt^{II} complexes containing aromatic linkers of different length, $\{[cis\text{-}Pt(NH_3)_2Cl]_2(4,4'\text{-methylene-dianiline})\}^{2+}$ (1) and $\{[cis\text{-}Pt(NH_3)_2Cl]_2\text{-}(\alpha,\alpha'\text{-diamino-p-xylene})\}^{2+}$ (2). These complexes belong to a new generation of promising polynuclear platinum drugs resistant to decomposition by sulfur nucleophiles which hampers clinical use of bifunctional polynuclear trans Pt^{II} complexes hitherto tested. Results obtained with the aid of methods of molecular biophysics and pharmacology reveal differences and new details

of DNA modifications by 1 and 2 and recognition of these modifications by cellular components. The results indicate that the unique properties of DNA interstrand cross-links of this class of polynuclear platinum complexes and recognition of these crosslinks may play a prevalent role in antitumor effects of these metallodrugs. Moreover, the results show for the first time a strong specific recognition and binding of high-mobility-group-domain proteins, which are known to modulate antitumor effects of clinically used platinum drugs, to DNA modified by a polynuclear platinum complex.

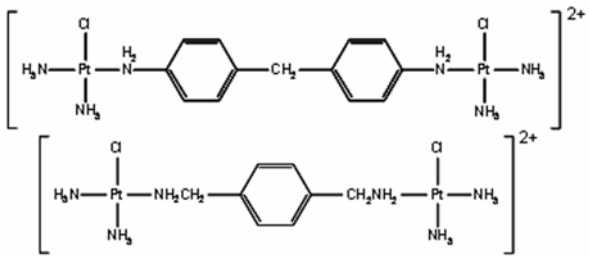

Figure 2: Structures of dinuclear cis Pt^{II} complexes containing aromatic linkers.

Cytotoxicity, cellular uptake, glutathione and DNA interactions of an antitumor large-ring Pt^{II} chelate complex incorporating the cis-1,4-diaminocyclohexane carrier ligand

Earlier studies have described promising antitumor activity of a large-ring chelate complex [$PtCl_2$(cis-1,4-DACH)] (DACH = diaminocyclohexane). Encouraging antitumor activity of this analogue of cisplatin prompted us to perform studies focused on the mechanistic basis of pharmacological effects of this complex. Four early steps in the mechanism of biological activity of cisplatin have been delineated: cell entry, reactions with sulfur-containing compounds, platinum-DNA binding along with processing platinated DNA by proteins (enzymes) and DNA repair. Here, we describe comparative experiments (involving also cisplatin) revealing: (i) improved cytotoxicity (3.4 – 5.4-fold) of [$PtCl_2$(*cis*-1,4-DACH)] in human tumor ovarian cell lines; (ii) enhanced cellular uptake (~1.5-fold) of [$PtCl_2$(*cis*-1,4-DACH)]; (iii) somewhat enhanced rate of reactions of [$PtCl_2$(*cis*-1,4-DACH)] with glutathione (~1.5-fold), but a similar rate of reactions with

metallothionenin-2; (iv) enhanced rate of DNA binding of [$PtCl_2$(*cis*-1,4-DACH)] in cell-free media (~2-fold); (v) similar sequence preference of DNA binding of [$PtCl_2$(*cis*-1,4-DACH)] in cell-free media; (vi) identical DNA interstrand crosslinking efficiency (6%); (vii) similar bending (32 °) and enhanced local unwinding (~1.5-fold) induced in DNA by the major 1,2-GG-intrastrand crosslink; (viii) markedly enhanced inhibiting effects of DNA adducts of [$PtCl_2$(*cis*-1,4-DACH)] on processivity of DNA polymerase; and (ix) a slightly lower efficiency of DNA repair systems to remove the adducts of [$PtCl_2$(*cis*-1,4-DACH)] from DNA.

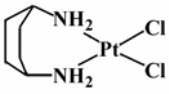

Figure 3: Structure of [$PtCl_2$(*cis*-1,4-DACH)].

Conformation and recognition of DNA modified by a new antitumor dinuclear Pt^{II} complex resistant to decomposition by sulfur nucleophiles

A detailed biochemical and molecular biophysics study of the molecular mechanism of action of antitumor dinuclear Pt^{II} complex $[\{PtCl(DACH)\}_2\ \mu\text{-}Y]^{4+}$ [DACH = 1,2-diaminocyclohexane, Y = $H_2N(CH_2)_6NH_2(CH_2)_2NH_2(CH_2)_6NH_2$] (complex 1) was performed. This new, long-chain bifunctional dinuclear Pt^{II} complex is resistant to metabolic decomposition by sulfur-containing nucleophiles. The results show that DNA adducts of 1 can largely escape repair and yet inhibit very effectively transcription so that they should persist longer than those of conventional cisplatin. Hence, they 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. In addition, the findings of the present work make new insights into mechanisms associated with antitumor effects of dinuclear/trinuclear Pt^{II} complexes possible.

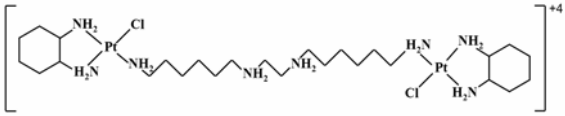

Figure 4: Structure of antitumor dinuclear Pt^{II} complex $[\{PtCl(DACH)\}_2\text{-}\mu\text{-}Y]^{4+}$.

Studies on cellular accumulation of satraplatin and its major metabolite JM118 and their interactions with glutathione

Before the active form of a Pt drug reaches its major pharmacological target in the cell nucleus, the Pt complex has to accumulate in cells, and during its transportation into cells and inside cells, it reacts with various biomolecules. Satraplatin is the first orally administered Pt drug under active clinical investigation. The major metabolite of this Pt^{IV} complex is its Pt^{II} analogue (JM118), which also has significant anticancer properties. Here we report the role of active transport in cellular entry of satraplatin and JM118 and interactions of these Pt complexes with glutathione. The results reveal that the organic cation transporters may play a more important role in the mechanism of cytotoxicity of JM118 than in the cytotoxicity of cisplatin. In contrast, satraplatin is a poor substrate of these transporters. In addition, satraplatin reacts with glutathione with the rate markedly lower than JM118 and cisplatin. Interestingly, satraplatin can be activated by glutathione allowing it to react with DNA, although to a much lower extent than in the case of another Pt^{IV} drug tetraplatin.

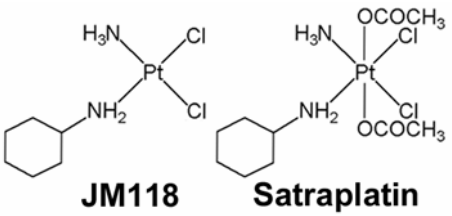

Figure 5: Structure of JM118 and satraplatin.

Different features of the DNA binding mode of antitumor

cis-amminedichlorido(cyclohexylamine)platinum(II) (JM118) and cisplatin *in vitro cis*-Amminedichlorido(cyclohexylamine)platinum(II) (JM118) is an antitumor Pt^{II} analogue of cisplatin exhibiting considerably higher activity than cisplatin in human tumor cells. JM118 is also the major metabolite of the first orally administered Pt^{IV} drug satraplatin. In an effort to design improved platinum antitumor agents, it is important to elucidate the biochemical factors that affect the cytotoxic properties of existing platinum drugs. Since DNA is considered the major pharmacological target of platinum drugs, the objective in the present work was to understand more fully the DNA binding mode of antitumor JM118. We examined the rate of aquation of the first chloride of bifunctional JM118 and found that it was considerably lower than that of cisplatin; consequently, the rate of the reaction of JM118 with DNA was lower compared to cisplatin. The influence of global modification by JM118 and its major site-specific adducts on DNA conformation by biochemical methods was investigated as well. While examination of the global modification revealed in several cases no substantial differences in the lesions induced by JM118 and cisplatin, DNA bending due to the 1,2-GG intrastrand adduct of JM118 was lower than that of cisplatin. The bending angles afforded by the adducts of JM118 were only slightly affected by the orientation of the cyclohexylamine ligand toward the 3' or 5' direction of the duplex. We also used *in vitro* assays that make it possible to monitor DNA repair synthesis by cell-free extracts and DNA-protein cross-linking to probe properties of DNA adducts of JM118. These results showed a higher DNA-protein cross-linking efficiency of JM118 and a less efficient removal from DNA of the adducts of JM118 in comparison with cisplatin. Thus, the results of the present work provide additional evidence that DNA binding of JM118 is in several aspects different from that of conventional cisplatin.

DNA photocleavage by DNA and DNA-LNA amino acid-dye conjugates

DNA photocleavage by triplex forming oligonucleotides (TFO) has potential implications in both biotechnology and medicine. A series of homopurine DNA and DNA/LNA 14-mers to which an amino acid (glycine or L-tryptophan) and a cyanine dye are covalently linked was synthesized. Two cyanine dyes were examined that include a quinolinium ring linked to a benzothiazolium ring through a monomethine (TO1) or trimethine (TO2)

linker. The 14-mer sequence was chosen to target mdm2, a ubiquitin ligase (E3) that regulates p53 by promoting its ubiquitylation and proteosomal degradation. Such inhibition has been previously proposed as a therapeutic approach to target wild-type p53-expressing cancers. To examine whether our TFO conjugates photocleave the mdm2 target, we incubated the various conjugates with the mdm2 plasmid and irradiated the samples with visible light. We show that only the TFO with the complementary sequence and with an intervening L-tryptophan leads to the linearization of the plasmid after a short irradiation time (10 min) exciting the dye (lambda(max)(TO1) = 500 nm and lambda(max)(TO2) = 630 nm) with visible light. Furthermore, the photoreactivity is more pronounced for the LNA/DNA conjugate, an observation that is consistent with improved hybridization to the DNA target. Sequence specificity of the photoreaction is further corroborated on a synthetic 44-mer duplex containing the TFO site. Evidence for a ROS-dependent mechanism is also given and discussed.

A metal-free DNA nuclease based on a cyclic peptide scaffold

The ability to cleave DNA with the aid of chemical nucleases has been a challenge in the scientific community, particularly in the absence of a redox active metal ion. Inspired by structural characterization of the active site found in Staphylococcal nuclease, a series of organic molecule comprising cyclic pentapeptides conjugated to a DNA intercalator (e.g., anthraquinone) was designed. The cyclic peptide is designed to cleave the phosphodiester backbone, whereas the intercalator is expected to improve binding affinity to the substrate (DNA). Our lead compound (1-AQ), composed of the cyclic peptide cyc-D-Lys-Gly-Arg-Ser-Arg conjugated to anthraquinone, degrades DNA into small fragments at physiologically relevant conditions (i.e., 37 degrees C, pH = 7.4). We find that 1-AQ is highly effective in degrading duplex DNA at micromolar concentrations as corroborated by agarose and polyacrylamide gel electrophoresis. Changing the DNA intercalator to acridine (1-Ac) renders the compound comparable in nuclease activity to 1-AQ. In comparison to control compounds (Lin-1 and 1) that lack either the cyclic scaffold or the DNA intercalator, our lead compound (1-AQ) is found to be significantly more active as a DNA chemical nuclease. We have studied the importance of the triad (Arg-Ser-Arg) as the designed module for DNA cleavage. Changing L-Ser to L-Glu (cyc-D-Lys-Gly-Arg-Glu-Arg, Glu-AQ) results in an inactive compound, whereas the cyclic peptide Gly-AQ (cyc-D-Lys-Gly-Arg-Gly-Arg, where glycine replaces L-serine) has similar DNA nuclease

activity to 1-AQ. In addition, changing the stereochemistry from D-lysine to L-lysine results in a cyclic peptide (1-L-AQ) exerting weak DNA nuclease activity, highlighting the importance of the cyclic backbone conformation for efficient DNA nuclease activity. The addition of ROS scavengers does not reduce DNA nuclease activity; an observation that supports a hydrolytic cleavage mechanism. Finally, we have estimated the kinetics of DNA cleavage of a 15-mer duplex DNA substrate by compound 1-AQ. By monitoring DNA duplex degradation by following the change in absorbance (hyperchromicity) at various 1-AQ concentrations, we report a maximal k(obs) value (as an underestimation of k(max)) of 1.62 h(-1) at a 7.5-fold of 1-AQ. We have also compared the other two active peptide conjugates, namely, 1-Ac and Gly-AQ to that of 1-AQ. Both compounds exert similar nuclease activity to that of 1-AQ. To the best of our knowledge, this is the most active metal-free DNA nuclease reported to date that exerts its DNA nuclease activity at biologically relevant conditions.

Granted projects

HHMI (USA), INTNL 55005613, Platinum and ruthenium compounds. From DNA damage to cancer chemotherapy. Principal investigator: J. Kašpárková, 2006 - 2010

Ministry of Education, Youth and Sports CR, ME, LC06030, Center of Basic Research, Biomolecular Center. Co-principal investigator: V. Brabec, 2006 - 2010

GA AS CR KAN200200651, Nanoparticle and supramolecular systems for targeted transport of therapeutic drugs. Co-principal investigator: V. Brabec, 2006 - 2010

GA AS CR IAA400040803, Mechanistic studies related to targeted cancer chemotherapy with light-activated platinum and ruthenium antitumor agents. Principal investigator: J. Kašpárková, 2008 - 2011

Ministry of Education, Youth and Sports CR, Kontakt, ME08017, Platinum metal complexes as DNA-protein cross-linking agents. Principal investigator: V. Brabec, 2008 - 2010

Ministry of Education, Youth and Sports CR, COST, OC08003, Structure, recognition and processing of DNA damage by antitumor metal-based drugs. Principal investigator: V. Brabec, 2008 - 2011

Ministry of Education, Youth and Sports CR, Kontakt, ME10066, Transition metal-based anticancer compounds. From mechanistic studies to innovatory cancer chemotherapy. Principal investigator: V. Brabec, 2010 - 2012

GA CR 30109/H004, Molecular and structural biology of selected antitumor drugs. From mechanistic studies to chemotherapy of tumors. Principal investigator: V. Brabec, 2009 - 2012

GA CR P305/10/P143, Isolation and identification of proteins in cancer cell extracts that bind to DNA modified by antitumor metallodrugs. Principal investigator: P. Machalová, 2010 - 2012

AS CR Program Support to project of international collaboration of AS CR M200040901, Antitumor transition metal-based complexes. From mechanistic studies to cancer chemotherapy. Principal investigator: J. Kašpárková, 2009 - 2013

GA CR P301/10/0598, Metallodrugs based on osmium, platinum and ruthenium complexes. From mechanistic studies to novel, more efficient chemotherapy of cancer. Principal investigator: V. Brabec, 2010 - 2014

European Commission, FP7, ERC-2009-AdG_20090325, Bioinorganic chemistry for the design of new medicines. Co-principal investigator: V. Brabec, 2010 – 2015

Publications

Alkhader, S., Ezra, A., Kasparkova, J., Brabec, V., Yavin, E.: *A metal-free DNA nuclease based on a cyclic peptide scaffold.* Bioconjugate Chem., 21, 2010, 1425-1431.

Zerzankova, L., Kostrhunova, H., Vojtiskova, M., Novakova, O., Suchankova, T., Lin, M., Guo, Z., Kasparkova, J., Brabec, V.: *Mechanistic insights into antitumor effects of new dinuclear cis PtII complexes containing aromatic linkers*. Biochem. Pharmacol., 80, 2010, 344-351.

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

Novakova, O., Malina, J., Suchankova, T., Kasparkova, J., Bugarcic, T., Sadler, P. J., Brabec, V.: *Energetics, conformation, and recognition of DNA duplexes modified by monodentate RuII complexes containing terphenyl arenes*. Chem. Eur. J., 16, 2010, 5744-5754.

Kostrhunova, H., Kasparkova, J., Gibson, D., Brabec, V.: *Studies on cellular accumulation of satraplatin and its major metabolite JM118 and their interactions with glutathione.* Mol. Pharmaceutics, 7, 2010, 2093-2102.

Kostrhunova, H., Vrana, O., Suchankova, T., Gibson, D., Kasparkova, J., Brabec, V.: *Different features of the DNA binding mode of antitumor cis-amminedichlorido(cyclohexylamine)platinum(II) (JM118) and cisplatin in vitro.* Chem. Res. Toxicol., 23, 2010, 1833-1842.

Kasparkova, J., Suchankova, T., Halamikova, A., Zerzankova, L., Vrana, O., Margiotta, N., Natile, G., Brabec, V.: *Cytotoxicity, cellular uptake, glutathione and DNA interactions of an antitumor large-ring PtII chelate complex incorporating the cis-1,4-diaminocyclohexane carrier ligand*. Biochem. Pharmacol., 79, 2010, 552-564.

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Biophysical Chemistry and Molecular Oncology

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Within the DBCMO, two partially autonomous research groups were involved in specifically oriented research. The group **"Analysis of proteins important in biomedicine"** led by Prof. Emil Paleček dealed mainly with peptides and proteins and particularly with their properties at electrically charged surfaces (mostly concentrated in field II, as specified below). The research was oriented towards a new method of electrochemical analysis based on the ability of proteins to catalyze hydrogen evolution at mercury electrodes. Such electrocatalysis is manifested by the so-called peak H which differs from the previously studied electrochemical signals of proteins particularly by its high sensitivity to local and global changes in protein structures. In addition, the group focused on the electrochemical analysis and chemical modification of polysaccharides.

The group **"Physics and Physical Chemistry of Biopolymers"** led by Dr. František Jelen was oriented towards (a) interactions of nucleic acids components with metal ions, such as copper; (b) development of electrochemical methods for microanalysis of nucleic acids components, metabolites and drugs; (c) application of elimination voltammetry (EVLS) in analysis of nucleic acids. Activities of the group belong mainly under the field I.

In 2010, a new group **"Molecular Oncology"** was established to improve efficiency of the department management in the molecular oncology area. The group, led by Dr. Václav Brázda, is oriented towards studies of DNA-protein interactions and their roles in cellular regulation pathways and specifically in development and defense against cancer. Activities of the group belong under the field III.

Summary of the results:

In 2010 the Department of Biophysical Chemistry and Molecular Oncology pursued research concentrated to three main fields (see below). Despite existence of the above mentioned groups, many results arose from collaboration of scientists through the whole Department.

Field I: Electrochemistry of natural, synthetic and chemically modified nucleic acids and their components, development of electrochemical DNA sensors and their applications in detection of DNA damage, DNA hybridization and in molecular diagnostics

Voltammetric study of aminopurines on pencil graphite electrode in the presence of copper ions

Electrochemical oxidations of aminopurines (adenine, 2-aminopurine, 2,6-diaminopurine) and their complexes with Cu(I) on a pencil graphite electrode were investigated by means of linear sweep voltammetry (LSV) and elimination voltammetry with linear scan (EVLS). The anodic process of the Cu(I)-aminopurine complex, corresponding to the oxidation of Cu(I) to Cu(II), takes place in the potential range between 0.4 and 0.5 V (vs. Ag/AgCl/3 M KCl). At more positive potentials the aminopurines provide voltammetric peaks resulting from the oxidation of the purine ring. The stability of the accumulated complex layer was investigated by the adsorptive transfer stripping technique.

Two-dimensional condensation of nucleobases: A comparative study of halogen derivatives of cytosine

The high ability of self-association of nucleic acid components leads to a two-dimensional (2D) condensation at electrode surfaces. The driving force of the process resides in the intermolecular interactions, such as dispersion forces, hydrogen bonding or electrostatic interactions. In this study, the condensation of 5-halogen cytosine derivatives (5-fluorocytosine, 5-bromocytosine and 5-iodocytosine) at the hanging mercury drop electrode was investigated to evaluate the influence of the different types of intermolecular interactions in the phenomenon. All of these derivatives form 2D capacitance pits, but in distinct conditions of pH, concentrations and temperature. Dispersion forces are identified as the main contributor to the 2D condensation of 5-iodocytosine, while for 5-fluorocytosine this is hydrogen bonding in hemiprotonated dimers. The third derivative, 5-bromocytosine, is an intermediate case between the two formers.

