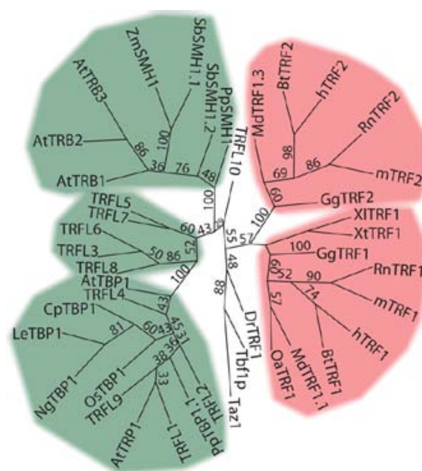
IBP

Institute of Biophysics

INSTITUTE OF BIOPHYSICS

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



RESEARCH REPORT 2011

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Introduction

In 2011, a research activity evaluation of the AS CR institutes was completed. The IBP Board and Council have elaborated a number of documents related to the evaluation. Their completion was considered to be a most crucial task, with a lot of effort put into, due to the earlier stated relation between the evaluation results and a financial support of the institute in the following period. In the evaluation, our institute took second place in its section, leaving other six institutes behind. In view of the rather good evaluation of the institute and considering the opinion of most heads of the IBP departments, the institute board has decided to pursue the existing internal evaluation system.

In the 2011 evaluation, the department of J. Šponer (names are cited without titles), took first place, as usual. Compared to 2010 and 2009, their IF in 2011 decreased, yet the citation index increased (a number of publications of 2006, which are crucial for the IBP, have been highly cited). M. Fojta´s department has improved, gaining on the leading team of J. Šponer. For the last four years, M. Fojta´s department has performed steadily, with the cumulative IF for the institute approaching ΣIF=50. The citation index is high and steadily rising; the department has a relatively low number of citations in magazines with a high IF. This year, M. Fojta, collaborating with a colleague from the IOCB, has managed to publish an article in Chem. Soc. Rev. with an IF=26.58. Following the departments of J. Šponer and M. Fojta, there came the departments of V. Brabec and S. Kozubek, with similar numbers of points. V. Brabec´s department has recorded a decrease in the cumulative IF for the institute yet a slow rise in the number of citations. These two parameters are offset by each other. The number of key papers with an IF>4 has been high for a lot of years (5 publications per year).

S. Kozubek´s department includes groups of E. Bártová, M. Falk, M. Hofer, M. Štros and J. Fajkus, out of which E. Bártová´s group contributes most, by far. This year, E. Bártová´s group has published seven papers with an IF above 4, out of which six belong to the IBP almost exclusively. The numbers of citations have increased significantly. The other groups contribute to the overall performance of the department too, yet noticeably less. Another department is A. Kozubík´s one, which has seen a steadily perceptible rise in both the cumulative IF and the number of citations. Their contribution to the overall number of points, derived from the growth dynamics, has still been the highest. The second highest contribution to the

overall number of department points comes from the cumulative IF and then from citations. The contribution derived from pedagogical activity is also high (the highest of the institute). There follow the departments of A. Lojek and B. Vyskot with similar numbers of points. Regarding B. Vyskot´s department, there has been a decrease in the performance measured by the cumulative IF for the institute calculated for the last six years, i.e. in the long term. On the contrary, the number of citations has grown for both departments. B. Vyskot´s department has recorded a high number of citations in magazines with a high IF while the number of these citations of A. Lojek´s department has been relatively low. A. Lojek´s department has participated in two publications with an IF>10. Both departments have recorded a significant contribution of pedagogical and other activities. Following them, there came the department of A. Kovařík, which has had steady, or slightly decreasing, values of the cumulative IF in the long term (approaching IF=10 for the institute per year). The number of key publications with an IF>4 is two papers per year and has been steady recently. An increase in the number of citations is insignificant, hence the overall dynamics parameter has been approaching zero. M. Vorlíčková´s department has seen a decrease in the cumulative IF, which has been offset by the increasing number of citations, yet the dynamics parameter has been approaching zero. Contributions of the other parameters have been balanced. The pedagogical activity contribution has been low.

The result of this evaluation has influenced both the amount of the subsidies granted to individual departments in 2012 and the yearly bonuses to their heads. I suppose that in the course of 2012 there will be specified the internal evaluation system, as well as other aspects of the institute development, such as potential reconstructions or further facilitating. In my opinion, the evaluation system applied since 2005 has played a positive role in the institute development. For the last five years, the institute has improved in important parameters almost twice, reaching the highest cumulative IF per staff within the section of biological and medical sciences of the AS CR.

Stanislav Kozubek

Molecular Biophysics and Pharmacology

Head
Viktor Brabec

Senior Scientist
Jana Kašpárková

Scientists
Hana Kostrhunová, Jaroslav Malina, Olga Nováková, Marie Vojtíšková, Oldřich Vrána

Postdocs
Anna Halámiková-Kisová, Jana Štěpánková, Lenka Zerzánková

PhD. Students
Jakub florián, Michaela Frýbortová, barbora Lišková, Jarmila Mlčoušková, Tereza Muchová, Vojtěch Novohradský, Radana Olivová, Jitka Prachařová, Tereza Suchánková

Technical assistant
Milada Kořínková

(Carboxydiamine)Pt(II) complexes of a combretastatin A-4 analogous chalcone: the influence of the diamine ligand on DNA binding and anticancer effects

The combretastatin A-4 analogue *m*-hydroxychalcone 2 was esterified with a (1-carboxyethane-2,3-diamine)dichloridoplatinum(II) fragment to give complex 6 which was more active against various cancer cell lines ($IC_{50} < 1$ μM) than its analogue 3 bearing a 6-aminomethylnicotinate ligand. Complex 6 bound to the same sites of DNA as cisplatin but caused a larger DNA unwinding angle and ten times more interstrand cross-links. Also, DNA lesions due to binding of 6 were only half as efficiently repaired as cisplatin–DNA adducts. Complex 6 also showed a much lower affinity to the platinum detoxifier glutathione.

Influence of pyridine versus piperidine ligands on the chemical, DNA binding and cytotoxic properties of light activated trans, trans, trans-[Pt$(N_3)_2(OH)_2(NH_3)$(L)]

The photocytotoxicity and photobiochemical properties of the new complex *trans, trans, trans*-[Pt$(N_3)_2(OH)_2(NH_3)$(piperidine)] (5) are compared with its analogue containing the less basic and less lipophilic ligand pyridine (4). The log P (n-octanol/water) values were of -1.16 and -1.84 for the piperidine and pyridine complexes, respectively, confirmed that piperidine increases the hydrophobicity of the complex. Density Functional Theory (DFT) and time-dependent density functional theory (TDDFT) calculations indicate that 5 has accessible singlet and triplet states which can promote ligand dissociation when populated by both UVA and visible white light. When activated by UVA or white light, both compounds showed similar cytotoxic potencies in various human cancer cell lines although their selectivity was different. The time needed to reach similar antiproliferative activity was noticeably decreased by introducing the piperidine ligand. Neither compound showed cross-resistance in three oxoplatin-resistant cell lines. Furthermore, both compounds showed similar anticlonogenic activity when activated by UVA radiation. Interactions of the light-activated complexes with DNA showed similar kinetics and levels of DNA platination and similar levels of DNA interstrand cross-linking (ca. 5 %). Also the ability to unwind double stranded DNA where comparable for the piperidine analogue (24°, respectively), while the piperidine complex showed higher potency in changing the conformation of DNA, as measured in an ethidium bromide binding assay. These results indicate that the nature of the heterocyclic nitrogen ligand can have subtle influences on both the phototoxicity and photobiochemistry of this class of photochemotherapeutic agents.

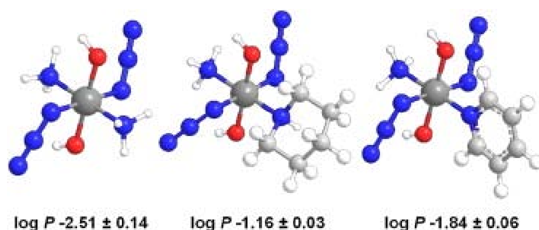

Figure 1: Structure–activity relationships show that neither the basicity nor the aromaticity of the liganding amine (L) in [Pt$(N_3)_2(OH)_2(NH_3)$(L)] is responsible for good photoactivation to cytotoxic species but rather the bulkiness of the amine ligand.