The reduction of doxorubicin at a mercury electrode and monitoring of its interaction with DNA using constant current chronopotentiometry

In this report, voltammetry with linear scan and chronopotentiometric stripping (CPS) with constant current were used for the analysis of doxorubicin (DOX) at a hanging mercury drop electrode (HMDE). CPS was used for the study of DOX *in situ* electrochemical reduction in adsorbed

state and for *ex situ* (adsorptive transfer) analysis of the drug. For the first time, CPS was used to study the reversible reduction of the DOX quinone moiety at –0.45 V (vs. Ag|AgCl|3 M KCl) as well as electrode processes giving rise to an irreversible signal around -1.45 V at the HMDE in 0.2 M acetate or Britton-Robinson buffers at different pH values. The dependence of the latter signal on pH revealed involvement of protonation equilibria; however, neither CV nor CPS data confirmed the catalytic character of the electrode reaction previously suggested by other authors. The CPS method was also applied to monitor the DOX interaction with double- (ds) and single-stranded (ss) DNA. In the presence of dsDNA, more pronounced changes in DOX signal intensity were observed, in agreement with a strong intercalation of the DOX redox centre into the DNA double helix.

Oxidation of protopine at a pyrolytic graphite electrode using cyclic and square-wave voltammetry

This paper describes oxidation of the isoquinoline alkaloid, protopine (PR) at a pyrolytic graphite electrode (PGE) using cyclic and square-wave voltammetry. In the alkaline range (pH 7.5–10.5) of a Britton–Robinson (B–R) buffer, a PR oxidation can be observed as a well-developed voltammetric peak around +0.9 V (vs. Ag|AgCl|3 M KCl). With increasing pH of the B–R buffer, the PR peak is shifted to less positive potentials. The acquired voltammetric data suggest that PR strongly adsorbs onto the surface of the pyrolytic graphite where it is subjected to irreversible electrochemical oxidation in its uncharged free (tricyclic) base form. The results are discussed in connection with the electrochemical oxidation of other isoquinoline alkaloids and the potential applications of these data.

A label-free electrochemical test for DNA-binding activities of tumor suppressor protein p53 using immunoprecipitation at magnetic beads In this paper we extend the application area of the label-free structure-sensitive electrochemical DNA sensing with mercury-based electrodes which is for the first time used, in combination with immunoprecipitation at magnetic beads (MB), for the probing of DNA interactions with tumor suppressor protein p53. The technique relies on capture of the p53–DNA complexes at MB via anti-p53 antibodies, followed by salt-induced dissociation of linear DNA from the complex and its voltammetric detection. Competitive binding of p53 to various plasmid DNA substrates, including lin or scDNAs with or without a specific target site, can easily be followed by *ex situ* electrochemical analysis of DNA recovered from the immunoprecipitated

complexes. 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 simple quantitation of DNA containing free ends at submicrogram levels. We demonstrate applicability of the proposed technique to monitor different DNA-binding activities of wild type and mutant p53 proteins.

Direct voltammetric analysis of DNA modified with enzymatically incorporated 7-deazapurines

Nucleic acids studies use 7-deazaguanine (G*) and 7-deazaadenine (A*) as analogues of natural purine bases incapable of forming Hoogsteen base pairs, which prevents them from being involved in DNA triplexes and tetraplexes. Reduced propensity of the G*- and/or A*- modified DNA to form alternative DNA structures is utilized, for example, in PCR amplification of guanine-rich sequences. Both G* and A* exhibit significantly lower potentials of their oxidation, compared to the respective natural nucleobases. At carbon electrodes, A* yields an oxidation peak which is by about 200-250 mV less positive than the peak due to adenine, but coincides with oxidation peak produced by natural guanine residues. On the other hand, oxidation signal of G* occurs at a potential by about 300 mV less positive than the peak due to guanine, being well separated from electrochemical signals of any natural DNA component. We show that enzymatic incorporation of G* and A* can easily be monitored by simple *ex situ* voltammetric analysis of the modified DNA at carbon electrodes. Particularly G* is shown as an attractive electroactive marker for DNA, efficiently incorporable by PCR. While densely G*-modified DNA fragments exhibit strong quenching of fluorescence of SYBR dyes, commonly used as fluorescent indicators in both gel staining and real time PCR applications, the electrochemical detection provides G*-specific signal suitable for the quantitation of the amplified DNA as well as for the determination of the DNA modification extent. Determination of DNA amplicons based on the measurement of peak G*ox is not affected by signals produced by residual oligonucleotide primers or primary templates containing natural purines.

Determination of the level of DNA modification with cisplatin by catalytic hydrogen evolution at mercury-based electrodes

Electrochemical methods proved useful as simple and inexpensive tools for the analysis of natural as well as chemically modified nucleic acids. In particular, covalently attached metal-containing groups usually render the DNA well-pronounced electrochemical activity related to redox processes of the metal moieties, which can in some cases be coupled to catalytic hydrogen evolution at mercury or some types of amalgam electrodes. In this paper we used voltammetry at the mercury-based electrodes for the monitoring of DNA modification with cisdiamminedichloroplatinum (cisplatin), a representative of metallodrugs used in the treatment of various types of cancer or being developed for such purpose. In cyclic voltammetry at the mercury electrode, the cisplatin modified DNA yielded catalytic currents the intensity of which reflected DNA modification extent. In square-wave voltammetry, during anodic polarization after prereduction of the cisplatinated DNA, a well-developed, symmetrical signal (peak P) was obtained. Intensity of the peak P linearly responded to the extent of DNA modification at levels relevant for biochemical studies (rb) 0.01-0.10, where rb is the number of platinum atoms bound per DNA nucleotide. We demonstrate a correlation between the peak P intensity and a loss of sequence specific DNA binding by tumor suppressor protein p53, as well as blockage of DNA digestion by a restriction endonuclease Msp I (both caused by the DNA cisplatination). Application of the electrochemical technique in studies of DNA reactivity with various anticancer platinum compounds, as well as for an easy determination of the extent of DNA platination in studies of its biochemical effects, is discussed.

Facile end-labeling of RNA with electroactive Os(VI) complexes

Ribose at the 3′-end of oligonucleotides (oligos) selectively modified by Os(VI)2,2′-bipyridine (bipy) produced two CV redox couples at pyrolytic graphite electrode. Using square wave voltammetry (SWV) 22-mer oligos can be detected down to 250 nM. At mercury electrodes the Os(VI)bipy-oligo adducts produced an electrocatalytic peak at ~−1.2 V allowing their determination down to picomolar concentrations. High specificity of Os(VI)bipy for ribose in nucleic acids and high sensitivity of the determination at mercury and solid amalgam electrodes give promise for new efficient methods of microRNA determination.

Preparation and properties of mercury film electrodes on solid amalgam surface

A simple apparatus (electrolyzer) and a reliable procedure were developed for the preparation of mercury films of exactly defined thickness on a silver solid amalgam substrate. Constant concentration of Hg(II) in the electrolyzer is ensured by dissolution of an anode from silver paste amalgam. Small volume of electrolyte, which can be used repeatedly many times, and paste amalgam preventing the spillage of liquid mercury substantially decrease the danger of environmental contamination with mercury. Parameters and behavior of mercury film electrodes on silver solid amalgam substrate (MF-AgSAE) were compared with polished silver solid amalgam electrode (p-AgSAE) which does not contain liquid mercury, with mercury meniscus modified silver solid amalgam electrode (m-AgSAE), and with hanging mercury drop electrode (HMDE). The height of anodic stripping voltammetric peaks divided by electrode area was highest for MF-AgSAE and the width of those peaks, which determines the resolution of the method, was minimal at MF-AgSAE. Available potential window of MF-AgSAE in different supporting electrolytes is comparable with that of HMDE.

Osmium tetroxide complexes as versatile tools for structure probing and electrochemical analysis of biopolymers

Osmium tetroxide complexes with nitrogenous ligands and analogous complexes of six-valent osmium proved excellent tools for selective labeling of biopolymers (nucleic acids, proteins and polysaccharides). Reactions of these species with target moieties within the biopolymer molecules (pyrimidine nucleobases, tryptophan residues or sugar moieties) are facile at physiological conditions and are in general structure-selective, allowing their application in DNA and protein structure probing. The modification products can be detected by a variety of widely accessible analytical techniques, including biochemical (enzymatic) approaches, immunoassays, chemical DNA sequencing, spectrophotometry and electrochemistry. Particularly the electrochemical techniques are promising for utilization in biosensors and routine bioassays due to the possibility of highly sensitive and selective detection of the labeled biopolymers adducts based on distinct electrochemical properties of the introduced osmium moieties. Utilization of the osmium tags in probing DNA structural

transitions, sensing of DNA hybridization, damage and DNA methylation, labeling of peptides and proteins, probing accessibility of tryptophan residues in proteins and their complexes, and labeling of sugar moieties, are reviewed.

Electrochemical nucleic acid-based biosensors: Concepts, terms, and methodology

An electrochemical nucleic acid (NA)-based biosensor is a biosensor that integrates a nucleic acid as the biological recognition element and an electrode as the electrochemical signal transducer. The present report provides concepts, terms, and methodology related to biorecognition elements, detection principles, type of interactions to be addressed, and construction and performance of electrochemical NA biosensors, including their critical evaluation, which should be valuable for a wide audience, from academic, biomedical, environmental, and food-testing, drug-developing, etc. laboratories to sensor producers.

Field II: Properties of peptides, proteins and polysaccharides at electrically charged surfaces, application of electrochemistry in development of novel micromethods for protein and polysaccharide analysis

Influence of the interfacial peptide organization on the catalysis of hydrogen evolution

The hydrogen evolution reaction is catalyzed by peptides and proteins adsorbed on electrode materials with high overpotentials for this reaction, such as mercury. The catalytic response characteristics are known to be very sensitive to the composition and structure of the investigated biomolecule, opening the way to the implementation of a label-free, reagentless electroanalytical method in protein analysis. Herein, it is shown using the model peptide Cys-Ala-Ala-Ala-Ala-Ala that the interfacial organization significantly influences the catalytic behavior. This peptide forms at the electrode two distinct films, depending on the concentration and accumulation time. The low-coverage film, composed of flat-lying molecules (area per molecule of <250-290 A° 2), yields a well-defined catalytic peak at potentials around -1.75 V. The high-coverage film, made of upright-oriented peptides (area per molecule of <43A° 2), is catalytically more active and the peak is observed at potentials less negative by <0.4 V.

The higher activity, evidenced by constant-current chronopotentiometry and cyclic voltammetry, is attributed to an increase in the acid dissociation constant of the amino acid residues as a result of the low permittivity of the interfacial region, as inferred from impedance measurements. An analogy is made to the known differences in acidic-basic behaviors of solvent-exposed and hydrophobic domains of proteins.

Fabrication and characterization of solid mercury amalgam electrodes for protein analysis

Gold and carbon electrodes have been largely used as transducers in protein and DNA sensors and arrays. Liquid mercury electrodes, with potential windows allowing detection of DNA and protein reduction processes at highly negative potentials, were considered as useless in such arrays. Here, we show that solid amalgam electrode (SAE) arrays can be prepared as a substitution of liquid mercury in the analysis of the above biomacromolecules. Vacuum metal sputtering on a glass substrate, photolithography, and galvanic mercury amalgam formation were used for fabrication of an inexpensive disposable electrode array. The resulting ultrathin (less than 1 μm) amalgam microelectrodes were characterized with respect to influence of the electrode composition and size on the reproducibility and stability of electrochemical signals. Further characterization was performed using electron microscopy and the well-established ruthenium electrochemistry. Final, optimized, design was applied in protein analysis employing the recently described electrocatalytic chronopotentiometric peak H.

Protein structure-sensitive electrocatalysis at dithiothreitol-modified electrodes

Dithiothreitol (DTT)-mercury and DTT-solid amalgam electrodes are proposed for protein microanalysis by means of constant current chronopotentiometric stripping (CPS). At the DTT-modified hanging mercury drop electrode (DTT-HMDE), proteins at nanomolar concentrations produce the CPS peak H, which is due to the protein catalyzed hydrogen evolution. Self-assembled monolayers (SAMs) of DTT at the electrode surface protected surface-attached proteins from the electric field-driven denaturation, but did not interfere with the electrocatalysis. Using CPS peak H, native and denatured forms of bovine serum albumin

(BSA) and of other proteins were easily distinguished. On the other hand, in usual slow scan voltammetry (scan rates between 50 mV/s and 1 V/s), the adsorbed BSA behaved as fully or partially denatured. BSA-modified DTT-HMDE was exposed to different potentials, EB for 60 s, followed by CPS measurement. Three *E*B regions were observed, in which either BSA remained native (A, -0.1 to -0.3 V), was denatured (B, -0.35 to -1.4 V), or underwent desorption (C, at potentials more negative than -1.4 V). At potentials more positive than the reduction potential of the DTT Hg-S bond (! -0.65 V against Ag|AgCl|3 M KCl), the densely packed DTT SAM was impermeable to $[Ru(NH_3)_6]^{3+}$. At more negative potentials, the DTT SAM was disturbed, but under conditions of CPS (with very fast potential changes), this SAM still protected the protein from surface-induced denaturation. Thiol-modified Hg electrodes in combination with CPS represent a new tool for protein analysis in biomedicine and proteomics.

Polylysine-catalyzed hydrogen evolution at mercury electrodes

It has been shown that peptides and proteins produce at nanomolar concentrations a structure-sensitive chronopotentiometric peak H at mercury electrodes, which is due to the catalytic hydrogen evolution reaction (HER). Herein, we use for the first time poly(amino acids) to obtain information about the role of individual amino acid residues in the HER. At pH 6 polylysine (polyLys), polyarginine and tryptophan yield a peak H, in agreement with their ionization state, while polyglutamic acid gives no catalytic response. PolyLys catalyzes hydrogen evolution in its adsorbed state. Even at potentials negative to the potential of zero charge, hydrophobic interactions could be involved in polyLys adsorption.

From polarographic presodium wave of proteins to electrochemistry of biomacromolecules

History of electrochemistry of proteins and nucleic acids is briefly reviewed. The ability of proteins to catalyze hydrogen evolution at Hg electrodes was discovered almost 80 years ago in J. Heyrovský's laboratory. This phenomenon was not sufficiently appreciated for several decades. Recently it has been shown that using constant current chrono-potentiometric stripping (CPS) with hanging mercury drop, solid amalgam or Hg-film electrodes the CPS peak H is obtained with nanomolar concentrations of peptides and proteins. This peak is derived from the

presodium wave but it has some new properties useful in protein research. It is sensitive to changes in protein structures and to protein redox states, representing a new tool for protein analysis applicable in biomedicine. Electroactivity of nucleic acids was discovered about 50 years ago. Electrochemistry of DNA and RNA is now a booming field because of its potential use in sensors for DNA hybridization and DNA damage. Quite recently it has been shown that electrochemistry can be applied also in polysaccharide analysis.

Voltammetry of Os(VI)-modified polysaccharides

Polysaccharides (PSs), such as dextran, yeast mannan, starch and amylose, were modified with complexes of sixvalent osmium with nitrogen ligands [Os(VI)L] and voltammetric behavior of PS-Os(VI)L adducts was studied at mercury and carbon electrodes. Using Os(VI)temed as a modification agent and adsorptive transfer stripping (*ex situ*) method it was possible to determine PSs at submicromolar concentrations directly in the reaction mixture in an excess of monomeric glucose or sucrose both at Hg and carbon electrodes. Conventional (*in situ*) PS determination in the reaction mixture was possible only with mercury electrodes. The above methods have great potenciality in biological research.

Diffractive-optics-based sensor as a tool for detection of biocompatibility of titanium and titanium-doped hydrocarbon samples

Adsorption of the elongated human plasma fibrinogen (HPF) and globular human serum albumin molecules on a titanium-based surface is monitored by analyzing permittivity and optical roughness of protein-modified surfaces by using a diffractive optical element (DOE)-based sensor and variable angle spectro-ellipsometry (VASE). Both DOE and VASE confirmed that fibrinogen forms a thicker and more packed surface layer compared to a more porous and weakly adsorbed albumin layer. A linear relation of the permittivity (ε0) and dielectric loss (ε00) was found for some of the dry titanium-doped hydrocarbon (TDHC) surfaces with excellent HPF adsorption ability. We discuss some aspects of TDHC's aging and its possible effects on fibrinogen adsorption.

Field III: Structure and interaction of DNA and proteins in oncological research, especially with respect to the p53-family proteins

The potential of the cruciform structure formation as an important factor influencing p53 sequence-specific binding to natural DNA targets

p53 is one of the most important tumor suppressors which responds to DNA damage by binding to DNA and regulating the transcription of genes involved in cell cycle arrest, apoptosis, or senescence. As it was shown previously, p53 binding to DNA is strongly influenced by DNA topology. DNA supercoiling is fundamentaly important for a wide range of biological processes including DNA transcription, replication, recombination, control of gene expression and genome organization. In this study, we investigated the cruciform structures formation of various inverted repeats in p53-responsive sequences from p21, RGC, mdm2 and GADD45 promoters under negative superhelical stress, and analyzed the effects of these DNA topology changes on p53-DNA binding. We demonstrated using three different methods (gel retardation analyses, ELISA and magnetic immunoprecipitation assay) that the p53 protein binds preferentially to negatively supercoiled plasmid DNAs with p53-responsive sequence presented as a cruciform structure. Not only the appearance of the cruciform structures within naked supercoiled DNA, but also the potential of the binding sites for adopting the non-B structures can contribute to a more favorable p53-DNA complex.

Selective binding of tumor suppressor p53 protein to topologically constrained DNA: Modulation by intercalative drugs

Selective binding of the wild type tumor suppressor protein p53 to negatively and positively supercoiled (sc) DNA was studied using intercalative drugs chloroquine (CQ), ethidium bromide, acridine derivatives and doxorubicin as a modulators of the level of DNA supercoiling. 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. Formation of positive superhelices (due to further increasing intercalator concentrations) rendered the circular duplex DNA to be preferentially bound by the p53 again. CQ at concentrations modulating the closed circular DNA topology did not

prevent the p53 from recognizing a specific target sequence within topologically unconstrained linear DNA. Experiments with DNA topoisomer distributions differing in their superhelix densities revealed the p53 to bind selectively DNA molecules possessing higher number of negative or positive superturns. Possible modes of the p53 binding to the negatively or positively supercoiled DNA and tentative biological consequences are discussed.

Satellite glial cells express IL-6 and corresponding signal-transducing receptors in the dorsal root ganglia of rat neuropathic pain model

There is a growing body of evidence that cytokines contribute to both induction and maintenance of neuropathic pain derived from changes in dorsal root ganglia (DRG), including the activity of the primary sensory neurons and their satellite glial cells (SGC). We used immunofluorescence and *in situ* hybridization methods to provide evidence that chronic constriction injury (CCI) of the sciatic nerve induces synthesis of interleukin-6 (IL-6) in SGC, elevation of IL-6 receptor (IL-6R) and activation of signal transducer and activator of transcription 3 (STAT3) signaling. Unilateral CCI of the rat sciatic nerve induced mechanoallodynia and thermal hyperalgesia in ipsilateral hind paws, but contralateral paws exhibited only temporal changes of sensitivity. We demonstrated that IL-6 mRNA and protein, which were expressed at very low levels in naïve DRG, were bilaterally increased not only in L4-L5 DRG neurons but also in SGC activated by unilateral CCI. Besides IL-6, substantial increase of IL-6R and pSTAT3 expression occurred in SGC following CCI, however, IL-6R associated protein, gp130 levels did not change. The results may suggest that unilateral CCI of the sciatic nerve induces bilateral activation of SGC in L4-L5 DRG to transdukce IL-6 signaling during neuroinflammation.