Functionalization of osmium arene anticancer complexes with (poly)arginine: Effect on cellular uptake, internalization, and cytotoxicity

Attaching peptides to metallodrugs may result in improved biological properties of the complexes. The potential use of cell penetrating peptides (CPPs) as cell delivery vectors is attractive, since directed cell uptake of (metallo)drugs remains a major challenge in anticancer drug design. In this work, we report the synthesis of peptide conjugates of the organometallic Os^{II} anticancer complex [(η^6-biphenyl)Os(picolinate)Cl] with different arginine (Arg) chain lengths. Complexes conjugated to Arg5 or Arg8 at the 5-position of the picoline ring increase Os uptake into A2780 human ovarian cancer cells by ca. 2× and 10×, respectively, whereas a single Arg had no effect. Furthermore, a 15-fold increase in binding of Os to DNA, a potential target for these complexes, was observed for Arg8 compared to the Arg1 conjugate. The Arg5 and Arg8 conjugates exhibited fast kinetics of binding to calf thymus DNA and an ability to precipitate DNA at very low concentrations. In serum-free medium, the Arg8 complex was cytotoxic (IC_{50} 33 μM) and appears to be a rare example of a bioactive organometallic peptide conjugate. Experiments on CHO cells deficient in DNA repair suggested that unrepaired DNA damage contributes to the cytotoxicity of the Arg5 and Arg8 conjugates. These studies demonstrate the potential for use of cell- and nucleus-penetrating peptides in targeting organometallic arene anticancer complexes.

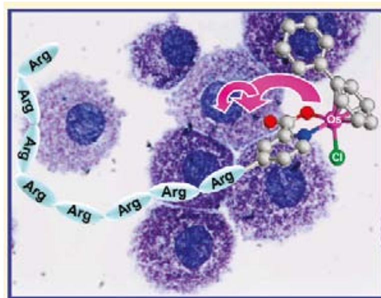

Figure 2: Attaching arginine chains to the organometallic Os^{II} anticancer complex may result in improved biological properties of the complexes.

DNA binding selectivity of oligopyridine-ruthenium(II)-lysine conjugate

The synthesis, characterization and DNA binding properties of the complex $[Ru(terpy)(4,4'-(COLysCONH_2)_2bpy)Cl]^{3+}$ (1) have been studied. Complex (1) hydrolyzes to (2) with a calculated rate constant $K_h = 2.35 \pm 0.08 \times 10^{-4}$ s^{-1} and binds coordinatively to ct-DNA, with a saturation r-value at about 0.1. Stabilization of the ct-DNA helix at low electrolyte ($NaClO_4$) concentration (10 mM) and destabilization at higher electrolyte concentrations (50-200 mM) was observed. Circular dichroism studies indicate that the hydrolyzed complex binds to DNA, increasing the unwinding of the DNA helix with an unwinding angle calculated as Phi = 12 ± 2 degrees. The positive LD signal observed at 350 nm indicates some kind of specificity in complex orientation towards the global DNA axis. Complex (2) binds specifically to G4 on the central part of the oligonucleotide duplexes $d(CGCGCG)_2$ and $d(GTCGAC)_2$, as evidenced by NMR spectroscopy. Both lysine moieties were found to interact most likely electrostatically with the DNA phosphates, assisting the coordinative binding and increasing the DNA affinity of the complex. Photoinduced DNA cleavage by (2), upon UVA irradiation was observed, but despite its relative high DNA affinity, it was incomplete (similar to 12%).

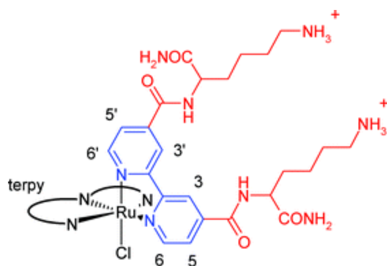

Figure 3: Schematic illustration of the complex 1.

DNA interactions of 2-pyrrolinodoxorubicin, a distinctively more potent daunosamine-modified analog of doxorubicin

It was shown earlier that 2-pyrrolinodoxorubicin was 500–1000 times more active towards human and mouse cancer cells *in vitro* than parental doxorubicin. However, the biochemical factors responsible for such a large

difference in potency between doxorubicin and 2-pyrrolinodoxorubicin are not clear at the molecular level. To provide this information, we have investigated in cell-free media by biochemical and biophysical methods interactions of both anthracyclines with DNA, effects of these interactions on activity of human topoisomerase II, human Bloom's syndrome helicase and prokaryotic T7 RNA polymerase, and the capability of these drugs to form DNA interstrand cross-links in formaldehyde-free medium. Experiments aimed at understanding the properties of double-helical DNA in the presence of doxorubicin and 2-pyrrolinodoxorubicin revealed only small differences in DNA modifications by these anthracyclines and resulting conformational alterations in DNA. Similarly, the ability of 2-pyrrolinodoxorubicin modifications of DNA to inhibit catalytic activity of topoisomerase II does not differ significantly from that of doxorubicin. On the other hand, we demonstrate that an important factor responsible for the markedly higher antiproliferative potency of DNA modifications by 2-pyrrolinodoxorubicin is capability of these modifications to inhibit downstream cellular processes which process DNA damaged by this drug and involve separation of complementary strands of DNA, such as DNA unwinding by helicases or RNA polymerases. In addition, the results are also consistent with the hypothesis that in particular the capability of 2-pyrrolinodoxorubicin to readily form DNA interstrand cross-links is responsible for inhibition of these processes in the cells treated with this analogue of doxorubicin.

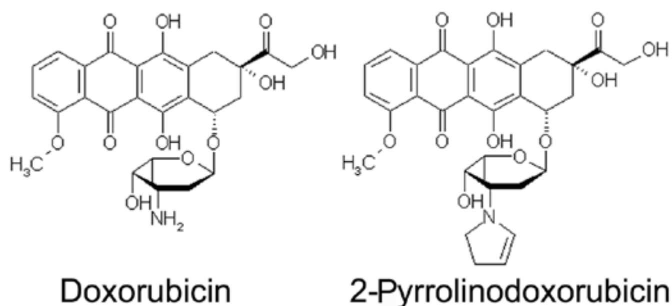

Figure 4: Structures of anthracyclines.

Walking of antitumor bifunctional trinuclear Pt^{II} complex on double-helical DNA

The trinuclear BBR3464 ([{trans-PtCl(NH_3)$_2$}$_2$μ-(trans-Pt(NH_3)$_2$(H_2N(CH_2)$_6$$NH_2$)$_2$)]$^{4+}$) belongs to the polynuclear class of platinum-based anticancer agents. DNA adducts of this complex differ significantly in structure and type from those of clinically used mononuclear platinum complexes, especially, long-range (Pt, Pt) intrastrand and interstrand cross-links are formed in both 5'-5' and 3'-3' orientations. We show employing short oligonucleotide duplexes containing single, site-specific cross-links of BBR3464 and gel electrophoresis that in contrast to major DNA adducts of clinically used platinum complexes, under physiological conditions the coordination bonds between platinum and N7 of G residues involved in the cross-links of BBR3464 can be cleaved. This cleavage may lead to the linkage isomerization reactions between this metallodrug and double-helical DNA. Differential scanning calorimetry of duplexes containing single, site-specific cross-links of BBR3464 reveals that one of the driving forces that leads to the lability of DNA cross-links of this metallodrug is a difference between the thermodynamic destabilization induced by the cross-link and by the adduct into which it could isomerize. The rearrangements may proceed in the way that cross-links originally formed in one strand of DNA can spontaneously translocate from one DNA strand to its complementary counterpart, which may evoke walking of the platinum complex on DNA molecule.

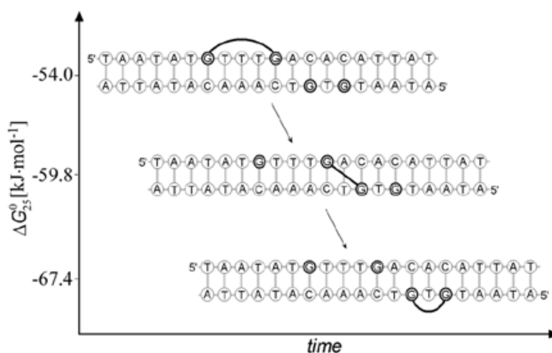

Figure 5: Scheme of the linkage isomerization reactions between trinuclear platinum compound BBR3464 and double-helical DNA substrate.