Spatio-temporal changes of SDF1 and its CXCR4 receptor in the dorsal root ganglia following unilateral sciatic nerve injury as a model of neuropathic pain

There is a growing evidence that chemokines and their receptors play a role in inducing and maintaining neuropathic pain. In the present study, unilateral chronic constriction injury (CCI) of rat sciatic nerve under aseptic conditions was used to investigate changes for stromal derived factor-1 (SDF1) and its CXCR4 receptor in lumbal (L4–L5) and cervical (C7–C8)

dorsal root ganglia (DRG) from both sides of naïve, CCI-operated and sham-operated rats. All CCI-operated rats displayed mechanical allodynia and thermal hyperalgesia in hind paws ipsilateral to CCI, but forepaws exhibited only temporal changes of sensitivity not correlated with alterations in SDF1 and CXCR4 proteins. Naïve DRG displayed immunofluorescence for SDF1 (SDF1-IF) in the satellite glial cells (SGC) and CXCR4-IF in the neuronal bodies with highest intensity in small- and medium-sized neurons. Immunofluorescence staining and Western blot analysis confirmed that unilateral CCI induced bilateral alterations of SDF1 and CXCR4 proteins in both L4–L5 and C7–C8 DRG. Only lumbal DRG were invaded by ED-1+ macrophages exhibiting SDF1-IF while elevation of CXCR4-IF was found in DRG neurons and SGC but not in ED-1+ macrophages. No attenuation of mechanical allodynia, but reversed thermal hyperalgesia, in ipsi- and contralateral hind paws was found in CCI-operated rats after i.p. administration of CXCR4 antagonist (AMD3100). These results indicate that SDF1/CXCR4 changes are not limited to DRG associated with injured nerve but that they also spread to DRG non-associated with such nerve. Functional involvement of these alterations in DRG non-associated with injured nerve in neuropathic pain remains to be elucidated.

Fetal colon cell line FHC exhibits tumorigenic phenotype, complex karyotype, and TP53 gene mutation

Stable cell lines obtained by spontaneous immortalization might represent early stages of malignant transformation and be useful experimental models for studies of mechanisms of cancer development. The FHC (fetal human cells) cell line has been established from normal fetal colonic mucosa. Detailed characterization of this cell line and mechanism of spontaneously acquired immortality have not been described yet. Therefore, we characterized the FHC cell line in terms of its tumorigenicity, cytogenetics, and TP53 gene mutation analysis. FHC cells displayed capability for anchorage-independent growth in semisolid media *in vitro* and formed solid tumors after transplantation into SCID (severe combined immunodeficiency) mice. This tumorigenic phenotype was associated with hypotriploidy and chromosome number ranging from 66 to 69. Results of comparative genetic hybridization arrays showed that most chromosomes included regions of copy number gains or losses. Region 8q23~8q24.3 (containing, e.g., MYC proto-oncogene) was present in more than 20 copies per nucleus. Moreover, we identified mutation of TP53 gene in codon 273;

triplet CGT coding Arg was changed to CAG coding His. Expression of Pro codon 72 polymorphic variant of p53 was also detected. Mutation of TP53 gene was associated with abolished induction of p21Waf1/Cip1 and MDM-2 proteins and resistance to apoptosis after genotoxic treatment. Because of their origin from normal fetal colon and their relative resistence to the induction of apoptosis, FHC cells can be considered a valuable experimental model for various studies.

Granted projects

GA AS CR IAA500040701, Interactions of wild type and mutant p53 proteins with damaged DNA and their roles in cellular response to anticancer chemotherapy. Principal investigator: M. Fojta, 2007 - 2010

GA AS CR IAA400040901, DNA labeling with redox markers for electrochemical sensing. Applications in analysis of nucleotide sequences and molecular diagnostic. Principal investigator: M. Fojta, 2009 - 2013

GA AS CR IAA400040804, Application of electrochemical methods focused on the microanalysis of nucleic acids bases and oligonucleotides. Principal investigator: F. Jelen, 2008 - 2010

AS CR M200040904, Complex interaction of oncology-related important transcription factors with target DNA *in vitro* and *in vivo*. Principal investigator: V. Brázda, 2009 - 2012

GA AS CR KAN400310651, Nanotechnologies for protein and gene diagnostics. Principal investigator: F. Foret, Principal co-investigator: E. Paleček, 2006 - 2010

GA AS CR KAN200040651, Electrochemical and optical analysis of biomacromolecules at the microelectrodes modified by an electroactive material nanolayer. Principal investigator: S. Hasoň, 2006 - 2010

GA CR 203/09/0317, Construction of novel functional nucleic acids for applications in chemical biology, catalysis and self assembly. Principal investigator: M. Hocek, Principal co-investigator: M. Fojta, 2009 - 2013

GA CR 301/10/1211, Transcriptional activities of wild-type and mutant p53, decision between cell proliferation, cell cycle arrest and apoptosis. Principal investigator: V. Brázda, 2009 - 2012

GA CR 204/08/1570, *In vitro* and in silico identification of non-canonical DNA structures in genomic DNA sequences. Principal investigator: M. Brázdová, Principal co-investigators: M. Lexa, O. Fučík, 2008 - 2010

GA CR 202/08/1688, Utilization of physical methods of investigation of nucleic acid and protein adsorption at interfaces in medical diagnosis and biocompatibility study. Principal investigator: V. Vetterl, 2008 - 2010

GA AS CR IAA400040903, Interfacial and electrochemical behavior of synthetic oligonucleotides: effects of nucleotide sequence, conformation and chemical modification. Principal investigator: L. Havran, 2009 - 2011

GA CR 203/08/P598, Electrochemical tools for detection of point mutations and polymorphisms in DNA. Principal investigator: P. Kostečka, 2008 - 2010

GA CR P301/10/P548, Reaction of six-valent and eight-valent osmium complexes with biomacromolecules and their application in biomedicine: Principal investigator: M. Trefulka, 2010 - 2012

Ministry of Education, Youth and Sports of the CR - Research centre LC06035, Centre of biophysical chemistry, bioelectrochemistry and bioanalysis. New tools for genomics, proteomics and biomedicine. Coordinator: M. Fojta, 2006 - 2010

Ministry of Education, Youth and Sports of the CR - ME09038, Interactions of proteins and peptides with surfaces. New tools for biomedicine. Principal investigator: E. Paleček, Principal co-investigator: J. Wang, 2009 - 2012

Ministry of Education, Youth and Sports of the CR - 1M0528, Stomatological research center. Principal investigator: J. Vaněk, Principal co-investigator: V. Vetterl, 2005-2011

CZ.1.07/2.3.00/09.0046 (OP VK), Modern biophysical methods: advanced education and training in experimental biology. Principal investigator: M. Fojta, 2010 - 2012

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MOLECULAR EPIGENETICS

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Repeated reunions and splits feature the highly dynamic evolution of 5S and 35S ribosomal RNA genes (rDNA) in the *Asteraceae* family
(Aleš Kovařík)

In flowering plants and animals the most common ribosomal RNA genes (rDNA) organisation is that in which 35S (encoding 18S-5.8S-26S rRNA) and 5S genes are physically separated occupying different chromosomal loci. However, recent observations established that both genes have been unified to a single 35S-5S unit in the genus *Artemisia* (*Asteraceae*), a genomic arrangement typical of primitive eukaryotes such as yeast, among others. Here we aim to reveal the origin, distribution and mechanisms leading to the linked organisation of rDNA in the *Asteraceae* by analysing unit structure (PCR, Southern blot, sequencing), gene copy

number (quantitative PCR) and chromosomal position (FISH) of 5S and 35S rRNA genes in approximately 200 species representing the family diversity and other closely related groups. Dominant linked rDNA genotype was found within three large groups in subfamily Asteroideae: tribe Anthemideae (93% of the studied cases), tribe *Gnaphalieae* (100%) and in the "*Heliantheae* alliance" (23%). The remaining five tribes of the Asteroideae displayed canonical non linked arrangement of rDNA, as did the other groups in the *Asteraceae*. Nevertheless, low copy linked genes were identified among several species that amplified unlinked units. The conserved position of functional 5S insertions downstream from the 26S gene suggests a unique, perhaps retrotransposon-mediated integration event at the base of subfamily *Asteroideae*. Further evolution likely involved divergence of 26S-5S intergenic spacers, amplification and homogenisation of units across the chromosomes and concomitant elimination of unlinked arrays. However, the opposite trend, from linked towards unlinked arrangement was also surmised in few species indicating possible reversibility of these processes. Our results indicate that nearly 25% of *Asteraceae* species may have evolved unusual linked arrangement of rRNA genes. Thus, in plants, fundamental changes in intrinsic structure of rDNA units, their copy number and chromosomal organisation may occur within relatively short evolutionary time. We hypothesize that the 5S gene integration within the 35S unit might have repeatedly occurred during plant evolution, and probably once in *Asteraceae*.

Similar patterns of rDNA evolution in synthetic and recently formed natural populations of *Tragopogon* (*Asteraceae*) allotetraploids
(Roman Matyášek)

Tragopogon mirus and T. miscellus are allotetraploids (2n = 24) that formed repeatedly during the past 80 years in eastern Washington and adjacent Idaho (USA) following the introduction of the diploids *T. dubius*, *T. porrifolius*, and *T. pratensis* (2n = 12) from Europe. In most natural populations of *T. mirus* and *T. miscellus*, there are far fewer 35S rRNA genes (rDNA) of *T. dubius* than there are of the other diploid parent (*T. porrifolius* or *T. pratensis*). We studied the inheritance of parental rDNA loci in allotetraploids resynthesized from diploid accessions. We investigate the dynamics and directionality of these rDNA losses, as well as the contribution of gene copy number variation in the parental diploids to rDNA variation in the derived tetraploids. Using Southern blot hybridization and fluorescent *in situ* hybridization (FISH), we analyzed

copy numbers and distribution of these highly reiterated genes in seven lines of synthetic *T. mirus* (110 individuals) and four lines of synthetic *T. miscellus* (71 individuals). Variation among diploid parents accounted for most of the observed gene imbalances detected in F1 hybrids but cannot explain frequent deviations from repeat additivity seen in the allotetraploid lines. Polyploid lineages involving the same diploid parents differed in rDNA genotype, indicating that conditions immediately following genome doubling are crucial for rDNA changes. About 19% of the resynthesized allotetraploid individuals had equal rDNA contributions from the diploid parents, 74% were skewed towards either *T. porrifolius* or *T. pratensis*-type units, and only 7% had more rDNA copies of *T. dubius*-origin compared to the other two parents. Similar genotype frequencies were observed among natural populations. Despite directional reduction of units, the additivity of 35S rDNA locus number is maintained in 82% of the synthetic lines and in all natural allotetraploids. Uniparental reductions of homeologous rRNA gene copies occurred in both synthetic (Fig. 1) and natural populations of *Tragopogon* allopolyploids. The extent of these rDNA changes was generally higher in natural populations than in the synthetic lines. We hypothesize that locus-specific and chromosomal changes in early generations of allopolyploids may influence patterns of rDNA evolution in later generations.

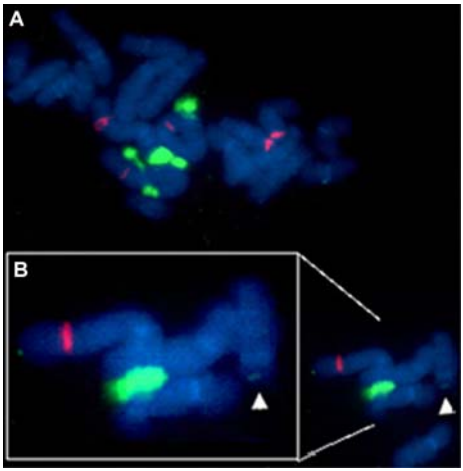

Figure 1: Fluorescence *in situ* hybridization to metaphase spreads of synthetic *T. mirus* The 35S and 5S rDNA probes were labeled in green and red, respectively. Individual 73-14 had four strong plus 0-2 very weak (minute) 35S signals. (B) is an expanded region of (A) showing the large locus of *T. dubius* origin and the minute locus of *T. porrifolius* origin (arrowheads) left after the deletion of the majority of genes from the array.

Inhibition of SAH-hydrolase activity during seed germination leads to deregulation of flowering genes and altered flower morphology in tobacco (Jaroslav Fulneček)

Developmental processes are closely connected to certain states of epigenetic information which, among others, rely on methylation of chromatin. S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) are key cofactors of enzymes catalyzing DNA and histone methylation. To study the consequences of altered SAH/SAM levels on plant development we applied 9-(S)-(2,3-dihydroxypropyl)-adenine (DHPA), an inhibitor of SAH-hydrolase, on tobacco seeds during a short phase of germination period (6 days). The transient drug treatment induced: (1) dosage-dependent global DNA hypomethylation mitotically transmitted to adult plants; (2) pleiotropic developmental defects including decreased apical dominance, altered leaf and flower symmetry, flower whorl malformations and reduced fertility (Fig. 2); (3) dramatic upregulation of floral organ identity genes NTDEF, NTGLO and NAG1 in leaves. We conclude that temporal SAH-hydrolase inhibition deregulated floral genes expression probably via chromatin methylation changes. The data further show that plants might be particularly sensitive to accurate setting of SAH/SAM levels during critical developmental periods.

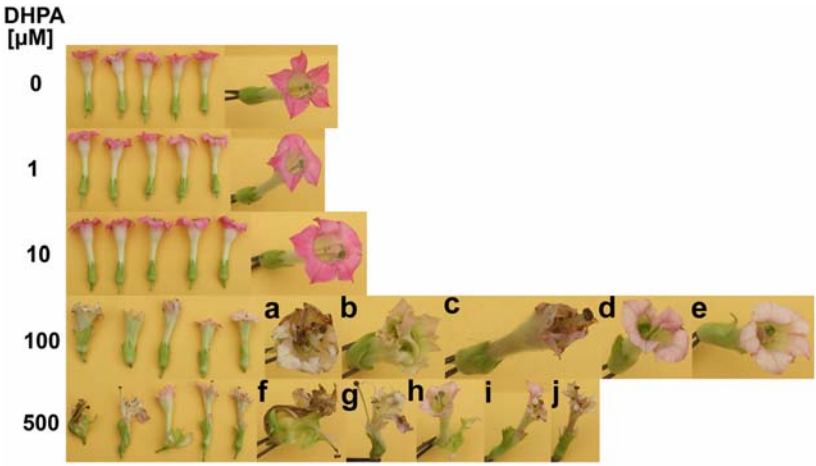

Figure 2: The flower phenotypes resulting from SAH-hydrolase inhibition during seed germination. The aberrant flowers are from plants whose seeds were treated with 100 µM DHPA (a-e) and 500 µM DHPA (f-j) for 6 days. Flowers were observed after 110 days of plant development.

Granted projects

GA CR 206/09/1751, The impact of genomic shock associated with interspecific hybridization and polyploidization on evolution of rDNA loci in young invasive weeds. Principal investigator: R. Matyášek, 2009 - 2013

GA CR P501/10/0208, Genome unions in plants: from DNA to chromosomes and reverse. Principal investigator: A. Kovařík, 2010 - 2012

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Malinská, H., Tate, J.A., Matyášek, R., Leitch, A.R., Soltis, D.E., Soltis, P.S., Kovařík, A.: *Similar patterns of rDNA evolution in synthetic and recently formed natural populations of Tragopogon (Asteraceae) allotetraploids*. BMC Evolutionary Biology, 10 (291), 2010, 1-17.

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PhD. thesis defended in 2010

Mgr. Kateřina Křížová, PhD., Dynamics of epigenetic changes of transgenic loci in Nicotiana tabacum

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Early cytokinin response proteins and phosphoproteins of *Arabidopsis thaliana*

Cytokinins (CKs) are plant hormones involved in regulation of diverse developmental and physiological processes in plants whose molecular mechanisms of action are being intensely researched. However, most rapid responses to CK signals at proteomic and phosphoproteomic levels were unknown. We investigated early CK responses through proteome-wide expression profiling based on image and mass spectrometric (MS) analysis of two-dimensionally separated proteins and phosphoproteins. Effects of 15-minute treatments of *Arabidopsis thaliana* seedlings with four main CKs representing hydroxyisopentenyl, isopentenyl, aromatic and urea-derived type CKs were compared to help elucidate their common and specific function(s) in regulating plant development. In proteome and phosphoproteome maps, significant differences were reproducibly observed for 53 and 31 protein spots, respectively. Of these, 96 proteins were identified by MS, providing a snapshot of early links in CK-regulated signaling circuits and cellular processes, including light signaling and photosynthesis, nitrogen metabolism, the CLAVATA pathway, and protein and gene expression regulation, in accordance with previously described CK functions. Furthermore, they indicate novel links between temperature and CK signaling, and an involvement of calcium ions in CK signaling. Most of the differentially regulated proteins and phosphoproteins are located in chloroplasts, suggesting an as yet uncharacterized direct signaling chain responsible for CK action in chloroplasts. Finally, first insights into the degree of specificity of CK receptors on phosphoproteomic effects were obtained from analyses of CK action in a set of CK receptor double mutants.

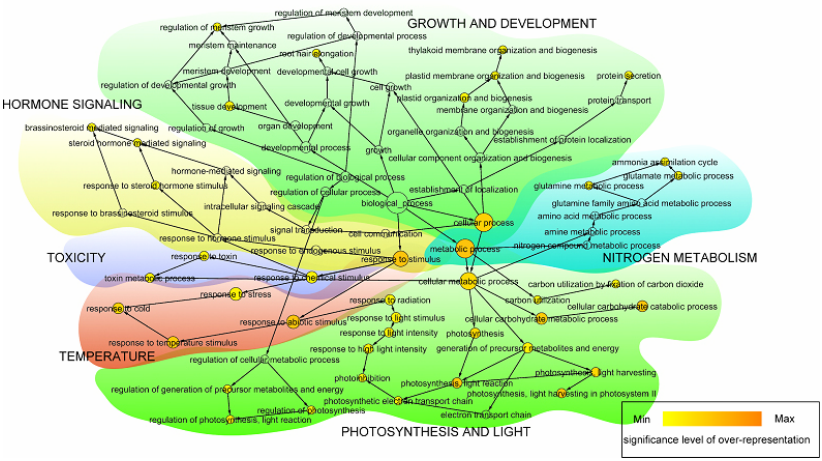

Figure 1: Gene ontology (GO) analysis of the early cytokinin response proteins in *Arabidopsis*. GO categories that were significantly over-represented among the differentially expressed proteins were identified. The yellow to orange color of the circles indicates the level of significance of over-represented categories (P=0.05, yellow; P=10^{-7}, orange). The size of the circles is proportional to the number of proteins in each category. Links with low significance were removed manually to reduce complexity of the image.

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MOLECULAR CYTOLOGY AND CYTOMETRY

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Properties of JMJD2B histone demethylase, epigenetics of embryonic stem cells

We studied JMJD2b histone demethylase that antagonizes H3K9me3 at pericentric heterochromatin (Fodor et al., Genes Dev. 20: 557−1562, 2006). The occurrence of JMJD2b in distinct nuclear compartments and JMJD2b kinetics were analyzed in tumor cells and in mouse fibroblasts with reduced pericentric H3K9 di- and tri-methylation as a consequence of deficiency of SUV39h histone methyltransferases. In these cells, the level of full-length JMJD2b (JMJD2b-GFP-1086) at centromeric clusters was reduced, which corresponds to global JMJD2b decrease. Chromatin of

ribosomal genes in fibroblasts, dense on H3K9 methylation, was relatively absent of JMJD2b-GFP-1086, but mutant and truncated forms of this protein appeared in this fibrillarin-positive compartment (Fig. 1).

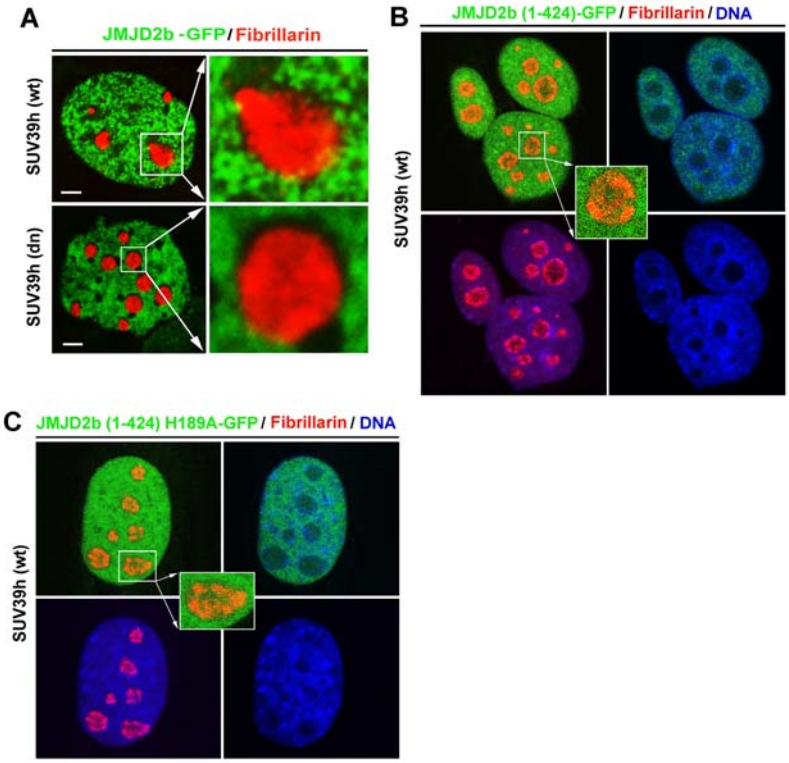

Figure 1. (A) Fibroblasts transiently expressing full length JMJD2b-GFP-1086 (green) were fixed and stained by antibody against nucleolar protein fibrillarin (red). Subtle overlapping between JMJD2b-GFP-1086 and fibrillarin-positive compartment of nucleoli was found in SUV39h wt cells, but not at SUV39h dn fibroblasts. Appearance of (B) truncated JMJD2b(1-424)-GFP and (C) mutant JMJD2B(1-424)H189A-GFP (green) in compartment of fibrillarin-postive nucleoli (red) of SUV39h wt cells. Nuclei were stained by DAPI (blue).

Androgen-dependent prostate cancer cells were characterized by significantly high level of JMJD2b-GFP-1086 at nucleoli. Kinetic properties of JMJD2b-GFP-1086 were similar in nucleoli and nucleoplasm of normal and tumor cells; ~50% recovery of the pre-bleached intensity was reached after 0.7 s. However, maximal recovery of the JMJD2b-GFP-1086 increased especially in SUV39h deficient cells, when compared with wt fibroblasts. Similarly, mobile fraction of mutant JMJD2b(1-424)H189A-GFP and especially truncated form of JMJD2b(1-424)-GFP, that did not accumulate into foci, was higher then in full-length protein. Together, we implied that nucleoli are the sites of only aberrant function of JMJD2b, which kinetics properties can be influenced by mutant background.

Other experiments were aimed at the study of protein dynamics. We used fluorescence recovery after photobleaching (FRAP) to analyze the kinetics of 18 proteins and determine the relationships between nuclear arrangement, protein molecular weight, global transcription level, and recovery kinetics. In particular, we studied heterochromatin-specific HP1 beta, BMI1, and TRF1 proteins, and nucleolus-related proteins, UBF and RPA194. We asked whether the trajectories and kinetics of particular proteins change in response to histone hyperacetylation by HDAC inhibitors or after suppression of transcription by actinomycin D. We showed that protein dynamics can be influenced by many factors and events, including nuclear pattern and transcription activity. A slower recovery after photo-bleaching was found when proteins, such as HP1 beta, BMI1, TRF1, and others accumulated at specific foci. In identical cells, proteins that were evenly dispersed throughout the nucleoplasm recovered more rapidly. Distinct trajectories for HP1 beta, BMI1, and TRF1 were observed after induced hyperacetylation or suppression of transcription. The relationship between protein trajectory and transcription level was confirmed for telomeric protein TRF1, but not for HP1 beta or BMI1 proteins. Moreover, heterogeneity of foci movement was especially observed when we made distinctions between centrally and peripherally positioned foci. Based on our results, we propose that protein kinetics are likely influenced by several factors, including chromatin condensation, differentiation, local protein density, protein binding efficiency, and nuclear pattern. Thus, we discuss if these factors and events likely cooperate to dictate the mobility of particular proteins.

The third part of our experiments was aimed at the study of mouse embryonic stem cells. Embryonic stem cells (ESCs) maintain their pluripotency through high expression of pluripotency-related genes. We

showed that the global gene expression pattern in the mouse ESC (mESC) population is not solely responsible for pluripotency. We observed differing levels of OCT4, NANOG, and c-MYC proteins among the individual cells of mESC colonies, and fluctuations in these levels did not disturb mESC pluripotency. Cells with strong expression of endogenous GFP-OCT4 had low levels of NANOG and c-MYC proteins, and vice versa. In addition, cells with high levels of NANOG tended to occupy interior regions of mESC colonies. In contrast, peripherally positioned cells within colony had dense H3K27 trimethylation (H3K27me3). We also observed distinct levels of GFP-OCT4 in particular cell cycle phases and measured the highest levels of OCT4 in G2 phase, which correlated with the pKi-67 nuclear pattern. Intriguingly, the OCT4 protein, but not H3K9me3, was bound to mitotic chromosomes. These data showed that there must be an endogenous mechanism that prevents the induction of spontaneous differentiation, despite fluctuations in protein levels within a mESC colony. Based on the results presented here, it is likely that cells within a colony support each other in the maintenance of pluripotency.

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Response of normal skin fibroblasts to fractionated irradiation with γ-rays

Our results show that similarly as the lymphocytic leukemia MOLT4 cells, normal skin fibroblasts are mostly unable to repair all DSBs before the application of the next fractionated dose of γ-rays inducing new DNA damage. The progressive accumulation of DSBs and the inability to repair this damage triggers in non-confluent fibroblasts premature quiescence that probably precedes premature senescence. These cells, similarly as the cells in premature senescence are able to remain alive for months.

Contrary to lymphocytic leukemia cells, normal skin fibroblasts with unrepaired DSBs do not die either after small fractionated doses or after a high single radiation dose. They persist long time in quiescence without signs of senescence (morphology, SA-βgal activity). We presume that the long persistence of fibroblasts with unrepaired DSBs in the G0/G1 phase might be related to their natural capacity to stay in quiescence after reaching confluence *in vitro*. Fibroblasts that have not yet consumed their limited life-span *in vitro* are able to restart proliferation after subculture; however, the cells exposed to low doses of fractionated irradiation lose the proliferation capacity during subculture. While the lymphocytic leukemia cells irradiated with 4x1 Gy and a single dose of 4 Gy have very similar survival, there is a big difference between human fibroblasts irradiated with 4x 1.5 Gy and a single dose of 6 Gy, to the fractionated dose advantage.

These results suggest that exponentially growing lymphocytic leukemia cells, in common with fast proliferating tumors are not very sensitive to a fraction size in contrast to the more slowly growing fibroblasts and most

late responding (radiation therapy dose limiting) normal tissues which have a low proliferating index. This result is very important in light of recent developments of optimized fractionation regimes of radiotherapy.

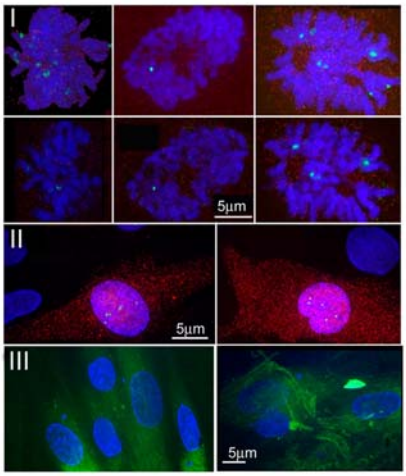

Figure 2: Examples of some responses of human skin fibroblasts to fractionated irradiation with the dose of 1.5 Gy delivered in the interval of 24 h. (I) Mitoses containing DSBs (green spots of γH2AX) at 20 h after the second irradiation. Upper row represents the total image composed of 40 slices of 0.2 µm, the lower row represents the central slices. (II) Fibroblasts expressing a higher amount of cyclin B1(red) indicating their presence at G2 phase (γH2AX- green spots). The fibroblasts that started to express a high amount of cyclin B1 entered mitosis rapidly and were not detained in this state unlike MOLT4 cells. (III) Fibroblasts with active SA-βgal (SA-βgal) (green) at 72 h after the fourth fractionated dose of 1.5 Gy, detected at pH 6 with 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG). These senescent fibroblasts were not frequent at 72 h after irradiation.

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HMGB proteins: Interaction with DNA and chromatin

Impact of *HMGB1*-knockout on gene expression. In order to understand possible biological functions of HMGB-type proteins, we have carried out MALDI-TOF analysis and DNA microarrays of *HMGB1*-knockout murine cells to understand involvement of HMGB1 in intracellular signaling pathways such as cell cycle pathways and apoptosis. Our data obtained from Affimetrix Gene Chips indicated that *HMGB1* knockout in murine cells resulted in *up*- or *down*-regulation of a number of genes, in particularly genes involved in protein-folding (chaperones), transcription and apoptosis.

Regulation of human *topo IIalpha* gene promoter. Our project is based on our recently published (Štros et al., Nucleic Acids Res. 37:2070-2086, 2009) and preliminary data suggesting a possible relationship between chromosomal HMGB1/2 proteins and DNA topoisomerase IIα (topo IIalpha). HMGB1/2 proteins could up-regulate cellular expression of the human topo IIα in cells lacking Rb gene. Using ChiPs (Chromatin Immuno Precipitation) we have demonstrated diminished (> 3-fold) binding of transcription factor NF-Y to ICE1-4 elements within the human topo IIalpha

gene promoter. I tis possible that HMGB-type proteins and also other the HMGB-type proteins could have a significant impact on the formation/stability of topoII-DNA cleavable complexes, and correspondingly on the efficacy of anticancer drugs specifically targeting the latter enzymes.

Chronic lymphocytic leukemia (CLL) as a model to study Rb1 gene *in vivo*. A suitable model to study Rb1 gene *in vivo* is chronic lymphocytic leukemia (CLL). Therefore a number of samples from patients with chronic lymphocytic leukemia (CLL) have been collected (under informed consent signature) and analyzed as a collaboration project with Department of Internal Medicine - Hematooncology, University Hospital Brno. Multiple Ligation Probe Analysis (MLPA) method was used to distinguish patients with and without Rb1 deletion. Cytogenetic array analysis (Affymetrix) was employed to detect single nucleotide polymorphism (SNP), and 35 patients with *Rb1* gene deletion were characterized. Data obtained from microarray analysis are subject of detailed analysis.

Dysfunction of chromatin assembly factor 1 induces shortening of telomeres and loss of 45S rDNA in *Arabidopsis thaliana*

Chromatin Assembly Factor 1 (CAF1) is a three-subunit H3/H4 histone chaperone responsible for replication-dependent nucleosome assembly. It is composed of CAC 1-3 in yeast; p155, p60, and p48 in humans; and FASCIATA1 (FAS1), FAS2, and in *Arabidopsis thaliana*. We report that disruption of CAF1 function by *fas* mutations in *Arabidopsis* results in telomere shortening and loss of 45S rDNA, while other repetitive sequences (5S rDNA, centromeric 180-bp repeat, CACTA, and Athila) are unaffected. Substantial telomere shortening occurs immediately after the loss of functional CAF1 and slows down at telomeres shortened to median lengths around 1 to 1.5 kb. The 45S rDNA loss is progressive, leaving 10 to 15% of the original number of repeats in the 5th generation of mutants affecting CAF1, but the level of the 45S rRNA transcripts is not altered in these mutants. Increasing severity of the fas phenotype is accompanied by accumulation of anaphase bridges, reduced viability, and plant sterility. Our results show that appropriate replicationdependent chromatin assembly is specifically required for stable maintenance of telomeres and 45S rDNA.

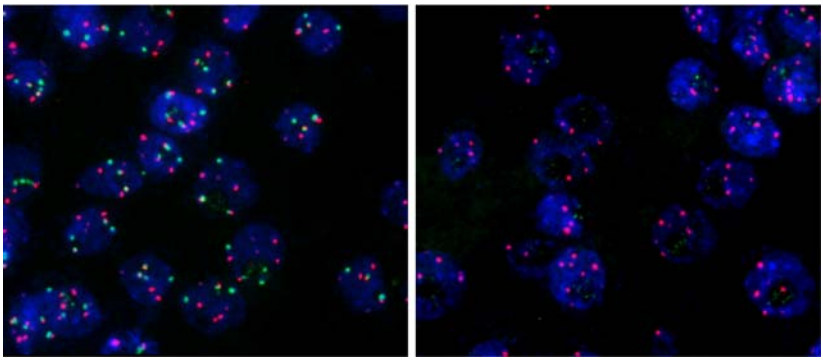

Figure 3: Comparison of the 5S (red) and 45S (green) rDNA loci in G5 *FAS1-4* metaphase and interphase nuclei. *FAS/FAS* – wild type, *fas/fas* – mutant.

Telomere maintenance in liquid crystalline chromosomes of dinoflagellates

The organisation of dinoflagellate chromosomes is exceptional among eukaryotes. Their genomes are the largest in the Eukarya domain, chromosomes lack histones and may exist in liquid crystalline state. Therefore, the study of the structural and functional properties of dinoflagellate chromosomes is of high interest. We have analysed the telomeres and telomerase in two Dinoflagellata species, Karenia papilionacea and Crypthecodinium cohnii. Active telomerase, synthesising exclusively Arabidopsis-type telomere sequences, was detected in cell extracts. The terminal position of TTTAGGG repeats was determined by *in situ* hybridisation and BAL31 digestion methods and provides evidence for the linear characteristic of dinoflagellate chromosomes. The length of telomeric tracts, 25–80 kb, is the largest among unicellular eukaryotic organisms to date. Both the presence of long arrays of perfect telomeric repeats at the ends of dinoflagellate chromosomes and the existence of active telomerase as the primary tool for their high-fidelity maintenance demonstrate the general importance of these structures throughout eukaryotes. We conclude that whilst chromosomes of dinoflagellates are unique in many aspects of their structure and composition, their telomere maintenance follows the most common scenario.

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Significance of adenosine receptor signaling in hematopoiesis

Some of our experiments were aimed at testing the ability of N^6-(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA), an adenosine A_3 receptor agonist, to modulate hematopoiesis in sublethally irradiated mice, when administered alone or in a combination with granulocyte colony-stimulating factor (G-CSF) in a two-day postirradiation treatment regimen. A complete analysis of hematopoiesis including determination of numbers of bone marrow hematopoietic progenitor and precursor cells, as well as of numbers of peripheral blood cells, has been performed. The outcomes of the treatment were assessed at days 3 to 22 after irradiation. IB-MECA alone has been found to induce a significant elevation of numbers of bone marrow granulocyte-macrophage progenitor cells (GM-CFC) and peripheral blood neutrophils. IB-MECA given concomitantly with G-CSF increased significantly bone marrow GM-CFC and erythroid progenitor cells (BFU-E) in comparison with the controls and with animals administered each of the drugs alone.

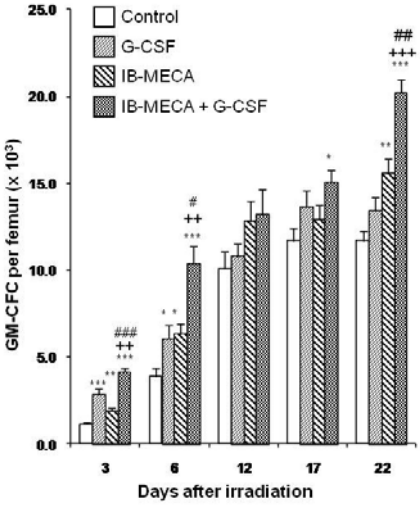

Figure 4: Numbers of GM-CFC per femur in sublethally irradiated mice. IB-MECA and/or G-CSF were given on days 1 and 2 after irradiation with a dose of 4 Gy γ rays. *, **, * - P < 0.05, P < 0.01, P < 0.001, respectively, vs. pertinent control group; ++, +++ - P < 0.01, P < 0.001, respectively, vs. G-CSF-treated mice; #, ##, ### - P < 0.05, P < 0.01, P < 0.001, respectively, vs. IB-MECA-treated mice (Tukey post hoc test).**

The findings suggest the ability of IB-MECA to stimulate hematopoiesis and to support hematopoiesis-stimulating effects of G-CSF in sublethally irradiated mice

Another studies were aimed at determination of adenosine receptor mRNA expression in various hematopoietic cells. First, four mouse bone marrow or thymus cell populations, namely granulopoietic/monocytopoietic, erythropoietic, B-lymphopoietic, and T-lymphopoietic precursor cells have been assayed by RT-PCR technique for the presence and relative amounts of adenosine A_1, A_{2a}, A_{2b}, and A_3 receptor mRNA. It has been found that (i) all four populations studied express all four adenosine receptor subtypes, (ii) the A_1 receptor is the least expressed in all populations studied, (iii) the A_3 receptor is markedly expressed in the populations of granulo-

poietic/monocytopoietic and erythropoietic cells, (iv) the A_{2a} receptor is markedly expressed in the populations of B-lymphopoietic and T-lymphopoietic cells, and v) the A_{2b} receptor does not predominate in any of the precursor cells studied. These data offer a new possibility for the assessment of the readiness of these cells to respond, by receptor-mediated mechanisms, to adenosine or its analogs present in the tissues as a result of endogenous processes and/or following their administration. Second, expression of mRNA for adenosine receptor subtypes A_1, A_{2a}, A_{2b}, and A_3 in normal and lipopolysaccharide (LPS)-activated murine RAW 264.7 macrophages has been investigated using the method of quantitative real-time polymerase chain reaction. The results have shown a very low, unquantifiable expression of adenosine A_1 receptor mRNA both in normal and LPS-activated macrophages. The other three adenosine receptor mRNAs have been found to be expressed at various but always quantifiable levels. Activation of the macrophages with LPS induced upregulation of the expression of adenosine receptor A_{2a} and A_{2b} mRNA whereas the expression of adenosine receptor A_3 mRNA was downregulated. Unstimulated macrophages exhibited a high expression of the A_{2b} adenosine receptor mRNA. The findings were discussed from the point of view of the antiinflammatory and hematopoiesis-stimulating roles of the adenosine receptor signaling.

In a separate series of experiments we examined differences in selected indices of granulopoiesis in outbred, F_1 hybrid, and inbred mouse strains. Specifically, serum granulocyte colony-stimulating factor (G-CSF) levels, numbers of marrow granulocyte-macrophage progenitor cells and morphologically recognizable proliferative marrow granulocytic precursor cells were evaluated. These parameters were determined in untreated controls, and in mice exposed either to a non-specific stimulus (injection of saline), or to a granulopoiesis-enhancing stimulus (administration of a cyclooxygenase-2 inhibitor, meloxicam). Lower levels of G-CSF were detectable in the outbred ICR mice, which also demonstrated an enhanced response to both types of the stimuli. Considering the fact that outbred mice are closer to natural mammalian populations, including human ones, the possibility of using outbred mice, instead of the often used inbred strains, for experiments evaluating the effects of pharmacological interventions on hematopoiesis should be investigated.