DNA interstrand cross-links of an antitumor trinuclear platinum(II) complex: Thermodynamic analysis and chemical probing

The trinuclear platinum compound $[\{trans\text{-}PtCl(NH_3)_2\}_2(\mu\text{-}transPt(NH_3)_2\{NH_2(CH_2)_6NH_2\}_2)]^{4+}$ (BBR3464) belongs to the polynuclear class of platinum-based anticancer agents. These agents form in DNA long-range (Pt,Pt) interstrand cross-links, whose role in the antitumor effects of BBR3464 predominates. Our results show for the first time that the interstrand cross-links formed by BBR3464 between two guanine bases in opposite strands separated by two base pairs (1,4-interstrand cross-links) exist as two distinct conformers, which are not interconvertible, not only if these cross-links are formed in the 5′-5′, but also in the less-usual 3′-3’ direction. Analysis of the conformers by differential scanning calorimetry, chemical probes of DNA conformation, and minor groove binder Hoechst 33258 demonstrate that each of the four conformers affects DNA in a distinctly different way and adopts a different conformation. The results also support the thesis that the molecule of antitumor BBR3464 when forming DNA interstrand cross-links may adopt different global structures, including different configurations of the linker chain of BBR3464 in the minor groove of DNA. Our findings suggest that the multiple DNA interstrand cross-links available to BBR3464 may all contribute substantially to its cytotoxicity.

Organometallic half-sandwich iridium anticancer complexes

The low-spin $5d^6$ Ir^{III} organometallic half-sandwich complexes $[(\eta^5\text{-}Cp^x)Ir(XY)Cl]^{0/+}$, Cp^x = Cp*, tetramethyl(phenyl)cyclopentadienyl (Cp^{xph}), or tetramethyl(biphenyl)cyclopentadienyl (Cp^{xbiph}), XY = 1,10-phenanthroline (4-6), 2,2′-bipyridine (7-9), ethylenediamine (10 and 11), or picolinate (12-14), hydrolyze rapidly. Complexes with N,N-chelating ligands readily form adducts with 9-ethylguanine but not 9-ethyladenine; picolinate complexes bind to both purines. Cytotoxic potency toward A2780 human ovarian cancer cells increases with phenyl substitution on Cp*: Cp^{xbiph} > C^{pxph} > Cp*; C^{pxbiph} complexes 6 and 9 have submicromolar activity. Guanine residues are preferential binding sites for 4-6 on plasmid DNA. Hydrophobicity (log P), cell and nucleus accumulation of Ir correlate with cytotoxicity, 6 > 5 > 4; they distribute similarly within cells. The ability to displace DNA intercalator ethidium bromide from DNA correlates with cytotoxicity and viscosity of Ir-DNA adducts.

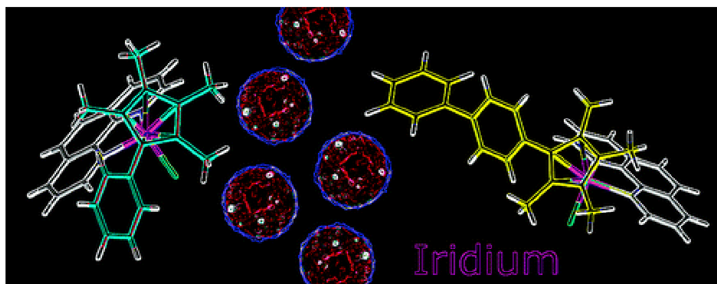

Figure 6: Organometallic half-sandwich iridium anticancer complexes. The hydrophobicity and intercalative ability of C^{pxph} and C^{pxbiph} make a major contribution to the anticancer potency of their Ir^{III} complexes.

Replacement of a thiourea with an amidine group in a monofunctional platinum-acridine antitumor agent. Effect on DNA interactions, DNA adduct recognition and repair

A combination of biophysical, biochemical, and computational techniques was used to delineate mechanistic differences between the platinum-acridine hybrid agent $[PtCl(en)(L)](NO_3)_2$ (complex 1, en = ethane-1,2-diamine, L = 1-[2-(acridin-9-ylamino)ethyl]-1,3-dimethylthiourea) and a considerably more potent second-generation analogue containing L′ = *N*-[2-(acridin-9-ylamino)ethyl]-*N*-methylpropionamidine (complex 2). Calcula-tions at the density functional theory level provide a rationale for the binding preference of both complexes for guanine-N7 and the relatively high level of adenine adducts observed for compound 1. A significant rate enhancement is observed for binding of the amidine-based complex 2 with DNA compared with the thiourea-based prototype 1. Studies conducted with chemical probes and on the bending and unwinding of model duplex DNA suggest that adducts of complex 2 perturb B-form DNA more severely than complex 1, however, without denaturing the double strand and significantly less than cisplatin. Circular and linear dichroism spectroscopies and viscosity measurements suggest that subtle differences exist between the intercalation modes and adduct geometries of the two complexes. The adducts formed by complex 2 most efficiently inhibit transcription of the damaged DNA by RNA polymerase II. Not only do complexes 1 and 2 cause less distortion to DNA than cisplatin, they also do not compromise the thermodynamic stability of the modified duplex. This leads to a decreased or negligible affinity of HMG domain proteins for the adducts formed by either Pt-acridine complex. In a DNA repair synthesis

assay the lesions formed by complex 2 were repaired less efficiently than those formed by complex 1. These significant differences in DNA adduct formation, structure, and recognition between the two acridine complexes and cisplatin help to elucidate why compound 2 is highly active in cisplatin-resistant, repair proficient cancer cell lines.

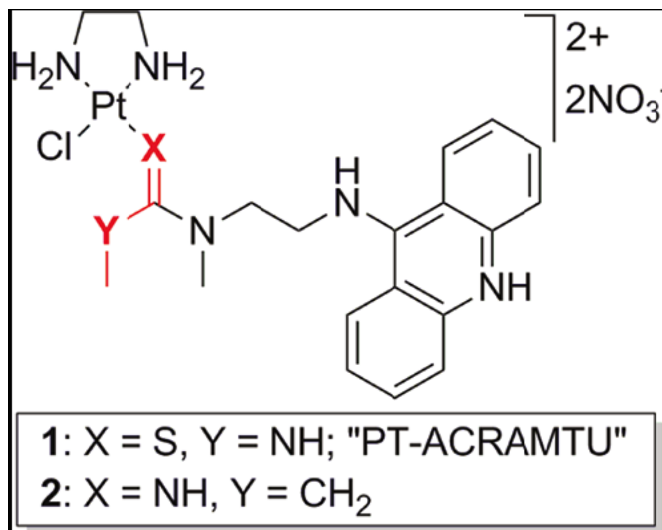

Figure 7: Structures of the platinum-acridine hybrid agents.

Differences in the cellular response and signaling pathways between cisplatin and monodentate organometallic Ru(II) antitumor complexes containing a terphenyl ligand

The new monofunctional Ru(II)-arene complex $[(\eta^6\text{-arene})Ru(II)(en)Cl]^+$, where en = 1,2-diaminoethane and the arene is para-terphenyl (complex 1) exhibits promising cytotoxic effects in human tumor cells including those resistant to conventional cisplatin (J. Med. Chem.2008, 51, 5310). The present study is focused on the cellular pharmacology of 1 to elucidate more deeply the mechanisms underlying its antitumor effects. We have identified several cellular mechanisms induced by 1 in human ovarian carcinoma cells, including inhibition of DNA synthesis, overexpression and activation of p53, expression of proapoptotic proteins $p21^{WAF1}$ and Bax, G_0/G_1 arrest, and nuclear fragmentation as a result of apoptotic, and, to a much lower

extent, also necrotic processes. Thus, 1 inhibits growth of the cancer cells through induction of apoptotic cell death and G_0/G_1 cell cycle arrest. Further investigations have shown that 1 induces apoptosis by regulating the expression of Bcl-2 family proteins. There were significant differences in cellular responses to the treatment with 1 and with conventional cisplatin, particularly in the kinetics and the extent of these responses. In addition, the distinct p53 activation profile of 1 compared with cisplatin provides an explanation for the activity of this ruthenium drug against cisplatin-resistant cells. Hence complex 1 may provide an alternative therapy in patients with acquired cisplatin resistance, particularly with respect to its very low mutagenicity and different mode of action compared to platinum antitumor drugs in clinical use.

Unique DNA binding mode of antitumor trinuclear tridentate platinum(II) compound

The new trinuclear tridentate Pt^{II} complex $[Pt_3Cl_3(hptab)]^{3+}$ (**1**; hptab = *N*,*N*,*N′*,*N′*,*N″*,*N″*-hexakis(2-pyridylmethyl)-1,3,5-tris(aminomethyl)benzene) exhibits pro cytotoxic effects in human and mouse tumor cells including those resistant to conventional cisplatin. The present study is focused on the molecular pharmacology of **1**, in particular on its interactions with DNA (which is the major pharmacological target of platinum antitumor drugs), to elucidate more deeply the mechanism underlying its antitumor effects. Results obtained with the aid of methods of molecular biophysics and pharmacology reveal new details of DNA modifications by **1**. Complex **1** binds to DNA forming in the absence of proteins and molecular crowding agents mainly trifunctional intrastrand cross-links. In these DNA adducts all three Pt^{II} centers of **1** are coordinated to DNA base residues, which leads to extensive conformational alterations in DNA. An intriguing aspect of the DNA-binding mode of this trinuclear Pt^{II} complex **1** is that it can cross-link proteins to DNA. Even more interestingly, **1** can cross-link in the presence of molecular crowding agent, which mimics environmental conditions in cell nucleus, two DNA duplexes in a high yield - a feature observed for the first time for antitumor trinuclear platinum complexes. Thus, the concept for the design of agents capable of forming intramolecular tridentate DNA adducts, DNA–protein and interduplex DNA–DNA cross-links based on trinuclear tridentate Pt^{II} complexes with semirigid aromatic linkers may result in new compounds which exhibit a variety of biological effects and can be also useful in nucleic acids research.

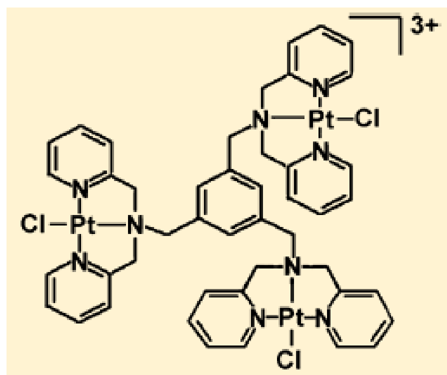

Figure 8: Structure of the new antitumor trinuclear tridentate platinum(II) complex.

Granted projects

Ministry of Education, Youth and Sports CR, ME, LC06030, Center of Basic Research, Biomolecular Center. Co-principal investigator: V. Brabec, 2006 - 2011

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, 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 301/09/H004, Molecular and structural biology of selected antitumor drugs. From mechanistic studies to chemotherapy of tumors. Principal investigator: V. Brabec, 2009 - 2012

GA CR P303/10/P047, Molecular and cellular pharmacology of novel class of antitumor iridium complexes. Implications for innovations in cancer chemotherapy. Principal investigator: A. Kisová, 2011 - 2013

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 - 2012

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

GA CR P205/11/0856, Thermodynamics of DNA damaged by physical and chemical agents. Principal investigator: J. Malina, 2011 - 2015

Publications

Zoldakova, M., Biersack, B., Kostrhunova, H., Ahmad, A., Padhye, S., Sarkar, F. H., Schobert, R., Brabec, V.: *(Carboxydiamine)Pt(II) complexes of a combretastatin A-4 analogous chalcone: the influence of the diamine ligand on DNA binding and anticancer effects.* Med. Chem. Commun., 2, 2011, 493-499.

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PhD. thesis defended in 2011

Mgr. Jana Štěpánková, PhD.: Mechanistic studies of biological effects of selected antitumor drugs

Mgr. Lenka Zerzánková, PhD.: Study of interactions of antitumor effective metal based complexes with biomacromolecules

Biophysical Chemistry and Molecular Oncology

Head
Miroslav Fojta

Group Leaders
Emil Paleček, František Jelen, Václav Brázda

Senior Scientists
Stanislav Hasoň, Luděk Havran, Veronika Ostatná, Hana Pivoňková, Vladimír Vetterl

Scientists
Marie Brázdová, Eva Brázdová-Jagelská, Hana Černocká, Lukáš Fojt

Postdocs
Pavel Kostečka, Mojmír Trefulka, Petra Horáková

Specialists
Alena Kouřilová, Ivana Mužíková, Lucie Navrátilová, Zdeněk Pechan

PhD. Students
Martin Bartošík, Monika Hermanová, Petr Orság, Peter Šebest, Vlastimil Tichý, Pavlína Vidláková, Helena Fridrichová, Medard Plucnara, Jan Špaček, Veronika Vargová, Zdenka Vychodilová

Undergraduate Students
Jan Coufal, Jana Čechová, Tomáš Komárek, Magda Vodičková, Veronika Lýčková, Matěj Adámek, Simona Svitáková, Ivana Kozáková, Robert Helma, Aneta Krejčí, Alena Polášková, Marek Petr

Technical Assistants
Yvonna Koudelková, Petra Mittnerová, Ludmila Římánková, Hana Vejvodová

External Co-Workers
Miloslava Fojtová, Raji Heyrovská, Petr Pečinka

Within the DCBMO, three 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.
The "**Molecular Oncology**" 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 of and defense against cancer. Activities of the group belong mainly under the field III.

Summary of the results:

In 2011 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 DNA-drug interactions and in molecular diagnostics

Square wave stripping voltammetry of unlabeled single- and double-stranded DNAs

We show that, in difference to previously applied electrochemical methods working with stationary electrodes, square wave voltammetry produces well-developed peaks IISW (specific for dsDNA) and IIISW yielded by ssDNA at hanging mercury drop electrode (HMDE) and solid amalgam electrodes (SAEs). Using these peaks various kinds of DNA structural transitions can be studied, including unwinding of dsDNA at negatively charged electrode surfaces. The sensitivity of the DNA analysis is much better than that obtained with guanine oxidation signals at carbon electrodes. Both carbon electrodes and SAEs appear attractive as transducers in label-free RNA and DNA sensors.

Ternary monolayers as DNA recognition interfaces for direct and sensitive electrochemical detection in untreated clinical samples

Detection of specific DNA sequences in clinical samples is a key goal of studies on DNA biosensors and gene chips. Herein we present a highly sensitive electrochemical genosensor for direct measurements of specific DNA sequences in undiluted and untreated human serum and urine samples. Such genosensing relies on a new ternary interface involving hexanedithiol (HDT) co-immobilized with the thiolated capture probe (SHCP) on gold surfaces, followed by the incorporation of 6-mercapto-1-hexanol (MCH) as diluent. The performance of ternary monolayers prepared with linear dithiols of different lengths was systematically examined, compared and characterized by cyclic voltammetry and electrochemical impedance spectroscopy, with HDT exhibiting the most favorable analytical performance. The new SHCP/HDT +MCH monolayer led to a 80-fold improvement in the signal-to-noise ratio (S/N) for 1 nM target DNA in undiluted human serum over the common SHCP+MCH binary alkanethiol interface, and allowed the direct quantification of the target DNA down to 7 pM (28 amol) and 17pM (68 amol) in undiluted/untreated serum and urine, respectively. It also displayed attractive antifouling properties, as indicated from the favorable S/N obtained after a prolonged exposure (24 h) to untreated biological matrices. These attractive features of the SHCP/HDT +MCH sensor interface indicate considerable promise for a wide range of clinical applications.

Alkylsulfanylphenyl derivatives of cytosine and 7-deazaadenine nucleosides, nucleotides and nucleoside triphosphates: synthesis, polymerase incorporation to DNA and electrochemical study

Aqueous Suzuki–Miyaura cross-coupling reactions of halogenated nucleosides, nucleotides and nukleoside triphosphates derived from 5-iodocytosine and 7-iodo-7-deazaadenine with methyl-, benzyl- and tritylsufanylphenylboronic acids gave the corresponding alkylsulfanyl-phenyl derivatives of nucleosides and nucleotides. The modified nucleoside triphosphates were incorporated into DNA by primer extension by using Vent (exo-) polymerase. The electrochemical behaviour of the alkylsulfanylphenyl nucleosides indicated formation of compact layers on the electrode. Modified nucleotides and DNA with incorporated benzyl- or tritylsulfanylphenyl moieties produced signals in $[Co(NH_3)_6]^{3+}$ ammonium buffer, attributed to the Brdicka catalytic response, depending on the negative potential applied. Repeated constant current chronopotentiometric scans in this medium showed increased Brdicka catalytic response, which suggests the deprotection of the alkylsulfanyl derivatives to free thiols under the conditions.