Granted projects

ME CR LC06027, Monoclonal gammapathy and multiple myeloma, basic research centre. Principal investigator: R. Hájek, Principal co-investigator: E. Bártová, 2006 - 2010

ME CR ME919, Mapping of fragile sites in human genome. Principal investigator: E. Bártová, 2007 - 2011

GA AS CR IAA500040802, New mechanisms of the oncoprotein functions in the genesis of promyelocytic leukemia. Principal investigator: M. Falk, 2008 - 2011

ME CR LC535, Dynamics and organization of chromosomes in the cell cycle under standard and pathological conditions. Principal investigator: I. Raška, Principal co-investigator: S. Kozubek, 2006 - 2010

GA CR P302/10/1022, Changes in chromatin structure at sites of DNA double-strand breaks and their necessity for DSB repair. Principal investigator: S. Kozubek, 2010 - 2012

ME CR LC06004, Integrated research of the plant genome. Principal investigator: B. Vyskot, 2005 - 2010

GA CR 204/08/1530, Explanation of the role of HMGB1 protein in keeping the genome stability. Principal investigator: M. Štros, 2008 - 2010

GA CR P301/10/0590, Czech Science Foundation, Principal investigator: M. Štros, 2010 - 2012

GA AS CR IAA500040801, Telomeres and telomerase: from molecular biology to structural biology. Principal investigator: E. Sýkorová, 2008 - 2012

GA CR 521/09/1912, Telomeres of algae. Principal investigator: E. Sýkorová, 2009 - 2012

GA CR 306/08/0158, Activation of adenosine receptors combined with cyclooxygenase inhibition in modulation of radiation-induced myelo-suppression. Principal investigator: M. Hofer, 2008 - 2012

Ministry of Defense 1001 8 5090, Adenosine A_3 receptor agonist in therapy of acute radiation disease. Principal investigator: M. Hofer, 2010 - 2012

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PhD. thesis defended in 2010

Mgr. Martina Dvořáčková, Telomere proteins and their interactions

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Department of Cytokinetics focuses on the research in the field of cellular signaling and physiology relevant to cancer and developmental biology, with a particular focus on potential role of lipids and their derivatives in these processes. A special attention is paid to interactions of lipid dietary components (e.g. polyunsaturated fatty acids and butyrate), pharmaceuticals (cytostatics) and environmental pollutants (e.g. polycyclic aromatic hydrocarbons, dioxins) with physiological regulators of cytokinetics,

including tumor necrosis factor (TNF), tumor growth factor- β (TGF- β), fibroblast growth factor (FGF), and Wnt families of signaling proteins. The results are exploited especially in the fields of cancer prevention/therapy and toxicology.

Cellular and molecular physiology of lipids (Jiřina Hofmanová)

Among luminal nutrients, the types of dietary fat and fibre are thought to have the most significant impact on the health of colon tissue. Particularly, short-chain fatty acid butyrate (produced by microbial fibre fermentation) and bioactive ω-3 essential polyunsaturated fatty acids (PUFAs) are assumed to interact and to have beneficial effects in colon inflammation and carcinogenesis (Hofmanová et al., review Curr Pharmacol Biotechnol, in press). We investigated precise mechanims of fatty acid interactive effects differently influencing cytokinetics of colon cancer epithelial cell lines with various malignant potential and the ability of PUFAs to modulate the effects of endogenous apoptotic regulator TNF-related apoptosis-inducing ligand (TRAIL).

i/ The effects and interaction of fatty acids in colon cancer cells
Detailed analyses of cellular lipid content, composition and metabolism (measured by (LC/MS/MS a GC/MS) after treatment with NaBt, AA, sodium butyrate (NaBt), PUFAs (ω-6 arachidonic acid -AA, ω-3 docosahexaenoic acid- DHA), and/or their combinations showed an important association between cellular lipid alterations and differentiation/apoptotic response of human colon fetal (FHC) and adenocarcinoma (HCT-116) cells. PUFAs were efficiently incorporated and metabolized in both cell types, which influenced spectrum of total fatty acids and significantly increased level of AA and DHA in individual types of phospholipids. Newly, we observed that NaBt and its combination with PUFAs differently modulated fatty acid endogenous synthesis in FHC but not in HCT-116 cells. Fatty acid also differently modulated membrane fluidity, accumulation of cytoplasmic lipid droplets, mitochondrial membrane potential and reactive oxygen species (ROS) production. Evaluation by two-way ANOVA confirmed the most significant participation of ROS production, differentiation and apoptosis, the highest effects of combination NaBt and PUFAs, and lower sensitivity of HCT-116 cells compared to FHC cells (Hofmanová et al., J Nutr Biochem, in press).

ii/ The mechanisms of TRAIL effects and possibilities of their modulation by PUFAs in colon cancer cells

We investigated sensitizing effects of DHA on apoptosis triggered by TRAIL in otherwise resistant SW620 epithelial cell line derived from human colon cancer metastasis. TRAIL is a tumor necrosis factor (TNF) family member, known for its ability to selectively induce apoptosis in cancer cells, but not in most of normal cells. However, many cancer cells including colon are still resistant to cytotoxic effects of TRAIL. We showed that a pretreatment with physiologically-relevant concentrations of DHA resulted in increased apoptosis of the resistant colon cancer cells triggered by TRAIL. Our results showed important role of caspases and their endogenous inhibitors, pro- and anti-apoptotic Bcl-2 family proteins, and mitochondria in the observed effects. Our results suggest that DHA plays a role of an effective modulator of epithelial colon cancer cell sensitivity towards apoptosis, and we detected several potential molecules, which could be targeted in future therapeutic interventions.

iii) The factors modulating sensitivity of colon epithelial cells to TRAIL

Our further results highlighted significant differences between attached and detached epithelial colon cancer cells with regard to their response to cytotoxic agents. We detected some molecular mechanisms underlying the different sensitivity of adherent and non-adherent human epithelial colon cells to TRAIL-induced apoptosis, including different activation of PI3K/Akt and MAPK pathways. These findings are especially important, as they suggest that these factors can contribute to modulation of sensitivity of epithelial cancer cells in various stages of tumor development to the same anticancer agent.

Since the interactions between epithelial cells and extracellular matrix (ECM) are essential for organization of colon crypt and they play a role in signaling of important cytokines, we also explored the impact of the anchorage type in adherent cells, and its role in cell adhesion, cytokinetic parameters, and response to TRAIL. Our data showed that TRAIL-resistant populations of HCT-116 colon cancer cells show significant changes in the expression of adhesive molecules, when compared to sensitive ones, which can further be modulated by cultivation surface type, and have impact on final cell survival.

Growth factors in cancer cell signaling (Karel Souček)

Growing evidence suggests that the tissue microenvironment affects not only cancer development and progression, but also the response to anti-cancer therapy. Presently, understanding of this phenomenon represents the most challenging field of cancer biology. Primary research direction of our group is to elucidate mechanisms regarding how the tissue microenvironment affects precancerous and cancerous cells and apply our findings directly to clinically relevant problems (e.g., dissemination of cancer cells, development of androgen-β -independent prostate cancer, or resistance to anti-cancer therapy). Growth/differentiation factor-15 (GDF-15) is a cytokine that is abundantly induced in the tissue microenvironment during cancer progression and therapy; however its functional role is not known in all details. In 2010 we continued with studies focused on (1) functional role of GDF-15 (member of transforming growth β family), (2) molecular mechanisms of TGF-β-induced epithelial-mesenchymal transition in epithelial cells, (3) mechanisms of neuroendocrine differentiation (NED) of prostate cancer. Study investigating GDF-15 has shown its abundant presence in human seminal plasma; moreover we demonstrated its immunomodulatory properties. Our study proved novel properties of GDF-15 which can help to clarify its role in human reproduction but also in cancer progression (Soucek et al., 2010). TGF-β cytokines belongs to the important inductors of epithelial-mesenchymal transition (EMT). This process is crucial for dissemination of cancer cells and metastasis development. In our work we have shown that SNAI2/Slug is a crucial regulator of TGF-β1-induced EMT in benign prostate epithelial cells and that the expression of SNAI2/Slug is increased in tumorigenic clones compared to parental cells (Slabáková et al., The Prostate, in press). Moreover we demonstrated that a TGF-β1 signaling pathway inhibits IL-6-induced STAT3 phosphorylation through donregulation of Jak2 expression (Staršíchová et al., 2010). NED of epithelial prostate cancer cells is phenomena associated with anti-androgen therapy of prostate cancer and disease progression. We identified that induction of the senescence-associated secretory phenotype by androgen depletion was mediated by down-regulation of S-phase kinase-associated protein 2 (Skp2), whereas the neuroendocrine differentiation of prostate cancer cells was under separate control. These data demonstrate a previously unrecognized link between inhibition of androgen receptor signaling, down-regulation of Skp2, and the appearance of secretory, tumor-promoting senescent cells in prostate tumors. We propose that ADT may contribute to the development of androgen-independent prostate cancer through modulation of the tissue

microenvironment by senescent cells (Pernicová et al., Neoplasia, in press). The successful completion of these studies will help to understand mechanisms of cancer progression and reveal innovative strategies for treating prostate cancer in terminal stages.

Molecular mechanisms of Wnt and fibroblast growth factor (FGF) signaling (Vítěslav Bryja, Pavel Krejčí)

FGF signaling activates Wnt/β-catenin signaling via ERK MAP kinase-mediated phosphorylation of Wnt co-receptor Lrp6: When probing chondrocytes for effects of FGFR signaling on other signaling systems, we found increased FGF2-mediated activation of the Topflash luciferase reporter, which records the transcriptional activation of canonical Wnt/β-catenin signaling. When combined, Wnt3a and FGF2 caused a surprisingly potent Topflash activation, exceeding, in some cases, Topflash activation caused by Wnt3a alone by more than 100 fold (Červenka et al., 2011).
To test whether the Wnt/β-catenin activation occurs with FGFR3 harboring pathogenic mutations, we used six activating FGFR3 mutants (N540K, G380R, R248C, Y373C, K650M, K650E), associated with skeletal dysplasia and cancer. Ectopic expression of the FGFR3 mutants results in their ligand-independent activation via spontaneous dimerization, with the relative activating potential of the given mutation easily appreciated by the corresponding level of Erk MAP kinase activation. Topflash activation by FGFR3 mutants significantly exceeded that of wt FGFR3. Moreover, Topflash experiments using cells treated with Wnt3a demonstrated that FGFR3 mutants also potently sensitize cells to Wnt3a stimulus, similar to the FGF2-mediated activation of endogenous wt FGFR3. We next excluded the PI3K/Akt pathway from being candidate for the FGF2-mediated upregulation of Wnt/β-catenin signaling in chondrocytes, suggesting a novel mechanism. When searching different components of the Wnt/β-catenin pathway for their FGF2-mediated phosphorylation in RCS chondrocytes, we found phosphorylation of the Wnt co-receptor Lrp6 at Ser1490, which lies within one of five conserved PPPS/TP motifs present in the intracellular domain of Lrp6. To test whether the effect of FGF on Wnt/β-catenin signaling depends on phosphorylation within the PPPS/TP motifs, we transfected chondrocytes with a Lrp6 mutant in which the Ser/Thr in each particular PPPS/TP motif was replaced by Ala (Lrp6-5A). Lrp6-5A showed a dominant-negative effect on both FGF2 and/or Wnt3a-mediated Topflash activation, contrasting with overexpression of wt Lrp6, which led to

spontaneous Lrp6 phosphorylation at Ser1490 and significantly enhanced FGF2-mediated Topflash activation (Krejčí et al., submitted).
Altogether, we showed that activatory FGF effect on the Wnt/β-catenin pathway requires Lrp6 phosphorylation at its PPPS/TP motifs. Recently, we showed that all three MAP kinases, i.e. JNK, p38 and ERK, are capable of phosphorylating the PPPS/TP motifs in Lrp6 (Červenka et al., Mol Cell Biol 2011). In RCS chondrocytes, only Erk is strongly activated by FGF2, and chemical inhibition of the ERK pathway by U0126 suppresses FGF2-mediated Lrp6 phosphorylation. Similarly, FGF2 but not Wnt3a-mediated Topflash activation was sensitive to U0126, confirming the role of the ERK pathway in the FGF2 and Wnt3a/β-catenin signaling cross-talk. To further test whether ERK acts as an Lrp6 kinase, we immunoprecipitated active ERK from FGF2-treated chondrocytes, and subjected the immuno-complexes to a kinase assay with recombinant Lrp6 intracellular domain (ICD) as a substrate. We show that ERK phosphorylates Lrp6 at Ser1490. As the latter experiment does not rule-out the possibility of another ERK-associated kinase to co-immunopurify and phosphorylate Lrp6, we carried-out the kinase assays in a cell-free fashion, using recombinant ERK and recombinant Lrp6-ICD. Five Lrp6-ICD mutants were produced, each containing one intact PPPS/TP motif, with the other 4 mutated by replacing Ser or Thr with Ala. Using antibody against phosphorylated Ser1490, we detected ERK-mediated phosphorylation only in wt Lrp6-ICD and the mutant containing an intact Ser1490 PPPS/TP motif. Since Ser1490 antibody is designed to detect phosphorylation only at the first motif, we used mass-spectrometry (MS) to probe wt Lrp6-ICD for ERK-mediated phosphorylation at the remaining four motifs. We identified phosphorylation on the first four N-terminal PPPS/TP motifs.
Moreover, in 2010 we continued our work on the molecular analysis of signal transduction in the Wnt pathway. The most important findings show that (i) Wnt receptor Ror2 binds key cytoplasmic protein Dishevelled, and that this interaction is required for negative effects of Ror2 on the canonical Wnt pathway (Witte et al., 2010), (ii) that Vangl2, important component of non-canonical Wnt signaling mediates its effects by recruiting small GTPase Rac1 to the membrane (Lindqvist et al., 2010), and (iii) that mitogen-activated protein kinases (MAPKs) are capable of phosphorylation of Lrp6, creating a mechanism for the interaction between Wnt and mitogen signaling (Červenka et al., 2011). In collaboration with other labs we were able to study some properties of the Wnt ligands themselves - for example, we showed that preparations of recombinant Wnt3a are contaminated by so far unidentified activators of the Akt pathway (Čajánek et al., 2010). We have applied our findings also to clinical problems. We have shown that

casein kinase 1 epsilon (CK1e), which is absolutely required for the activation of the Wnt pathway, is mutated in breast cancer, and that breast cancer specific mutations in CK1e are inactivating. As such they block transduction in the Wnt/β-catenin pathway and at the same time cause activation of the non-canonical Wnt pathway, which in turn promotes cell motility. We believe that this mechanism underlies the aggressive behaviour and invasiveness of breast cancer cells (Foldynová-Trantírková et al., 2010). In this project we discovered and characterized a novel way of interaction between FGF and Wnt/β-catenin pathways. Our results are of significant interest to the scientific community since the mechanism we describe might represent a general way how growth factors interact with Wnt/β-catenin signaling. At the same time, our data suggest that activation of Wnt/β-catenin may play an important role among the mechanisms of pathological FGFR3 signaling in skeletal dysplasia.

Cellular and molecular toxicology (Jan Vondráček)

The principal aim of our studies is to contribute to understanding of molecular and cellular effects of environmental organic pollutants linked to carcinogenesis, reproductive or developmental impairment. These toxicological data help us to identify and analyze key signaling proteins affected by environmental toxicants. Complementary approaches also help us to understand physiological role of these proteins, such as the aryl hydrocarbon receptor (AhR), in organism. We investigated the effects of potential endogenous ligand indirubin on the AhR signaling, with a special focus on the AhR-dependent gene expression and cell cycle progression in rat liver progenitor cells (Procházková et al., 2011). Our data indicated that the AhR signaling activated by indirubin differs from the known AhR signaling pattern induced by toxic exogenous ligands, such as dioxin. In contrast to dioxin, endogenous doses of indirubin induced only a transient nuclear translocation of AhR and they had only limited effects on AhR degradation or expression of AhR target genes. In contrast, the effects of high micromolar doses of indirubin closely resembled those of dioxin, including AhR translocation, degradation and immediate up-regulation of AhR target genes. The opposite effects of endogenous vs. high micromolar concentrations of indirubin on expression of xenobiotic-metabolism unrelated gene (e.g. Jup gene), indicated that toxic exogenous AhR ligands might disrupt normal regulatory role of endogenous ligands of AhR in specific processes, such as intercellular communication. This fact might help to further understand the role of AhR e.g. in regulation of tumor

development, as the Jup is an important tumor suppressor (Procházková et al., 2011).
In collaboration with the Department of Toxicology, Pharmacology an Immunotherapy (Veterinary Research Institute, Brno), we participated in the study, which described the impact of model genotoxic AhR ligand, benzo[a]pyrene (BaP), on prostate epithelial cells (Hrubá et al., 2010). Our data demonstrated, for the first time, that although genotoxic BaP (as well as other genotoxic polycyclic aromatic hydrocarbons) produced significant levels of DNA adducts in the model of human prostate carcinoma cells, they do not activate the mechanisms leading to elimination of cells with significant DNA damage, presumably due to their failure to activate the p53-dependent DNA damage response. The inefficiency of p53 response, which contributes both to elimination of cells with considerable DNA damage and DNA repair, may further enhance these processes contributing to prostate malignancies (Hrubá et al., 2010).
The present results contribute to our understanding of the possible mechanisms of toxic actions of various classes of AhR ligands, including both endogenous and genotoxic compounds. This information help us to understand the nature of endogenous AhR signaling (in case of indirubin), as well as the impact of carcinogenic AhR agonists on specific epithelial cell types.

Mechanisms of the effects of platinum derivatives (Alois Kozubík)

The main goal of anticancer therapy is to selectively and effectively induce apoptosis in cancer cells while sparing surrounding healthy tissues. One promising approach is selective triggering of apoptotic pathway in cancer cells by TRAIL, a member of TNF superfamily. However, many tumor cells can still be resistant to the effects of TRAIL due to various deficiencies in apoptotic pathways. In some resistant cells, combined treatment with platinum-based chemotherapeutic drugs has been reported to overcome TRAIL resistance in many cancer cell types.
We showed that cisplatin or a more potent platinum(IV) complex LA-12 (used in twenty-fold lower concentration) enhanced killing effects of TRAIL in human colon and prostate cancer cell lines via stimulation of caspase activity and overall apoptosis. Investigation of molecular mechanisms responsible for these effects showed that both platinum complexes increased surface expression of TRAIL death receptor DR5 in colon cancer cells. The functional importance of this receptor was subsequently validated using siRNA-mediated DR5 silencing, which

rescued cells from sensitizing effects of platinum drugs on TRAIL-induced caspase-8 activation and apoptosis. Both platinum drugs also induced the relocalization of DR4 and DR5 receptors into lipid rafts, and stimulated TRAIL internalization. Our results highlighted the crucial importance of modulations of the initial steps of the extrinsic apoptotic pathway at the level of DR5 and plasma membrane in sensitization of colon cancer cells to TRAIL-induced apoptosis mediated by LA-12 and cisplatin. These findings have interesting implications in future therapeutic strategies, which enable to target specific molecules of apoptotic signaling pathways in colon cancer cells (Vondálová Blanářová et al., Carcinogenesis 32: 42, 2011).