Tail-labelling of DNA probes using modified deoxynucleotide triphosphates and terminal deoxynucleotidyl tranferase. Application in electrochemical DNA hybridization and protein-DNA binding assays

A simple approach to DNA tail-labelling using terminal deoxynucleotidyl transferase and modified deoxynucleoside triphosphates is presented. Amino- and nitrophenyl-modified dNTPs were found to be good substrates for this enzyme giving 3ˇ-end stretches of different lengths depending on the nucleotide and concentration. 3-Nitrophenyl-7-deazaG was selected as the most useful label because its dNTP was efficiently incorporated by the transferase to form long tail-labels at any oligonucleotide. Accumulation of many nitrophenyl tags per oligonucleotide resulted in a considerable enhancement of voltammetric signals due to the nitro group reduction, thus improving the sensitivity of electrochemical detection of the tail-labelled probes. We demonstrate a perfect discrimination between complementary and non-complementary target DNAs sequences by tail-labelled hybridization probes as well as the ability of tumour suppressor p53 protein to recognize a specific binding site within tail-labelled DNA substrates, making the methodology useful in electrochemical DNA hybridization and DNA-protein interaction assays.

Redox labels and indicators based on transition metals and organic electroactive moieties for electrochemical nucleic acids sensing

Natural nucleobases are electrochemically active, producing analytically useful oxidation and reduction signals (usually measured at carbon or mercury-based electrodes, respectively). Electroactive indicators and labels are used in electrochemical nucleic acids sensing to improve selectivity and sensitivity of the biosensors and bioassays and to expand the palette of applicable electrode materials. Electrochemical activity of these species is conferred by either redox-active transition metals, or organic electroactive moieties. The noncovalently interacting indicators interact with DNA in a structure-selective manner, usually preferring double-stranded DNA, *via* groove binding or intercalation between base pairs within the DNA double helix. These indicators are used mostly in DNA hybridization sensors due to their ability to discriminate between single-stranded probes and probe•target hybrid duplexes. A variety of electroactive moieties has been attached to nucleic acids covalently to create labeled DNA targets or signaling probes. Covalent DNA labeling offers a clear discrimination between complementary strands forming the DNA double helix, which further improves selectivity of the hybridization assays. Moreover, redox coding of particular nucleobases can be utilized in electrochemical DNA sequencing and single nucleotide polymorphism typing. Besides classical synthesis of modified nucleic acids *via* solid phase phosphoramidite methodology, more facile and versatile approaches have been introduced, based on either chemical modification of natural DNA components (such as thymine bases with osmium tetroxide complexes), or enzymatic incorporation of modified nucleotides using deoxynucleotide triphosphate conjugates bearing electroactive labels. Examples of electroanalytical applications of labeled nucleic acids are discussed.

Sensing mispaired thymines in DNA heteroduplexes using an electro-active osmium marker: towards electrochemical SNP probing

A complex OsO_4, 2,2′-bipyridine (Os,bipy), has been used for electroactive labeling of biopolymers as well as for probing of nucleic acids and protein structure and interactions. In DNA, Os,bipy forms electrochemically active adducts with pyrimidine nucleobases, exhibiting highly selective modification of thymine residues in single-stranded DNA. Here, we show that modification of rare thymine residues (one thymine among several tens of unreactive purine bases) can easily be detected by means of a simple ex

situ voltammetric analysis using carbon electrodes. Based on this remarkable sensitivity of detection, Os,bipy has been used as an electroactive probe for unpaired and/or mismatched thymine residues within DNA heteroduplexes. Site-specific chemical modification of the DNA with the Os,bipy has allowed a clear distinction between perfectly base-paired DNA homoduplexes and mismatched heteroduplexes, as well as discrimination among heteroduplexes containing one or two mispaired thymines, a single thymine insertion, or combination of a mispair and an insertion.

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.

Oxidation of sanguinarine and its dihydro-derivative at a pyrolytic graphite electrode using ex situ voltammetry. Study of the interactions of the alkaloids with DNA

This study describes the oxidation of sanguinarine (SG) and its metabolite dihydrosanguinarine (DHSG) on the surface of a basal-plane pyrolytic graphite electrode (PGE). Since both alkaloids strongly adsorb onto the

surface of pyrolytic graphite, measurements were performed using ex situ voltammetric methods, adsorptive transfer (AdT) cyclic voltammetry (CV) and square-wave voltammetry (SWV). Oxidation peaks of SG (peak A) and DHSG (peak A*) were observed around the potential of +0.7 V (vs. Ag/AgCl/3 M KCl), depending on the experimental conditions. The voltammetric peaks A and A* are probably related to the oxidation of N-methylphenanthridine nitrogenous heterocycle of SG and oxidation of DHSG back to SG, respectively. The electrochemical results and optimized AdT SWV were subsequently applied to the study of the interactions of SG and DHSG with DNA *in vitro*. Analysis of the alkaloid/DNA interactions was based on observing heights of oxidation peaks A and A* after incubation of SG and/or DHSG with supercoiled (sc) DNA. Electrochemical study of the interactions was supported and complemented with measurements using gel electrophoresis (Topoisomerase I scDNA relaxation assay) and steady-state and time-resolved fluorescence spectroscopy. The results suggest that SG intercalates into the doublestranded structure of scDNA (the SG/base pair ratio is max. 1/4) while increased binding affinity was observed for quaternary cation (SG+). DHSG which, unlike SG+, does not possess a strictly planar molecular structure, did not show intercalative DNA binding in any of the three methods applied.

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

Directional sensing of protein adsorption on titanium with a light-induced periodic structure

Directional dependence of adsorbed human plasma fibrinogen (HPF) and human serum albumin (HSA) on highly ordered pyrolytic graphite (HOPG), on polished titanium (Tip), and on titanium with a light-induced periodic surface structure (TiLIPSS) was investigated. Analyses, in the temporal domain, were conducted with a diffractive optical element (DOE)-based sensor that senses both topography (optical roughness) and reflectance related (gloss) parameters of the biomolecule-covered surface in phosphate buffered saline. Optical analysis was conducted from data measured with DOE *in vitro*. During the measurements, the probing laser beam had three different angular directions of the electric field of photons to inspect the directional responses from the studied surface types. First, results showed

that the HOPG was found to be statistically inert for the measured biomolecule HPF adsorption, but with HSA, it was found to be adsorbed onto the HOPG surface. The Tip and TiLIPSS showed a more reactive adsorption process in comparison to the HOPG. Second, the directional dependence from the electric field angle of the probe beam had a clear effect only with the measured TiLIPSS surface, which was produced for this investigation. Third, the existence of trapped gas nanobubbles was observed on the HOPG and on the Tip sample surfaces with the HPF molecule. Fourth, the HPF and the HSA molecules showed different DOE responses on all studied surfaces, which was interpreted to be caused by the structural differences of these two proteins. Finally, ellipsometric measurements were performed to confirm the analysis of the DOE gloss signals.

On the mechanism of hydrogen evolution catalysis by proteins: A case study with bovine serum albumin

The catalysis of the hydrogen evolution reaction (HER) by proteins has been known for decades but was only recently found to be useful for electroanalytical purposes. The mechanism of the catalytic process is investigated at hanging mercury drop electrodes by cyclic voltammetry, with bovine serum albumin as a model system. It is shown that the catalyst is the protein in the adsorbed state. The influence of various parameters such as the accumulation time, scan rate or buffer concentration is studied, and interpreted in the framework of a surface catalytic mechanism. Under the experimental conditions used in the work, a “total catalysis” phenomenon takes place, the rate of HER being limited by the diffusion of the proton donor. The adequacy of the existing models is discussed, leading to a call for the development of more refined models.

Electrocatalytic monitoring of metal binding and mutation-induced conformational changes in p53 at picomole level

We developed an innovative electrochemical method for monitoring conformational transitions in proteins using constant current chronopotentiometric stripping (CPS) with dithiothreitol-modified mercury electrodes. The method was applied to study the effect of oncogenic mutations on the DNA binding domain of the tumor suppressor p53. The CPS responses of wild-type and mutant p53 showed excellent correlation with structural and stability data and provided additional insights into the

differential dynamic behavior of the proteins. Further, we were able to monitor the loss of an essential zinc ion resulting from mutation (R175H) or metal chelation. We envisage that our CPS method can be applied to the analysis of virtually any protein as a sensor for conformational transitions or ligand binding to complement conventional techniques, but with the added benefit that only relatively small amounts of protein are needed and instant results are obtained. This work may lay the foundation for the wide application of electrochemistry in protein science, including proteomics and biomedicine.