Granted projects

GA CR 301/07/1557, Novel anticancer platinum complexes - mechanisms of their action and innovative chemotherapy. Principal investigator: A. Kozubík, 2007 - 2011

GA CR 524/07/1178, Importance of cell lipid changes during differentiation and apoptosis of colon epithelial cells. Principal investigator: J. Hofmanová, 2007 - 2011

ESF - MEYS CZ.1.07/2.3.00/09.020, Expanding the qualification competencies of doctoral study graduates at FMD UP. Principal co-investigator: J. Hofmanová, 2009 - 2011

GA CR 303/09/H048, Molecular mechanisms of selected pathological processes in the cell. Principal co-investigator: J. Hofmanová, 2009 - 2012

GA CR 310/07/0961, The role of environmental pollutants in mechanisms regulating development of prostate carcinoma. Principal co-investigator: K. Souček, 2007 - 2010

MZD 9600-4/2008, Modulation of signaling pathways leading to neuroendocrine differentiation in prostate cancer. Principal investigator: K. Souček, 2008 - 2011

MZD 9956-4/2008, Significance of asporin and other extracellular matrix proteins in invasive carcinomas of breast and prostate. Principal co-investigator: K. Souček, 2008 - 2011

GA CR 524/09/1337, Interactions of Wnt and Ah receptor signaling in regulation of functions of liver cells. Principal investigator: J. Vondráček, 2009 - 2011

GA CR 305/09/1526, The role of extracellular matrix-mediated cell adhesion in maintenance of colonic tissue homeostasis and in colon carcinogenesis. Principal investigator: M. Hýžďalová, 2009 - 2011

GA CR 204/09/J030, The role of beta-arrestin in achieving Wnt-signaling specificity. Principal investigator: V. Bryja, 2009 - 2011

GA CR 204/09/H058, Intercellular signaling in development and disease. Principal co-investigator: J. Vondráček, 2009 - 2012

IGA MZ CR NT 11201-5/2010, New mechanisms of platinum-based drug action as a tool for anticancer therapeutic strategies. Principal investigator: A. Kozubík, 2010 - 2014

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PhD. thesis defended in 2010

RNDr. Lenka Švihálková Šindlerová, PhD., Genotoxic and nongenotoxic effects of polycyclic aromatic hydrocarbons on oval hepatic cells *in vitro*

FREE RADICAL PATHOPHYSIOLOGY

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The effect of lipid peroxidation products on reactive oxygen species formation and nitric oxide production in lipopolysaccharide-stimulated macrophages

The aim of the study was to investigate the effects of lipid peroxidation products (LPPs) - acrolein, 4-hydroxynonenal, and malondialdehyde - on ROS and NO production in RAW 264.7 macrophages and to compare these effects with the cytotoxic properties of LPPs. Macrophages were stimulated with lipopolysaccharide (0.1 mu g/ml) and treated with selected LPPs (concentration range: 0.1-100 mu M). ATP test, luminol-enhanced chemiluminescence, Griess reaction, Western blotting analysis, amperometric and total peroxyl radical-trapping antioxidant parameter assay were used for determining the LPPs cytotoxicity, ROS and NO production, inducible nitric oxide synthase expression, NO scavenging, and antioxidant properties of LPPs, respectively. Our study shows that the cytotoxic action of acrolein and 4-hydroxynonenal works in a dose- and time-dependent manner. Further, our results imply that acrolein, 4-

hydroxynonenal, and malondialdehyde can inhibit, to a different degree, ROS and NO production in stimulated macrophages, partially independently of their toxic effect. Also, changes in enzymatic pathways (especially NADPH-oxidase and nitric oxide synthase inhibition) and NO scavenging properties are included in the downregulation of reactive species formation.

Solid-phase extraction of berries' anthocyanins and evaluation of their antioxidative properties

Solid-phase extraction (SPE) was used to obtain anthocyanin-rich extracts from five berry species: chokeberry, elderberry, black currant, blackberry and blueberry. During SPE more than 94.4% of the sugars and more than 88.5% of the acids present in the crude extracts were separated. The SPE resulted in 90-95.6% anthocyanins recovery. The antioxidative properties of the anthocyanin-rich extracts were tested by measuring their oxygen radical absorption capacity (ORAC), hydroxyl radical averting capacity (HORAC), total peroxyl radical trapping antioxidant parameter (TRAP), scavenging of nitric oxide and inhibition of lipid peroxidation. Elderberry extract revealed the highest ORAC value. Chokeberry extract was the most potent inhibitor of lipid peroxidation and had the highest TRAP value. Blueberry extract had the highest HORAC result and was the most powerful scavenger of NO. The high antioxidant activity according to all antioxidant assays revealed opportunities to apply these preparations as antioxidants.

GROUP OF PATHOPHYSIOLOGY OF FREE RADICALS IN CELL INTERACTIONS

HEAD
MILAN ČÍŽ

Serotonin and its 5-HT$_2$ receptor agonist DOI-hydrochloride inhibit the oxidative burst in total leukocytes but not in isolated neutrophils

Serotonin (5-HT) is capable of reducing the oxidative burst of professional phagocytes. In this study, we investigated whether 5-HT mediates this modulation via 5-HT receptors (5-HTR) or whether this is due to 5-HT antioxidative properties. The leukocytes or polymorphonuclear leukocytes (PMNL) were isolated from human blood, and their ability to produce reactive oxygen species (ROS) after 5-HT or its agonist treatment was tested by luminol-enhanced chemiluminescence (CL) analysis. It was found that 5-HTR$_2$ 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. Platelets and activated PMNL are in tight contact at sites of inflammation. 5-HT released from platelets might have a protective function against PMNL-derived oxidative stress and oxidative damages.

Evaluation of antioxidant activity of medicinal plants containing polyphenol compounds. Comparison of two extraction systems

This study investigates the influence of extraction system on the extractability of polyphenol compounds and antioxidant activity of various medicinal plants. Oxygen radical absorbance capacity (ORAC) and total polyphenol content of 25 Bulgarian medicinal plants subjected to water or 80 % acetone extractions were investigated and compared. The type of extragent significantly influenced the efficiency of the polyphenol extraction and the antioxidant activity. In all cases ORAC results and total

polyphenol content were higher for acetone extraction than for water extraction. The acetone extract of peppermint had the highest ORAC value — 2917 μmol Trolox equivalent (TE)/g dry weight (DW) and polyphenol content — 20216 mg/100 g DW. For water extraction thyme exhibited the highest ORAC antioxidant activity — 1434 μmol TE/g DW. There was a significant linear correlation between the concentration of total polyphenols and ORAC in the investigated medicinal plants. It can be concluded that the solvent used affects significantly the polyphenol content and the antioxidant activity of the extract and therefore it is recommended to use more than one extraction system for better assessment of the antioxidant activity of natural products. Several of the investigated herbs contain substantial amounts of free radical scavengers and can serve as a potential source of natural antioxidants for medicinal and commercial uses.

GROUP OF FREE RADICALS IN REGULATION OF CELL PHYSIOLOGY

HEAD
LUKÁŠ KUBALA

Redox-sensitive regulation of macrophage-inducible nitric oxide synthase expression *in vitro* does not correlate with the failure of apocynin to prevent lung inflammation induced by endotoxin

Reactive oxygen and nitrogen species are among the crucial mediators in the development of the pathological inflammatory process in the lungs and contribute to the damage of lung epithelium. The aim of the present study was to evaluate the potential of selected antioxidants or inhibitors of NADPH oxidase (glutathione, N-acetyl cysteine, trolox, apocynin, and diphenyleneiodonium chloride) to modulate nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression by mouse macrophages induced by lipopolysaccharide (LPS) *in vitro* and to evaluate the potential of apocynin to modulate the course of LPS-induced lung inflammation *in vivo*. All the tested drugs revealed inhibitory effects on LPS-induced NO production and iNOS expression in RAW 264.7 macrophages. Further, apocynin significantly inhibited activation of nuclear factor kappa B induced by LPS. Ex vivo, diphenyleneiodonium chloride and apocynin significantly reduced ROS production by inflammatory cells isolated from bronchoalveolar lavage fluid. In contrast, *in vivo* intranasal application of apocynin did not exert any significant effect on the course of lung inflammation in mice induced by LPS that was evaluated based on the accumulation of cells, interleukine-6, interleukine-12, RANTES, tumor necrosis factor-alpha, and protein concentration in bronchoalveolar lavage fluid and expression of iNOS in lung tissue. Only effected were the levels of nitrites 36 h after induction of lung inflammation that were reduced in the apocynin-treated group. In conclusion, our data suggest that the inhibitors of NADPH oxidase possess inhibitory potential against LPS-induced NO production by mouse macrophages; however, apocynin failed to reduce LPS-induced lung inflammation in mice.

The potency of hyaluronan of different molecular weights in the stimulation of blood phagocytes

The regulatory functions of glycosaminoglycan hyaluronan (HA) are suggested to be dependent on its molecular weight (MW). Proinflammatory

and stimulatory effects are proposed mainly for the low MW HA. However, the complex response of blood phagocytes to HA of different MW is unclear. Herein, the effects of highly purified HA of precisely defined MW (52, 250, and 970 kDa) on human blood phagocytes were tested. All MW HA activated blood phagocytes, including the spontaneous production of ROS, degranulation, and the production of tumor necrosis factor alpha, with low MW HA 52 kDa having the highest potency and high MW HA 970 kDa having the lowest potency. Interestingly, HA inhibited ROS production stimulated by opsonized zymosan particles and, in contrast, potentiated starch-activated ROS production, mostly independent of MW. Data showed a significant effect of HA of different MW on blood phagocytes, including high MW HA.

New luminescence-based approach to measurement of luciferase gene expression reporter activity and adenosine triphosphate-based determination of cell viability

The assay employing firefly luciferase as the end-point reporter is one of the most popular gene reporter systems. However, the physiological conditions of cells may affect the reporter gene expression, which makes an assessment of cell viability desirable. Estimates of cell viability may be based on different principles. We tested for correlations between various cell viability assessments, including luminescent determination of adenosine triphosphate in whole-cell lysate, and the reporter luciferase activity in pluripotent embryonic and colon adenocarcinoma cells. Luciferase activity in cell lysate from both cell lines cultured under different conditions correlated with the amount of viable cells assessed by all of the methods employed. Importantly, it was also possible to carry out adenosine triphosphate determination in cell lysates prepared in the buffer originally designed for determining luciferase activity; it correlated significantly with adenosine triphosphate determination in cells lysed in the buffer originally designed for adenosine triphosphate determination. The results suggest that the assessment of live cells by determining adenosine triphosphate can be multiplexed with a luciferase reporter gene assay, which allows independent monitoring of both reporter expression and cell viability.

Myeloperoxidase attracts neutrophils by physical forces

Recruitment of polymorphonuclear neutrophils (PMNs) remains a paramount prerequisite in innate immune defense and a critical cofounder in inflammatory vascular disease. Neutrophil recruitment comprises

a cascade of concerted events allowing for capture, adhesion and extravasation of the leukocyte. Whereas PMN rolling, binding, and diapedesis are well characterized, receptor-mediated processes, mechanisms attenuating the electrostatic repulsion between the negatively charged glycocalyx of leukocyte and endothelium remain poorly understood. We provide evidence for myeloperoxidase (MPO), an abundant PMN-derived heme protein, facilitating PMN recruitment by its positive surface charge. *In vitro*, MPO evoked highly directed PMN motility, which was solely dependent on electrostatic interactions with the leukocyte's surface. *In vivo*, PMN 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. Given MPO's affinity to both the endothelial and the leukocyte's surface, MPO evolves as a mediator of PMN recruitment because of its positive surface charge. 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.

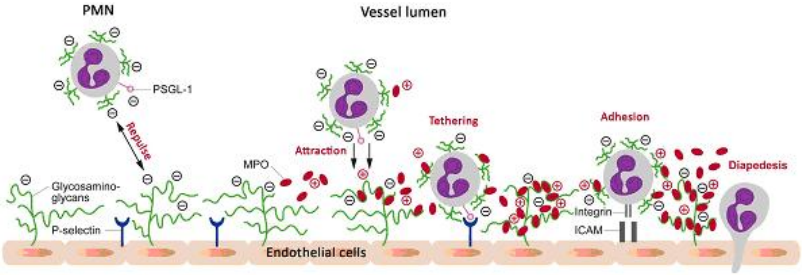

Figure 1: Scheme of the suggested mechanism of MPO-mediated PMN recruitment. Electrostatic repulsion between the negatively charged glycocalyx of PMN and the vessel wall prevents the interactions of PMN with the endothelium. Given the difference in height, the glycocalyx (~ 500 nm) shields selectins (~ 40 nm), which communicate the definite contact of PMN to the vessel wall (e.g. binding of constitutively expressed PSGL-1 on the PMN membrane with P-selectins on the endothelial surface). Thus binding of MPO to glycosaminoglycans reduces the negative surface charge and allows for electrostatic attraction of PMN to the endothelium, which then mediates receptor-ligand interactions resulting in PMN tethering and rolling, adhesion and diapedesis.

Granted projects

COST - MEYS OC 10044, Interactions among collagen, platelets and phagocytes in modulation of reactive oxygen and nitrogen species production. Principal investigator: M. Číž, 2010 - 2011

MEYS - MEB 0810013, Molecular biological aspects of pharmacological modulation of activation of professional phagocytes. Principal investigator: M. Číž, 2010 - 2011

GA CR 524/08/1753, The influence of L-arginine and its analogues on the generation of reactive oxygen and nitrogen species by professional phagocytes. Principal investigator: A. Lojek, 2008 - 2012

GA CR 305/08/1704, Role of hyaluronan of different molecular weight in the course of inflammation. Principal investigator: L. Kubala, 2008 - 2010

AS CR M200040908, international collaboration, Role of myeloperoxidase in the regulation of platelets physiology. Principal investigator: L. Kubala, 2009 - 2011

COST - MEYS OC 08058, Effects of polyunsaturated fatty acids and their metabolites on the physiological functions of professional phagocytes. Principal investigator: A. Lojek, 2010 - 2011

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Safrankova, B, Gajdova, S, Kubala, L.: *The potency of hyaluronan of different molecular weights in the stimulation of blood phagocytes*. Mediators Inflamm., 380948

PhD. thesis defended in 2010

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Mgr. Michaela Pekarová, PhD., Role of arginine and its metabolism in physiology of macrophages

Mgr. Daniela Viačková, PhD., The effect of different molecular weight hyaluronan on phagocyte physiology

STRUCTURE AND DYNAMICS OF NUCLEIC ACIDS

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We have carried out a wide range of investigations of structural dynamics and molecular interactions of nucleic acids, using a variety of methods such as long time-scale explicit solvent molecular dynamics (MD) simulations, quantum chemistry calculations and bioinformatics.

A-site finger (Helix 38) is an important dynamical functional segment of the ribosome, which is involved in regulation of ribosomal translocation and dynamical signaling between the ribosomal subunits. We carried out molecular dynamics simulations of the base of the A-site finger from archaeal and three bacterial large subunits. The study showed that despite noticeable differences in the secondary structures, the studied segments adopt almost identical fold and exhibit similar stochastic fluctuations. We compared geometries of segments from the simulations with structures obtained via cryo-electron microscopy by our partners. These experimental structures showed identical direction of fluctuations as the structures from the simulations. Based on our results we have suggested that dynamics of

the elbow segments is coupled with functional movement of the A-site finger in the course of protein synthesis. Our results thus show that although this ribosomal segment at first sight does not show any conservation on the level of sequences and secondary structures, there is a striking conservation of key properties of the RNA such as the topology and directional elasticity which are key for the function.

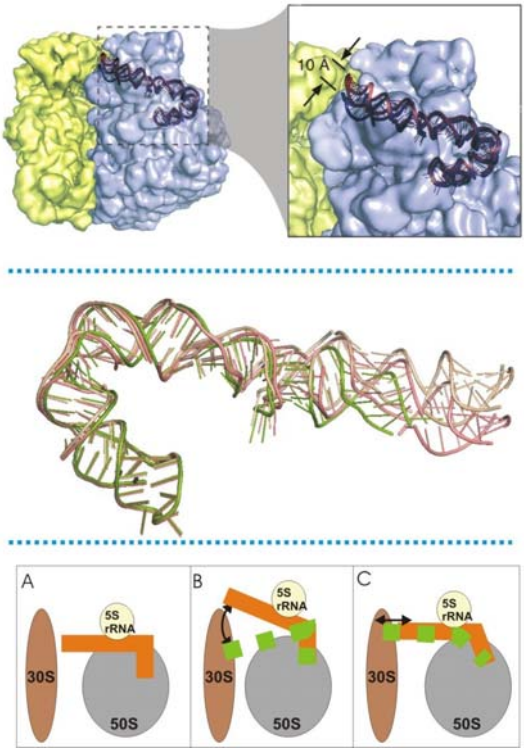

Figure 1: Top: Cryo-electron map of the ribosome with highlighted A-site finger (in ribbon representation), which leads from the large subunit (in blue) towards the small subunit (in yellow). The figure shows movement of top of the A-site finger in range of ca. 10 Å in the course of the elongation cycle. Middle: Detail of two structures obtained from cryo-electron microscopy (in dark and light pink) and X-ray structure (in green). Superposition of structures over one arm shows apparent movement of the second arm. Bottom: (A) Schematic representation of the A-site finger (in orange) with respect to the 5S rRNA and small (30S) and large (50S) subunits, other two pictures (B and C) show possible movements of the A-site finger in the ribosome. B shows movement up-and-down while C shows movement back-and-forth.

Using molecular dynamics and auxiliary techniques we studied 23S rRNA Helix 40 containing UAA/GAN internal loop. This motif is present in seven internal loops of 23S rRNA and also in other RNAs and exhibits characteristic spatial arrangement and secondary (2D) structure (it comprises trans Hoogsteen/Sugar edge A/G base pair followed by an unpaired stacked adenine, a trans Watson-Crick/Hoogsteen U/A base pair, and finally a bulged nucleotide (N)). The solution structure of an isolated UAA/GAA internal loop shows substantially rearranged base pairing with three consecutive non-Watson-Crick base pairs. The results showed that the NMR structure is entirely stable in simulations, while the simulated X-ray structure shows considerable widening of the major groove, a loss of base-phosphate interaction, and other instabilities. The X-ray geometry even undergoes a conformational transition toward the solution 2D structure. Free energy calculations confirm that the X-ray arrangement is less stable than the solution structure. LES, TMD, and NEB techniques provide a rather consistent pathway for interconversion between the X-ray and NMR structures. In summary, the simulations confirm that the UAA/GAN internal loop is a molecular switch RNA module that adopts its functional geometry upon specific tertiary contexts.

We continued in our molecular dynamics studies of RNA Kink-turn structures which represent recurrent elbow-like RNA motifs participating in protein-assisted RNA folding. We carried out a set of MD simulations using different variants of force field parameters (parm99 and parmbsc0) to investigate structural dynamics of the box C/D RNA and its complexes with two proteins: native archaeal L7ae protein and human 15.5 kDa protein, originally bound to very similar structure of U4 snRNA. The box C/D RNA forms K-turn with A-minor 0 tertiary interaction between its canonical (C) and noncanonical (NC) stems. The simulations reveal visible structural dynamics of this A-minor interaction involving six substates which substantially contribute to the elbow-like flexibility of the K-turn. The interaction can be temporarily shifted to the A-minor I type pattern; however, this is associated with distortion of the G/A base pair in the NC-stem. The simulations show reduction of the K-turn flexibility upon protein binding. In our MD simulations of free K-turn, we observed instability of the key signature interaction which was more stable in simulations of K-turns possessing A-minor I interaction. This signature interaction is stabilized by protein binding and stabilizing effect is more visible with human 15.5 kDa protein. The behavior of the A-minor interaction is force-field-dependent because the parmbsc0 force field attenuates the A-minor

fluctuations compared to parm99 simulations. Behavior of other regions of the box C/D RNA is not sensitive to the force field choice.