Electrocatalytic detection of polysaccharides at picomolar concentrations

Electroinactive polysaccharides (PS) modified by osmium(VI) complexes with nitrogenous ligands produce redox couples at carbon and mercury electrodes. We show that PS adducts with Os(VI) 2,20- bipyridine produce at -1.2 V (against Ag|AgCl|3 M KCl electrode) an additional peak at mercury and solid amalgam electrodes. This peak is due to the catalytic hydrogen evolution, allowing detection of PS (such as dextran and mannan) at picomolar concentrations.

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

Interferon-inducible protein 16: insight into the interaction with tumor suppressor p53

IFI16 is a member of the interferon-inducible HIN-200 family of nuclear proteins. It has been implicated in transcriptional regulation by modulating protein-protein interactions with p53 tumor suppressor protein and other transcription factors. However, the mechanisms of interaction remain unknown. Here, we report the crystal structures of both HIN-A and HIN-B domains of IFI16 determined at 2.0 and 2.35 A° resolution, respectively. Each HIN domain comprises a pair of tightly packed OB-fold subdomains that appear to act as a single unit. We show that both HIN domains of IFI16 are capable of enhancing p53-DNA complex formation and transcriptional activation via distinctive means. HIN-A domain binds to the basic C terminus of p53, whereas the HIN-B domain binds to the core DNAbinding

region of p53. Both interactions are compatible with the DNA-bound state of p53 and together contribute to the effect of full-length IFI16 on p53-DNA complex formation and transcriptional activation.

Cruciform structures are a common DNA feature important for regulating biological processes

DNA cruciforms play an important role in the regulation of natural processes involving DNA. These structures are formed by inverted repeats, and their stability is enhanced by DNA supercoiling. Cruciform structures are fundamentally important for a wide range of biological processes, including replication, regulation of gene expression, nucleosome structure and recombination. They also have been implicated in the evolution and development of diseases including cancer, Werner's syndrome and others. Cruciform structures are targets for many architectural and regulatory proteins, such as histones H1 and H5, topoisomerase IIb, HMG proteins, HU, p53, the proto-oncogene protein DEK and others. A number of DNA-binding proteins, such as the HMGB-box family members, Rad54, BRCA1 protein, as well as PARP-1 polymerase, possess weak sequence specific DNA binding yet bind preferentially to cruciform structures. Some of these proteins are, in fact, capable of inducing the formation of cruciform structures upon DNA binding. In this article, we review the protein families that are involved in interacting with and regulating cruciform structures, including (a) the junctionresolving enzymes, (b) DNA repair proteins and transcription factors, (c) proteins involved in replication and (d) chromatin-associated proteins. The prevalence of cruciform structures and their roles in protein interactions, epigenetic regulation and the maintenance of cell homeostasis are also discussed.

A dynamic programming algorithm for identification of triplex-forming sequences

Motivation: Current methods for identification of potential triplexforming sequences in genomes and similar sequence sets rely primarily on detecting homopurine and homopyrimidine tracts. Procedures capable of detecting sequences supporting imperfect, but structurally feasible intramolecular triplex structures are needed for better sequence analysis. Results: We modified an algorithm for detection of approximate palindromes, so as to account for the special nature of triplex DNA structures. From available

literature, we conclude that approximate triplexes tolerate two classes of errors. One, analogical to mismatches in duplex DNA, involves nucleotides in triplets that do not readily form Hoogsteen bonds. The other class involves geometrically incompatible neighboring triplets hindering proper alignment of strands for optimal hydrogen bonding and stacking. We tested the statistical properties of the algorithm, as well as its correctness when confronted with known triplex sequences. The proposed algorithm satisfactorily detects sequences with intramolecular triplex-forming potential. Its complexity is directly comparable to palindrome searching.

Granted projects

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

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 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 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 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 - 2011

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

GA CR P206/11/1638, Novel electrochemical sensors and sensing techniques for the analysis of nucleic acids structure and interactions. Principal investigator: M. Fojta, 2011 - 2013

GA CR P301/11/2076, DNA binding, stress-induced expression and transactivation activity of p73 protein isoforms. Principal investigator: H. Pivoňková, 2011 - 2013

GA CR P206/11/P739, Sequence-specific electrochemical sensing of PCR-amplified genomic DNA fragments. Principal investigator: P. Horáková, 2011 - 2013

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Molecular Epigenetics

Head
Aleš Kovařík

Senior Scientist
Roman Matyášek

Scientists
Jaroslav Fulneček, Zuzana Tesaříková

Postdoc
Kateřina Křížová

PhD. Students
Hana Malínská-Šrubařová, Lucie Crhák-Khaitová

Undergraduate Students
Květa Dofková, Eva Dobešová

Bc Students
Eva Kabáthová, Ivana Ježková

Technical Assistant
Jana Kaiserlichová

Paramutation of tobacco transgenes by small RNA-mediated transcriptional gene silencing (Kateřina Křížová)

Paramutation is the transfer of epigenetic information between homologous loci (allelic or non-allelic) that leads to a heritable change in expression of one of these loci. This process involves RNA signals and epigenetic modification of DNA and chromatin. Signals that trigger paramutation remain poorly understood. The repeats apparently trigger paramutation in certain endogenous loci of maize while in other systems, they do not seem to play a role in silencing. There are also conflicting reports as to whether transgene expression is influenced by specific structural DNA features such as matrix atttachment regions. To study transgeneration inheritance of RNA-triggered silencing and its paramutation effects in tobacco we

elaborated a hybrid system comprising a transcriptional silencer locus and a homologous target locus. The transcriptional silencer locus (271) induces silencing of the 35S promoter-linked genes. The two target loci contain neomycin phosphotransferase transgene driven by the same 35S promoter (35S:nptII) but at different positions and in a different epigenetic state. We addressed the questions whether 271 in trans silencing of both target genes did occur, how efficient, and when established, whether in trans TGS and promoter methylation were stable upon 271 segregation. Three generations of exposure to RNA signals from the 271 locus were required to complete silencing and methylation of the 35S promoter within locus 2. Segregating methylated locus 2 epialleles were obtained only from the third generation of hybrids, and this methylation was not correlated with silencing. Strikingly, only one generation was required for the PTGS locus 1 to acquire complete TGS and 35S promoter methylation. In this case, paramutated locus 1 epialleles bearing methylated and inactive 35S promoters segregated already from the first generation of hybrids. The results support the hypothesis that PTGS loci containing a palindrome structure and methylation in the coding region are more sensitive to paramutation by small RNAs and exhibit a strong tendency to formation of meiotically transmissible TGS epialleles. These features contrast with a non-methylated single copy transgenic locus that required several generations of contact with RNA silencing molecules to become imprinted in a stable epiallele.

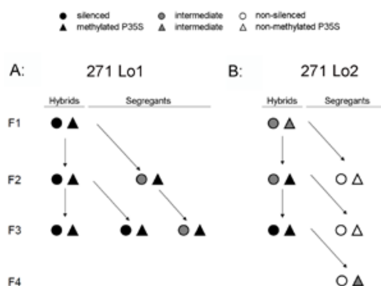

Figure 1: Schematic representation of the inheritance of silencing and methylation of tobacco transgene loci. Epigenetic patterns of targets in line 271/1 (A), 271/2 (B) and derived segregants. Symbols: circle – expression status of target (filled – fully silenced, empty - non-silenced and grey - intermediate); triangle – P35S methylation (filled – >40% Cs methylated, empty – non-methylated, grey – <40% methylation).

Analysis of two abundant, highly related satellites in the allotetraploid Nicotiana arentsii using double-strand conformation polymorphism analysis and sequencing (Roman Matyášek)

A significant evolutionary role has been assigned to allopolyploidy in the formation of flowering plant species. All plant genomes sequenced to date have signatures of whole-genome duplication(s) in their evolutionary history. The success of newly formed allopolyploids is partly attributable to their highly plastic genome and mating systems. Satellite DNA is a nearly universal component of eukaryotic genomes, and it consists of numerous tandem repeats that are arranged head to tail. These repeats are non-coding, late-replicating in the S-phase and mostly located in the constitutive, non-transcribed heterochromatin at centromeric and subtelomeric locations. Satellites often show enormous variability in nucleotide sequence and copy number, even among closely related species. Therefore, they are frequently utilised for taxonomic and phylogenetic studies and to follow hybridisation events between related species. In this work, we examined the fate of homologous subtelomeric satellites in intrasection allotetraploid Nicotiana arentsii formed from *N. undulata* (mother) and *N. wigandioides* (father) progenitors less than 200 thousand years ago. We cloned and sequenced a number of monomers from progenitors and the allotetraploid. Structural features of both cloned and genomic monomers were studied using double-stranded conformation analysis. Two homologous satellites were isolated from N. undulata (called NUNSSP) and N. wigandioides (NWISSP). While the NUNSSP monomers were highly homogeneous the NWISSP monomers formed two separate clades. Likewise, the genomic NUNSSP monomers showed less DNA conformation heterogeneity than NWISSP monomers, with distinct conformations. While both satellites predominantly occupy subtelomeric positions, a fraction of the NWISPP repeats was found in an intercalary location, supporting the hypothesis that dispersion prevents the repeats becoming homogeneous. Sequence, structural and chromosomal features of the parental satellites were faithfully inherited by N. arentsii, indicating the absence of repeat homogenisation after the allotetraploidisation event. Our study revealed that inter-genomic homogenisation of subtelomeric satellite repeats does not occur in *N. arentsii* allotetraploid. We propose that the sequence and structural divergence of subtelomeric satellites may render allopolyploid chromosomes less vulnerable to intergenomic exchanges.

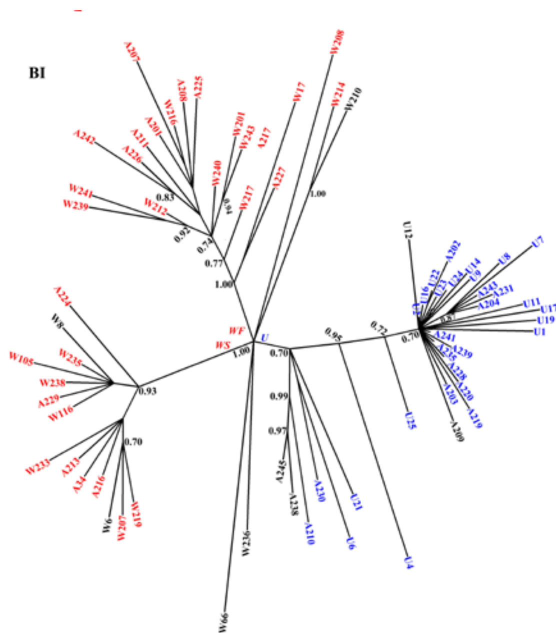

Figure 2: Phylogenetic relationships between the satellite monomers. Tree was constructed from aligned sequences using a BI algorithm. The sequences from *N. undulata* (symbol U followed by the number of the clone), *N. wigandioides* (W) and *N. arentsii* (A) are labelled in red, blue and green node marks, respectively. The three main clades U, WS and WF are visualised in both trees. Note: while sequences from the diploid parents *N. undulata* and *N. wigandioides* were well separated into U and WS+WF clades, respectively, sequences from the *N. arentsii* allotetraploid intermingled with sequences from both diploids and occurred in all major clades.

Epigenetic silencing precedes rDNA loci rearrangements during the stabilization of a polyploid species Brassica napus (Aleš Kovařík)

Epigenetic silencing of parental genes is an important feature of newly established allopolyploids believed to reconcile regulatory incompatibilities between parental subgenomes. Epigenetic changes at the rDNA loci following interspecific hybridization have been initially discovered by a Russian cytogenetict, Mikhail Navashin, almost 80 years ago. His careful cytological observations revealed inheritance of some chromosomal secondary constrictions in parental but not hybrid species. *Brassica napus* (oilseed rape, AACC, 2n=38) genome formed recently less than thousand years ago by interspecific hybridization of diploid progenitors close to

modern *B. rapa* (AA, 2n=2x=20) and *B. oleracea* (CC, 2n=2x=18). To determine genetic and epigenetic dynamics of rDNA loci in *Brassica napus* allotetraploid, we resynthesized *B. napus* from progenitor species, *B. oleracea* ”HDEM“ and *B. rapa* “Z1“. Two S0 synthetic lines were obtained through colchicine doubling or female unreduced gametes resulting in four lineages (S1, S2, S3). We carried out rDNA-FISH analysis with the 35S and 5S probes combined with BAC clone from *B. oleracea* library, named BoB014O06 which hybridizes specifically to all C-genome like chromosomes and allows visualization in *B. napus* C-genome for each plant. Starting already from F1, there was a strong uniparental silencing of *B. oleracea* genes in all tissues, and this was correlated with hyper-methylation of polymerase I promoters and increased condensation of the C-genome rDNA chromatin. After the first meiosis, and in subsequent generations (S1-S3), variation in 35S rDNA (12-16) and 5S rDNA (7-10) loci was observed. Both A genome and C genome loci were affected by rearrangements and in some lines recombinant chromosomes were observed. Collectively, some epigenetic and genetic modifications in synthetic lines resemble events that accompanied formation of natural species.

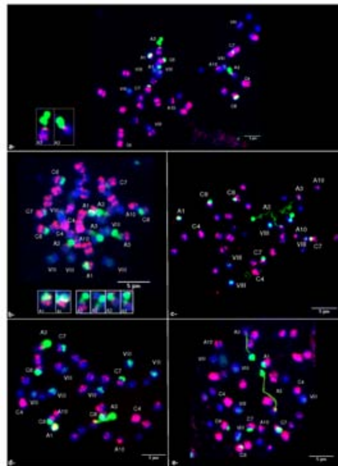

Figure 3: FISH analyses of somatic metaphase chromosomes of synthetic allotetraploids obtained from unreduced gametes (UG). The S0 generation individual is in (a); b, d – S1 generation of line 1 and 2, respectively; c, e - S3 generation of line 1 and 2, respectively. Detail in (a) shows recombinant (left) and normal (right) A3 chromosome. Details of A3 quadrivalents are in (b). Chromosomes counterstained with DAPI (blue) were hybridized simultaneously with the 35S, 5S and BoB014O06 probes. Bar represents 5 µm.

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

GA CR P501/11/P667, Reprogramming of epigenetic state of transgenic and endogenic loci during dedifferentiation and plant regeneration. Principal investigator: K. Křížová, 2011 - 2013

KONTAKT/MŠMT/Barrande, MEB-021114, Structure et expression des gènes ribosomiques chez une espèce allopolyploïde envahissante récemment formée en Europe: Spartina anglica. Principal investigator: A. Kovařík and M. Ainouche, 2011 - 2012

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PhD. thesis defended in 2011

Lucie Crhák-Khaitová, PhD., Characterization and expression of ribosomal RNA genes in allopolyploid roses (Caninae)

LABORATORY OF PLANT MOLECULAR BIOLOGY

(COMMON LABORATORY OF INSTITUTE OF BIOPHYSICS OF THE AS CR, V.V.I. AND FACULTY OF AGRONOMY, MENDEL UNIVERSITY IN BRNO)

LABORATORY LEADER

BŘETISLAV BRZOBOHATÝ

CO-WORKERS

NAGAVALLI SUBBANNA KIRAN, ŠÁRKA KOUKALOVÁ, PAVEL MAZURA, JAN NEJEDLÍK, PŘEMYSL SOUČEK

PHD. STUDENTS

JANA BALDRIANOVÁ, MARTIN ČERNÝ, TOMÁŠ FILIPI, MARTINA MAREČKOVÁ, JAN NOVÁK, JAROSLAV PAVLŮ, ALENA REKOVÁ

TECHNICIAN

IVETA VAŠÍNOVÁ

Engineering the cytokinin-glucoside specificity of the maize beta-D-glucosidase Zm-p60.1 using site-directed random mutagenesis

The maize beta-D-glucosidase Zm-p60.1 releases active cytokinins from their storage/transport forms, and its over-expression in tobacco disrupts zeatin metabolism. The role of the active-site microenvironment in fine-tuning Zm-p60.1 substrate specificity has been explored, particularly in the W373K mutant, using site-directed random mutagenesis to investigate the influence of amino acid changes around the 373 position. Two triple (P372T/W373K/M376L and P372S/W373K/M376L) and three double mutants (P372T/W373K, P372S/W373K and W373K/M376L) were prepared.

Their catalytic parameters with two artificial substrates show tight interdependence between substrate catalysis and protein structure. P372T/W373K/M376L exhibited the most significant effect on natural substrate specificity: the ratio of hydrolysis of cis-zeatin-O-beta-D-glucopyranoside versus the trans-zeatin-O-beta-D-glucopyranoside shifted from 1.3 in wild-type to 9.4 in favor of the cis- isomer. The P372T and M376L mutations in P372T/W373K/M376L also significantly restored the hydrolytic velocity of the W373K mutant, up to 60% of wild-type velocity with cis-zeatin-O-beta-D-glucopyranoside.