The aim of another study was to analyze the electronic structure and intrinsic energetics of the DNA sugar-phosphate backbone via highly accurate state-of-the-art quantum chemistry methods. In order to characterize biologically interesting backbone structures we have selected three different conformational substates of a DNA sugar-backbone: 1. canonical substate called BI-DNA, found in standard DNA double helical structures under physiological conditions, 2. pathological substate emerging in long molecular dynamic simulations and degrading the smooth B-DNA structure, and 3. experimentally determined substate found in human telomeric quadruplex DNA loops. In this study, we have demonstrated that it is unnecessary to utilize reference and computationally highly demanding coupled cluster (CC) methods in order to assess intrinsic stability of a given conformational type. This is due to the fact that the corrections for higher-order excitations in principle neglected within the Møller-Plesset second order perturbation theory (MP2) framework are rather conformational independent. As a consequence, MP2 level of theory along with sufficiently large basis set (ideally extrapolated to complete basis set) is adequate for electronic structure description of the sugar-phosphate backbone. In the case of density functional methods (DFT) we have stressed the necessity of dispersion interaction correction and showed the failure of the popular B3-LYP functional. We have also carried out atoms-in-molecules (AIM) analysis of the MP2-converged wavefunctions in order to reveal spurious contacts biasing the gas-phase potential energy surface of the model systems. Based on that we highlighted the effect of artificial and biologically irrelevant weak hydrogen bonds which influence the intrinsic energetics of the systems under study. Finally, we have tested and suggested a new model system, convenient for any subsequent DNA backbone studies not dealing with ε and ζ torsion angles.

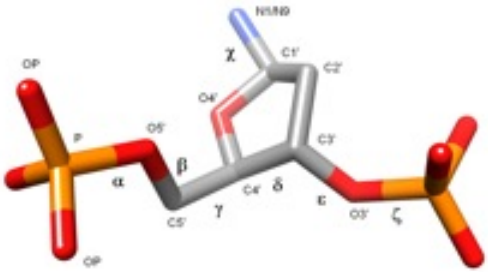

Figure 2: Atomic numbering and definition of the deoxyribonucleotide sugar-phosphate backbone torsion angles. The nucleotide backbone is described by the P-O5'-C5'-C4'-C3'-O3' linkage. The torsion angles represent the rotation around the given bond. It is conventional to describe the backbone torsion angles of ~ 60º as gauche+ (g+), of ~ 300º as gauche- (g-), and of ~ 180º as trans (t). The standard progression of NA chain is the 5' → 3' direction, which is from the left to the right in this particular Figure.

We performed explicit solvent molecular dynamics analysis of three RNA three-way junctions (3WJs) from the large ribosomal subunit: (1) the 3WJ formed by Helices 90-92 (H90–H92) from peptidyl transferase center, (2) the 3WJ formed by H42–H44 organizing the GTPase associated center (GAC) and (3) the 3WJ from 5S rRNA. Our study showed that all three 3WJs possess significant anisotropic hinge-like flexibility between their stacked stems. We have suggested, that these motions may play a role in functionally significant processes in the ribosome. In particular, the H90-H92 3WJ dynamics may facilitate accommodation of tRNA, while the 5S 3WJ flexibility appears to be essential for coordinated movements of A-site finger and 5S rRNA. The GAC 3WJ may support large-scale dynamics of the L7/L12-stalk region.

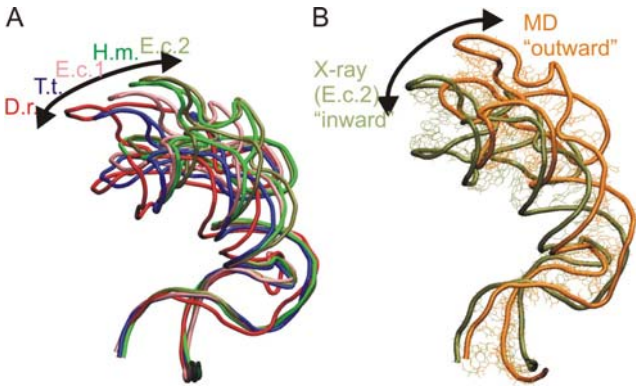

Figure 3: (A) Superposition of X-ray structures of three-way junction from the GTP-ase center (Deinococcus radiodurans – D.r., Thermus thermophilus - T.t., Escherichia coli - E.c., and Haloarcula marismortui – H.m.) illustrates the range of geometries in the X-ray structures. (B) Comparison of starting X-ray structure of three-way junction from E.c. and averaged geometry from MD simulation shows relaxation of the three-way junction during the simulation. The junction exhibits periodic anisotropic motions in the course of the simulations. Range of these motions is shifted with respect to the range of the experimental structures, i.e. junction adopts more open (outward) conformations in the simulations while experimental structures adopt more closed (inward) conformations, probably due to the surrounding ribosomal elements. The direction of motions predicted by simulations is entirely consistent with the experiment and the simulations explain the origin of the experimentally observed flexibility. These observed motions might play a role in the ribosome dynamics during protein synthesis cycle.

Invited feature article, which we have published, provides a side-by-side introduction for two research fields: quantum chemical calculations of molecular interactions in nucleic acids and RNA structural bioinformatics. Our main aim is to demonstrate that these research areas, while largely separated in contemporary literature, have substantial potential to complement each other that could significantly contribute to our understanding of the exciting world of nucleic acids. We identify research questions amenable to the combined application of modern ab initio methods and bioinformatics analysis of experimental structures, while also assessing the limitations of these approaches. The ultimate aim is to attain valuable physico-chemical insights regarding the nature of the fundamental molecular interactions to understand folding, function and evolution of RNA structures. It is a first such joint study in the literature.

Recent experimental studies on the Watson-Crick type base pairing of triazine and aminopyrimidine derivatives suggest that acid/base properties of the constituent bases might be related to the duplex stabilities measured in solution. We have used high-level quantum chemical calculations and molecular dynamics simulations to evaluate the base pairing and stacking interactions of seven selected base pairs, which are common in that they are stabilized by two N—H···O hydrogen bonds separated by one N—H···N hydrogen bond. We show that neither the base pairing nor the base stacking interaction energies correlate with the reported pKa data of the bases and the melting points of the duplexes. This suggests that the experimentally observed correlation between the melting point data of the duplexes and the pKa values of the constituent bases is not rooted in the intrinsic base pairing and stacking properties.

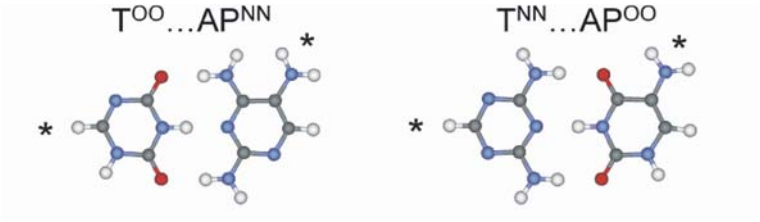

Figure 4: Representative examples of the triazine(T)-aminopyrimidine(AP) base pairs studied.

Granted projects

GA AS CR IAA400040802, Structure, dynamics and reaction mechanism of catalytic RNA. Principal investigator: J. Šponer, 2008 - 2011

ME CR LC06030, Biomolecular Center. Principal investigator: V. Sklenář, Principal co-investigator: J. Šponer, 2006 - 2011

GA CR 203/09/1476, Structural dynamics, molecular interactions and function of key RNA motifs. Principal investigator: J. Šponer, 2009 - 2012

GA CR 203/09/H046, Biochemistry on the crossroad from in silico to *in vitro*. Principal investigator: M. Otyepka, Principal co-investigator: J. Šponer, 2009 - 2012

GA AS CR KJB400040901, Computational study of RNA multiple junctions localized in functionally important sites of the ribosome. Principal investigator: K. Réblová, 2009 - 2011

GA CR P208/10/2302, Theoretical and experimental studies related to the prebiotic chemistry of nucleic acids. Principal investigator: J. E. Šponer, 2010 - 2013

GA CR P208/11/1822, Structure and dynamics of DNA. Advanced computational studies. Principal investigator: J. Šponer, 2011 - 2015

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CD Spectroscopy of Nucleic Acids

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Affection of the human telomeric quadruplex structure by modifications of guanine tetrads

Telomeres play an important role in cellular aging and cancer. Guanine-rich strands of telomeric DNA form quadruplexes, which are pivotal elements for maintaining telomere integrity and controlling cancer cell proliferation. DNA of all living organisms is constantly exposed to damages by endogenous oxidation, hydrolysis, and replication errors as well as exogenous genotoxic chemicals and physical agents that lead to mutations. The mutations also occur in telomeric DNA.

a) Abasic sites in telomeric DNA sequence

The study was performed to evaluate how the loss of a guanine base affects the structure of the three-tetrad G-quadruplex of the basic human telomere DNA sequence 5'-$dG_3(TTAG_3)_3$. None of the twelve possible abasic sites hindered quadruplex formation, but all reduced thermal stability of the parent quadruplex in NaCl and KCl (Figure 1). The base loss did not change the Na^+-stabilized intramolecular antiparallel architecture, based on CD spectra, but held up the conformational change induced in $dG_3(TTAG_3)_3$

upon addition of a physiological concentration of K^+ ions. The reduced stability and the inhibited conformational transitions observed here *in vitro* may indicate that unrepaired abasic sites in G-quadruplexes could lead to changes in the chromosome's terminal protection *in vivo*.

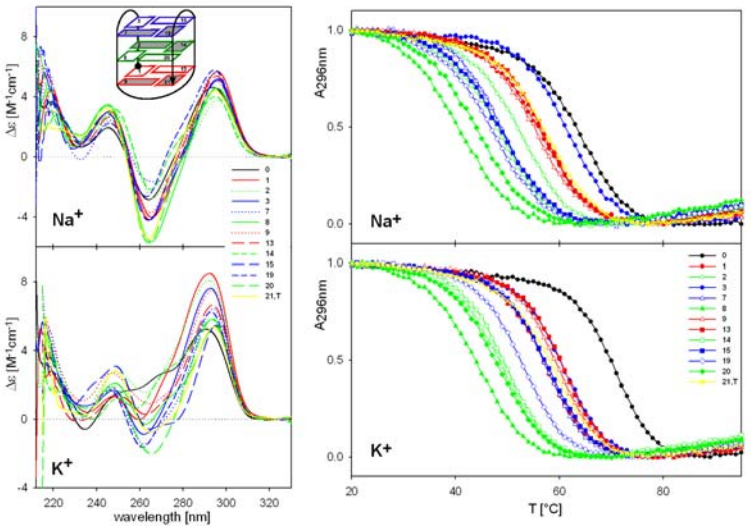

Figure 1: CD spectra (left) and melting curves (right) were measured in a buffered (pH 7) solution of 100 mM Na^+ or K^+, respectively. The spectral type remains preserved for the abasic analogs in the presence of Na^+. However, none of the analogs yields the CD spectrum characteristic of the unmodified (0) $dG_3(TTAG_3)_3$ structure stabilized by K^+. The abasic sites destabilize quadruplex structures, while the destabilization effect is larger in K^+ than in Na^+ ions. The analogs missing guanine base in the middle tetrad are the least stable, those missing guanine in the bottom tetrad (see the sketch) are the least destabilized.

b) A-for-G mutations in telomeric DNA sequence

Using CD spectroscopy, PAGE and dimethylsulphate probe (Fig. 2) we found that replacement of two to four guanines by adenines in the human telomere DNA repeat $dG_3(TTAG_3)_3$ did not hinder the formation of quadruplexes if the substitutions took place in the terminal tetrad bridged by the diagonal loop of the intramolecular antiparallel three-tetrad scaffold (Fig. 1, see the sketch). Thermodynamic data showed that in Na^+ solution the $dG_3(TTAG_3)_3$ quadruplex was destabilized, the least by the two

G:A:G:A tetrads, the most by the G:G:A:A tetrad in which the adenosines replaced syn- guanosines. In physiological K^+ solution the highest destabilization was caused by the 4A tetrad. In K^+ only the unmodified $dG_3(TTAG_3)_3$ quadruplex rearranged into a K^+-dependent quadruplex form, none of the multiple adenine-modified structures did so. This may imply biological consequences for nonrepaired A-for-G mutations.

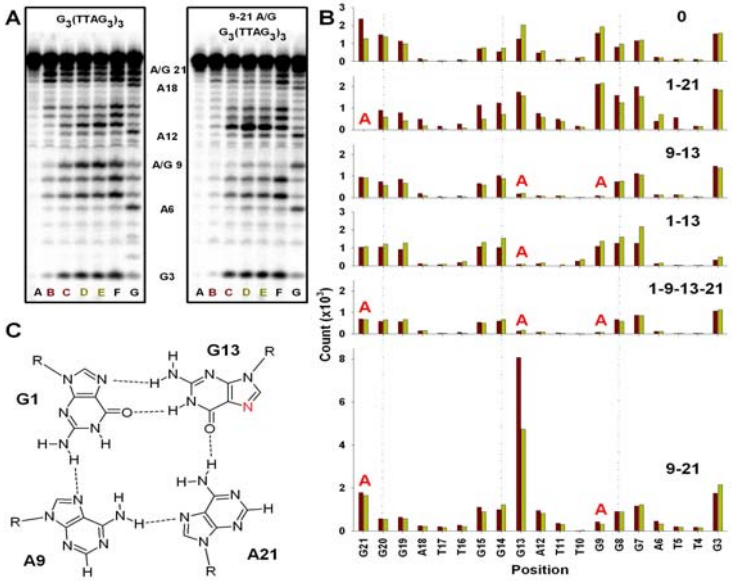

Figure 2: Dimethylsulphate interacts with free N7 group of guanines. DMS probing indicates that the telomeric sequence with G-for-A substitutions in the positions 9 and 21 contains (in contrast to the other studied sequences) the N7 group of G in the position 13 available to DMS interaction. We have suggested the structure of the tetrad (sketch).

CGG repeats associated with X chromosome fragility form left-handed Z-DNA structure

This work is a continuation of our effort to determine the structure responsible for expansion of the (CGG)n motif that results in fragile X chromosome syndrome. In our previous report we demonstrated that the structure adopted by an oligonucleotide with this repeat sequence is not a quadruplex as was suggested by others. Here we demonstrate that (CGG) runs adopt another anomalous arrangement - a left-handed Z-DNA

structure. The Z-DNA formation was induced by high salt and millimolar concentrations of Ni^{2+} ions and likelihood of its formation increased with increasing number of repeats. In an oligonucleotide in which the CGG runs were interrupted by AGG triplets, as is observed in genomes of healthy individuals, the hairpin conformation was stabilized and Z-DNA formation was hindered. We show here that methylation of the (CGG) runs markedly stabilized Z-DNA formation. We hypothesize that rather than in the expansion process the Z-DNA may be formed by long, expanded (CGG) stretches that become hypermethylated; this would inhibit transcription resulting in disease.

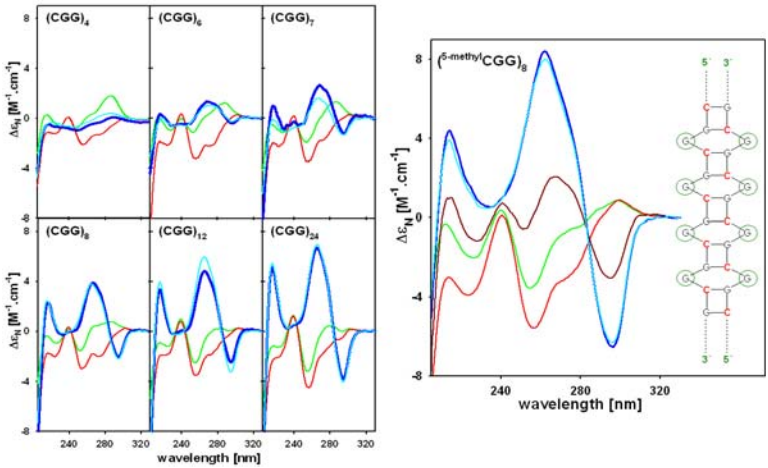

Figure 3: Formation of the left-handed Z-DNA reflected by its characteristic CD spectrum (blue) is stabilized (left) by increasing number of the (CGG) repeats, and mainly (right) by cytosine methylation. The sketch represents a suggested (CGG)n duplex structure enabling the switch into the left-handed conformation.

Granted projects

GA AS CR A100040701, Biophysical properties of biologically and medically important regions of human DNA. Principal investigator: M. Vorlíčková, 2007 - 2011

GA AS CR IAA500040903, Biophysics and bioinformatics of genome DNA fragments rich in guanine and adenine bases. Principal investigator: J. Kypr, 2009 - 2013

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Školáková, P., Bednářová, K., Vorlíčková, M.. Sagi, J.: *Quadruplexes of human telomere $dG_3(TTAG_3)_3$ sequences containing guanine abasic sites*. Biochemical and Biophysical Research Communications, 399, 2010, 203-208.

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PhD. thesis defended in 2010

MUDr. Markéta Fialová, Structural properties of biologically important quadruplexes

PLANT DEVELOPMENTAL GENETICS

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The mechanisms involved in sex determination are some of the most dynamic from an evolutionary point of view. Gonochorism prevails in current animal model species, although hermaphroditism is prevalent in angiosperm plants (approximately 90%). However, many plant families also include dioecious species, and dioecy is present in several crop species. The sex chromosomes in plants and animals have evolved independently, although the mechanisms of their evolution are probably very similar. The necessity of a synthesis of data obtained from various model species can be demonstrated by considering the research of the stepwise arrest of sex chromosome recombination. In general, it is hypothesized that sex chromosomes evolved from a specific pair of autosomes carrying some sex-determining gene(s). Subsequently, the newly-formed sex chromosomes stopped recombination in a small region around the sex-determining locus. Sex chromosomes in this early stage of evolution are not cytologically distinguishable (homomorphic). The process of recombination suppression then progresses through almost the entire sex chromosome.

The results of the human genome project have revealed a correlation between the localization of genes along the X chromosome and silent site sequence divergence from their Y homologues. This silent site sequence divergence serves as a measure of the time since the X and Y copy stopped

recombining. The regions containing genes with similar levels of divergence were named ‘evolutionary strata’ based on a metaphorical similarity to geological strata. Originally, the differences in silent site sequence divergence were explained by several large chromosome rearrangements, although later research showed that the boundaries between some strata are blurred in mammals, suggesting that epigenetic mechanisms or small inversions should also be taken into account. However, discrete strata, suggesting the role of chromosomal rearrangements in the arrest of recombination in birds, were identified in chickens. In humans, computer simulations of inversions followed by the analysis of putative breakpoints indicate that inversions played a crucial role in the origin of strata 4 and 5. Unfortunately, it was not possible to obtain clear results for stratum 3 because its age precludes successful use of the method. Results from nonvertebrate models suggest that the stepwise spread of a nonrecombining region is generally widespread process, probably connected with the existence of any nonrecombining region. Gradients in the silent site divergence have been found in plants (*Silene latifolia*) and fungi (*Microbotryum violaceum*). The dioecious plant *S. latifolia* has much younger sex chromosomes compared to humans but the strata are already present in these chromosomes, which suggests that *S. latifolia* is a promising model for the study of the initial mechanisms of recombination arrest. Despite substantial progress in the knowledge of sex chromosome evolution, there are still topics that are not well understood. One of them is why sex determination (including sex determination systems on a chromosomal level) is conserved in some taxonomic groups, whereas, in others, it is highly dynamic. We present the current status of knowledge of sex determination and sex determination plasticity in animal and plant models. We also discuss possible causes of differences and similarities between animal and angiosperm models. We then suggest a possible model of shifts in the sex-determining systems in plants from a typical plant sex determination system evolved from gynodioecy into sexdetermining systems more similar to the systems found in animal models (Fig. 1).

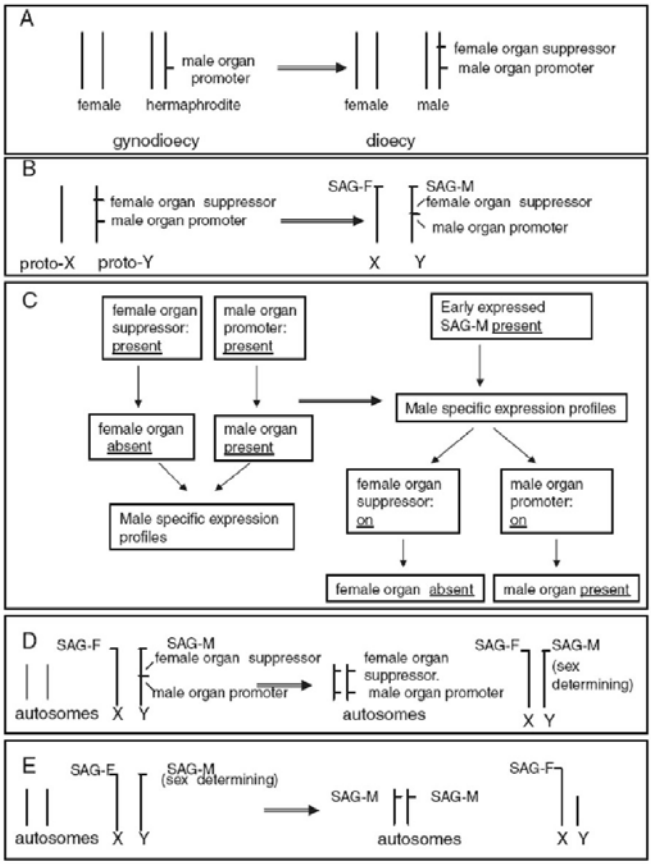

Figure 1: The evolution of the sex-determining pathways in plants. A, theory of origin of dioecy via male sterility. Both the female organ (gynoecium) suppressor and the male organ (anther) promoter promoter act independently but their coordination is achieved by their close location on the Y-chromosome or by their location in the nonrecombining region of the Y-chromosome. B, formation of sex chromosomes. Accumulation of sexually antagonistic genes and reduction of recombination frequency between female organ (gynoecium) suppressor and male fertility controlling genes creates sex chromosomes. For simplification, only one sexually antagonistic gene (SAG) is presented. SAG-F means sexually antagonistic alelle advantageous for females and SAG-M means male advantageous sexually antagonictic allele of the same gene. C, sexually antagonistic gene(s) based switch in the sex-determining pathway. Sexual dimorphism (i.e. controlled by SAG-M) is improved step by step and starts to act before the Y-linked genes involved in female and male organ development control. At certain stage, the expression of both the female organ suppressor and the male organ promoter becomes sex-limited as

a consequence of their adaptation to sex specific expression profiles of other genes. D, restructuration of sex chromosomes. Female organ suppressor and male organ promoting gene(s) are lost from the Y chromosome and transferred to autosome(s). E, origin of the X/A based sex-determining system. SAG-M is lost from Y-chromosome and transferred to an autosome. The X/A ratio becomes crutial for sex determination because SAG-M pushes development toward the male direction in contrast to SAG-F that pushes development toward female direction.

The evolution of sex chromosomes is a complex genetic and epigenetic process, which is often accompanied by structural rearrangements and accumulation of repetitive DNA in non-recombining regions. Moreover, intensive gene turnover within sex chromosomes is reflected by a high number of retroposed genes both on X and Y chromosomes. It is known that over the course of *Silene latifolia* sex chromosome evolution, many repetitive elements have accumulated on the Y chromosome. However, we still lack information about which elements are linked to degenerative processes in Y chromosome evolution by either genetic or epigenetic mechanisms, and little is known about the structural and functional role of repetitive DNA in Y linked genic regions of this plant. We unravel the structure and evolution of a sex linked gene, SlAP3, first reported as having originated by duplication from autosomes to the Y chromosome in *S. latifolia*. Since SlAP3Y is located close to the oldest stratum (4.5-7 MY) in the Y chromosome, this gene is a candidate to be affected by various degenerative processes. In our study, we did not find evidence for a duplication event in the case of this gene. Instead, we identified a new pair of sex linked alleles with no evidence for autosomal paralogues. We demonstrated the accumulation of retrotransposon sequences in an intron region of the Y linked allele (Fig. 2). We further analyzed expression patterns of individual elements identified in the Y copy of SlAP3 gene to reveal their role in Y chromosome evolution.

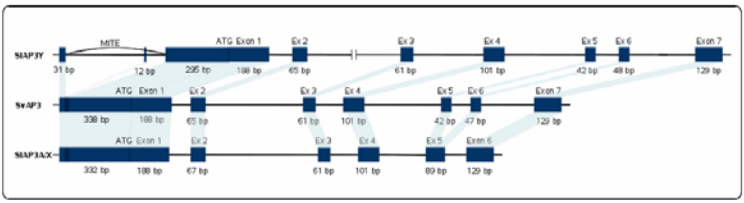

Figure 2: Alignment of a promoter and coding region of SlAP3X, SlAP3Y and SvAP3 genes. Rectangles represent exon regions of the genes. Corresponding coding sequences are indicated.

In *S. latifolia*, recombination between the sex chromosomes has ceased in three steps, and three groups of genes with different levels of divergence (also called strata) have been identified. The level of divergence between SlAP3 X and Y copies is about 13%, between the 20% X-Y divergence typical of the stratum 1 genes and the 10% X-Y divergence typical of the stratum 2 genes. We conducted a dN/dS analysis on the SlAP3 sequence to study the possibility of differences in intensity and form of selection in the X and Y copies of this gene (and also with autosomal orthologs). We included all available orthologous sequences from *Silene* species to have as many sequences as possible for the phylogenetic dN/dS analysis, which tends to give more accurate results with more sequences, and to have outgroups (the non-dioecious species *S. vulgaris* and *S. conica*). The dN/dS ratios in X and Y sequences among dioecious *Silene* species were not found to be significantly different, which does not provide clear evidence of the Y copy degeneration. However, dN/dS ratios were found to be significantly different (p-value = 0.0149) between dioecious and non-dioecious lineages. The higher ratio in the dioecious lineage suggests that selection has been relaxed in both the X and Y sequences compared to the autosomal copy in S. vulgaris and S. conica where dN/dS is much lower.

We also present an updated model (Fig. 3) of the sex chromosomes of *S. latifolia* combining previous data from genetic mapping, Y chromosome deletion mutants, FISH mapping, PCR on microdissected arms of the X chromosome, and DNA replication patterns and histone acetylation studies. The novelty of our model is that we propose a gathering of genes near the subtelomeres. This positioning is supported not only by early replication and histone acetylation of subtelomeres, but also by the FISH localization of DD44X/DD44Y and SlX4/SIY4 at the subtelomere of the q-arm of the X chromosome and the subtelomere of the p-arm of the Y chromosome. Our model contrasts with other previous models where SlY4 was localized on the q-arm of the Y chromosome. However, we have to keep in mind that the presence of more copies of this gene would explain this inconsistency between our models. Another novel feature of our model is the presence of more inversions occurring during the evolution of the Y chromosome. Comparisons of the *S. latifolia* X chromosome with its homologue in S. vulgaris showed that the Y chromosome, and not the X chromosome, was rearranged. We suggest that the older inversion covered only a small region and corresponds to the most diverged genes (SlY6a – SlY3). Localization of two initial mutations leading to dioecy within a small chromosome region was also proposed. The younger inversion in our model covers less diverged genes (DD44Y – SlssY). The less diverged genes (SlY6b – SlY1) on the

arms containing the PAR region were not inverted on the Y chromosome. Because the SlY6a gene is not included in any inversion in our model, its larger X/Y divergence could be explained by the presence of its paralogue SlY6b releasing the selective forces acting on this gene and allowing diversification of the SlY6a copy.

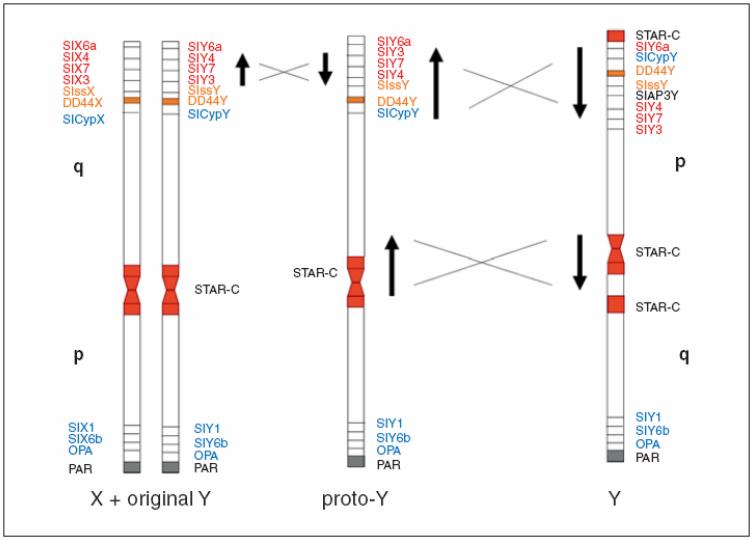

Figure 3: A scheme of sex chromosome evolution in *S. latifolia* based on recent data from genetic mapping, deletion mutants, FISH mapping, PCR on microdissected chromosomes, early DNA replication and histone acetylation. Three inversions are indicated, two of them formed the oldest evolutionary strata containing genes with highest divergence (in red) and medium divergence (in orange). The third inversion changed the submetacentric Y chromosome into a metacentric chromosome and introduced the STAR-C tandem repeat (red blocks) into a new location on the q-arm. Genes with lowest divergence (in blue) are located in subtelomeres of the q-arm that experienced no large inversion. The DD44 genes localized by FISH are represented by orange boxes. The pseudoautosomal region (PAR) is located at the distal end of the q-arm.

Granted projects

GA CR 204/09/H002, Plant developmental biology. Principal investigator: B. Vyskot, 2009 - 2012

MAYS LC06004, Integrative studies of plant genome. Principal investigator: B. Vyskot, 2006 - 2011

GA CR 521/08/0932, Horizontal gene transfer in plants. Principal investigator: B. Janoušek, 2008 - 2011

GA AS CR IAA600040801, Early phases of evolution of sex chromosomes: comparative study of *Silene otites, S. colpophylla* and *S. latifolia.* Principal investigator: B. Janoušek, 2008 - 2011

GA AS CR KJB600040801, Developmental pathways involved in the gynoecium suppression in dioecious plants. Principal investigator: J. Žlůvová, 2008 - 2010

GA AS CR M200040902, international collaboration, Structure and function of plant sex chromosomes. Principal investigator: B. Vyskot, 2009 - 2012

GA CR 522/09/0083, Isolation of genes linked to sex chromosomes and their use to study evolution of sex chromosomes in plants. Principal investigator: R. Hobza, 2009 - 2013

GA AS CR KJB600040901, *Silene vulgaris* as a model for comparative genomics. Principal investigator: R. Hobza, 2009 - 2011

GA AS CR M200040905, international collaboration, Genus *Silene* as a model for mating systems and adaptation mechanisms evolution - from ecology to genomics. Principal investigator: R. Hobza, 2009 - 2011

GA CR P501/10/0102, Comparative analysis of plant sex chromosomes. Principal investigator: B. Vyskot, 2010 - 2014

GA CR P305/10/0930, Sex chromosomes and dynamics of transposons. Principal investigator: E. Kejnovský, 2010 - 2014

GA CR P501/10/P483, Retrotransposon colonizing only recombining part of genome of dioecious plants. Principal investigator: Z. Kubát, 2010 - 2012

Publications

Kejnovsky, E., Vyskot, B.: *Silene latifolia: the classical model to study heteromorphic sex chromosomes*. Cytogenetic and Genome Research, 129, 2010, 250-262.

Vrbsky, J., Akimcheva, S., Watson, JM., Turner, TL., Daxinger, L., Vyskot, B., Aufsatz, W., Riha, K.: *siRNA-mediated methylation of Arabidopsis telomeres*. Plos Genetics, 6, 2010, e1000986.

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Cegan, R., Marais, G.A.B., Kubekova, H., Blavet, N., Widmer, A., Vyskot, B., Dolezel, J., Safar, J., Hobza, R.: *Structure and evolution of Apetala3, a sex-linked gene in Silene latifolia*. BMC Plant Biology, 10, 2010, e180.

Vyskot B.: *Epigenetika*. Učební text v češtině (152 stran), Vydavatelství Univerzity Palackého, Olomouc 2010 (ISBN 978-80-244-2534-4)

Hobza, R., Čegan, R., Vyskot, B., Nevrtalová, E.: *Molekulární mechanizmy rezistence k těžkým kovům a jejich akumulace v rostlinách. In: Současné možnosti fyziologie a zemědělského výzkumu přispět k produkci rostlin*. Praha Ruzyně, 2010, str. 121-138 (ISBN: 978-80-7427-023-9)

Teaching activities - semestral courses (lectures, seminars, practical classes)

Masaryk University, Brno

Viktor Brabec, Jana Kašpárková, Lenka Zerzánková, Jana Štěpánková: Seminar of Laboratory of molecular biophysics and pharmacology

Vítězslav Bryja, Pavel Krejčí: Developmental animal physiology I, II

Milan Číž, Lukáš Kubala, Antonín Lojek: Immunology

Milan Číž, Lukáš Kubala, Antonín Lojek: Free radicals in animal physiology

Jiří Fajkus: Journal Club

Jiří Fajkus, Miloslava Fojtová: Structure and function of eukaryotic chromosomes

Jiří Fajkus: Seminar of the Department functional genomics and proteomics

Jiří Fajkus, Miloslava Fojtová, Eva Sýkorová: Analysis of chromatin structure

Jiří Fajkus, Miloslava Fojtová: Applied genomics and proteomics

Miroslav Fojta: Molecular biology

Jiřina Hofmanová, Alois Kozubík: Health risks

Jiřina Hofmanová, Alois Kozubík: Special methods of animal physiology

Jiřina Hofmanová, Karel Souček, Alena Vaculová, Jan Vondráček, Pavel Krejčí: Molecular physiology of animals

Eduard Kejnovský, Roman Hobza: Evolutionary genomics

Aleš Kovařík: Specialized methods in microbiology

Stanislav Kozubek, Eva Bártová: Molecular physiology of the genome

Stanislav Kozubek, Martin Falk: Radiation biophysics

Alois Kozubík: Introduction to the study of general biology

Alois Kozubík, Jiřina Hofmanová: Physiology of cell systems

Alois Kozubík, Jiřina Hofmanová: Genotoxicity and carcinogenesis

Alois Kozubík, Jiřina Hofmanová, Karel Souček, Jan Vondráček: Modern methods of cell biology

Olga Nováková: Selected themes of application biophysics

Emil Paleček, Miroslav Fojta: Chemical properties, structure and interactions of nucleic acids; Chemistry of nucleic acids

Kamila Réblová, Naďa Špačková: Basics of molecule modelling and bioinformatics

Karel Souček: Molecular biology and genetics

Karel Souček: Journal club - Cancer biology I, I

Karel Souček, Eva Lincová, Alena Vaculová: Analytical cytometry

Jiří Šponer: Basics of molecular biophysics

Jiří Šponer: Molecular interactions in biology and chemistry

Jiří Šponer: Nucleic acids structure and dynamics

Alena Vaculová: Scientific work methodology

Vladimír Vetterl: Biophysics and biophysical chemistry

Vladimír Vetterl, František Jelen: Bioelectrochemistry 1

Vladimír Vetterl, František Jelen: Bioelectrochemistry 2

Marie Vojtíšková: Molecular biotechnology

Jan Vondráček: Applied chemistry and biochemistry

Jan Vondráček: Physiology of pharmaceuticals and toxic compounds

Oldřich Vrána: Biophysics

Oldřich Vrána, Olga Nováková, Marie Vojtíšková, Jaroslav Malina: Experimental methods of biophysics

Boris Vyskot: Developmental genetics

Boris Vyskot: Seminar for PhD. students

Palacký University Olomouc

Viktor Brabec: Biophysical seminar

Viktor Brabec: Structure and function of biomolecules

Viktor Brabec: Physical properties of nucleid acids

Viktor Brabec: Physics properties of nucleid acids II.

Jana Kašpárková: Molecular biophysics of mutagens, cancerogens and cytostatics

Jana Kašpárková: Molecular biophysics

Jiří Šponer: Structure and dynamics of nucleic acids

Boris Vyskot: Developmental biology

Boris Vyskot: Epigenetics

Mendel University of Agriculture and Forestry in Brno

Roman Hobza: Genetic engineering

Boris Vyskot: Genetic engineering II

University of Veterinary and Pharmaceutical Sciences Brno

Marie Brázdová: Biochemistry

Eduard Kejnovský, Roman Hobza: Evolutionary biology

Comenius University in Bratislava

Veronika Ostatná: Medical biophysics

University of South Bohemia in České Budějovice

Roman Hobza, Eduard Kejnovský: Evolutionary genomics

University of Ostrava

Boris Vyskot, Jiří Široký: Developmental biology

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Standard services of the Center of Information Technologies (CIT) include maintenance of the local area network (LAN), the connection of the IBP LAN to Brno Academic Computer Network and to the Internet, maintenance of exchange and IP telephony, maintenance of the IBP e-mail server, including antivirus and antispam systems, maintenance of the IBP web server including design and data update, development and maintenance of computer hardware and software jointly used by all laboratories (servers, graphic workstations, PCs with connected scientific instruments) running under UNIX, MS Windows 2000/XP/Vista/7. CIT also provides consulting services for individual scientists.
Library – a part of CIT takes care of online access to scientific journals over Internet, manages subscriptions to scientific informational resources, manages information exchange among libraries, takes care of printed versions of journals and books in the IBP and arranges access of users to them. Library also collects and archives research results of scientists of the IBP.

Main attention of CIT was devoted to the security issues. Security patches were installed in time and antivirus databases were regularly updated. All e-mails are monitored at the server by a virus scanner together with special software designed to detect and defang dangerous elements inside e-mail messages (dangerous attachments are renamed, so that they cannot be run automatically on PC). In addition, e-mails are scanned by antispam system.

In the 2010 there was extended EDUROAM (http://www.eduroam.org/, http://www.eduroam.cz/) network at the IBP. There were installed four new access points to manage user access to the IBP WiFi network.

There was installed a new central switch in the IBP computer network. Connection of the IBP LAN to Brno academic backbone network was upgraded from 1 Gbps to 10 Gbps and the LAN topology was readjusted to the new switch. The new central switch is prepared for IPv6 implementation.
Computer with IBP mailserver, nameserver and webserver was reinstalled in a virtual machine. A new design of web pages was implemented.

COST Action TD09/05 Meeting ”Epigenetics-Bench to Bedside”

The workshop was organized by Assoc.Prof. Eva Bártová at Institute of Biophysics AS CR, v.v.i. (November 22 to November 25, 2010; 31 participants from 17 countries including UK, France, Greece, Hungary, Czech Republic, Iceland, Lithuania, Switzerland, Croatia, Finland, Spain, Italy, Sweden, Holland, Poland, Germany and Slovenia). Workshop was aimed at epigenetics. Epigenetics is the science relating to changes in biological phenotype without an underlying change in the organism's genome. These epigenetic changes are orchestrated by a set of enzymes that modulate the structure of chromatin in eukaryotes, either by covalently altering DNA or histone proteins. These post-translational modifications are dynamic, and ultimately regulate the pattern of gene expression and repression. Progress in understanding the details of this process and their effects upon biological function requires an interdisciplinary approach. The goal of this COST network is to bring together synthetic chemists and chemical biologists working in epigenetics. COST Action TD09/05 provides European framework for networking and research collaborations in the area as well as encourage new participants. In addition to chemists, the network includes biologists, pharmacologists and clinicians from both academia and industry to provide a stimulating forum for cross-disciplinary interactions. In Brno meeting, all participants actively demonstrated their work during their oral presentation or during poster session.

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