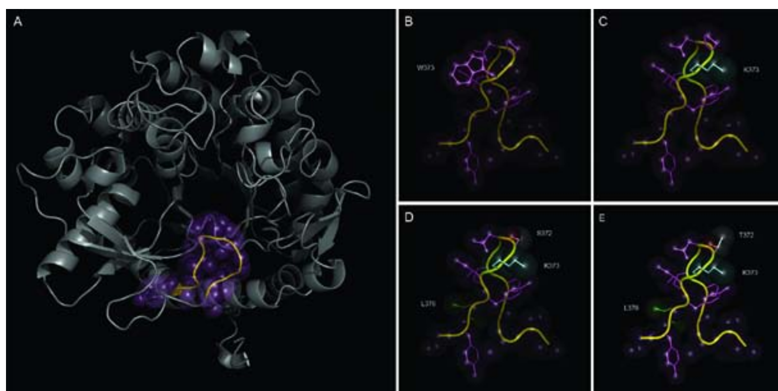

Figure 1: Tertiary structure of beta-D-glucosidase Zm-p60.1 wild-type and derived mutant forms. (A) Tertiary structure of Zm-p60.1; wild-type with colored loop III. (B) Detailed representation of loop III of wild-type Zm-p60.1. (C) Amino-acid alteration W373K. (D) Amino-acid alteration P372S/W373K/M376L. (E) Amino-acid alteration P372T/W373K/M376L. Amino-acid structures at mutated positions were inferred using PyMOL 1.1 (Molecular Graphic System; Schrödinger, LLC).

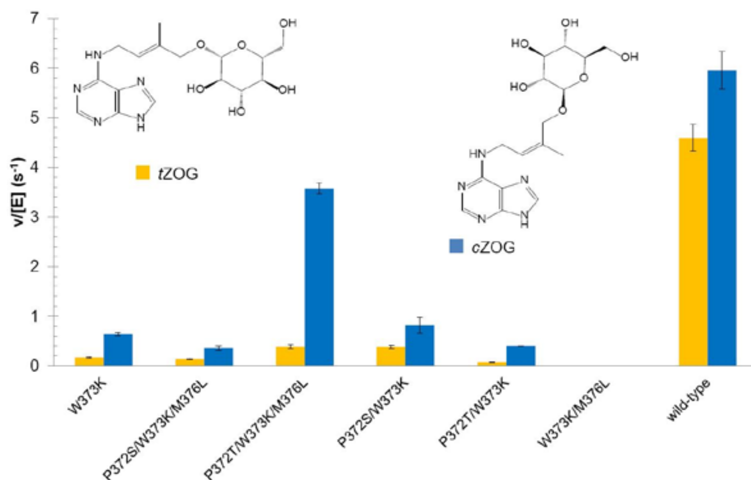

Figure 2: Hydrolysis of natural substrates [*trans*-zeatin-*O*-beta-D-glucopyranoside (*t*ZOG) and *cis*-zeatin-beta-D-glucopyranoside (*c*ZOG)] by wild-type and engineered Zm-p60.1 beta-D-glucosidases.

These findings reveal complex relationships among amino acid residues that modulate substrate specificity and show the utility of site-directed random mutagenesis for changing and/or fine-tuning enzymes. Preferential cleavage of specific isomer-conjugates and the capacity to manipulate such preferences will allow the development of powerful tools for detailed probing and fine-tuning of cytokinin metabolism in planta.

Focused directed evolution: theoretical versus real effectiveness of a minimal setup and simple robust screening. We have developed an optimized procedure to generate amino acid variations at specific site(s) of proteins, followed by a simple one-step screen for mutants with the desired beta-glucosidase activity. The procedure was evaluated by introducing sequence variation into a codon specifying a non-functional variant of the catalytic nucleophile (E401) of the maize beta-glucosidase Zm-p60.1.

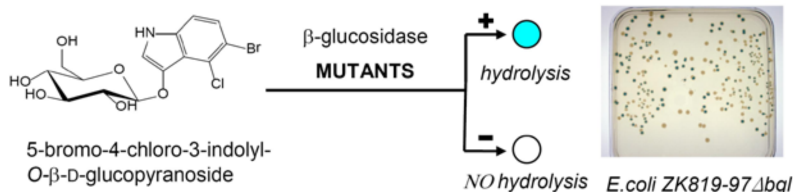

Figure 3: The simple screening system for visualization of beta-glucosidase activity in *E. coli*, based on cleavage of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-beta-D-glucopyranoside (X-Glc).

Observed and theoretically expected frequencies of the four possible variants of the codon and the two possible phenotypes (functional and non-functional) were investigated. Deviations in codon and phenotype frequencies were expressed as a coefficient. This coefficient was then used to estimate the extent of oversampling, of the mutant library, which would be necessary to compensate for the underrepresentation of some sequences. This evaluation of the overall performance of the method allows experimentally derived parameters to be incorporated into mutant library design. This method combines the application of a well-defined distribution of variability with a reliable screening process. Thus, it facilitates the production of novel functional variants of beta-glucosidases for either fundamental studies or potential biotechnological applications.

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Filipi, T., Mazura, P., Janda, L., Kiran, N.S., Brzobohatý, B.: *Engineering the cytokinin-glucoside specificity of the maize β-D-glucosidase Zm-p60.1 using site-directed random mutagenesis.* Phytochemistry, 74, 2012, 40-48.

Černý, M., Skalák, J., Kurková, B., Babuliaková, E., Brzobohatý, B.: *Using a commercial method for Rubisco immunodepletion in analysis of plant proteome.* Chemické Listy, 105 (8), 2011, 640-642.

Mazura, P., Filipi, T., Souček, P., Brzobohatý, B.: *Focused directed evolution: theoretical versus real effectiveness of a minimal setup and simple robust screening*. Carbohydrate Research, 346, 2011, 238-242.

PhD. thesis defended in 2011

Mgr. Martin Černý, PhD., Identification and characterization of proteins regulated by the plant hormone cytokinin

Molecular Cytology and Cytometry

Head
Stanislav Kozubek

Group of Structure and Function of the Cell Nucleus

Group Leader
Eva Bártová

Senior Scientist
Pavel Matula

Scientists
Gabriela Šustáčková, Lenka Stixová, Veronika Foltánková

PhD. Students
Soňa Legartová, Darya Orlova

Diploma Students
Alžběta Jugová, Petra Sehnalová, Jana Suchánková

Bc. Students
Terezia Přikrylová, Michal Franěk

Technical Assistant
Jana Poláková

Polycomb group (PcG) proteins

Polycomb group (PcG) proteins are negative regulators of transcription. They play important role during development, X-chromosome inactivation, and control of cell proliferation (Jacobs and van Lohuizen, 2002). Studies in *D. melanogaster* have revealed that PcG proteins are required for transcriptional repression of HOX genes during development (Ringrose and Paro, 2004). Recent observations have pointed towards an important role for PcG in the mammalian genome and increased expression of PcG proteins correlates with the malignancy and invasiveness of cancer cells (Jacobs and van Lohuizen, 2002; Sauvageau and Sauvageau, 2008).

PcG proteins are compartmentalized within the interphase nucleus into several distinct foci (Buchenau et al., 1998; Saurin et al., 1998) considered to be repression domains of gene transcription. In D. melanogaster, PcG proteins are organized into two main repression complexes, including Polycomb repressive complex 2 (PRC2), consisting of Enhancer of zeste (E(z)), Suppressor zeste 12 Su(z)12, Extra sex combs (Esc) proteins, and nucleosome remodeling factor Nurf55 (Czermin et al., 2002; Müller et al., 2002; Kuzmichev et al., 2004). The PRC1 complex consist of many proteins, including RING1 or BMI1 (Lund and Lohuizen, 2004; Ringrose and Paro, 2004). Specific protein of PRC2 complex is characterized by histone lysine methyltransferase (HMT) activity, directed towards methylation on histone H3 at lysine 27 (H3K27me3) (Kuzmichev et al., 2004). Moreover, Chou et al. (2010) showed PARP-dependent function of PcG proteins during DNA repair processes.

Therefore, here we addressed additional question whether acetylation events can influence the nuclear arrangement and function of the PcG-related BMI1 protein in UV-induced DNA lesions. Results were published by Šustáčková et al. (2012). In these experiments we used time-lapse confocal microscopy, micro-irradiation by UV laser (355 nm) and GFP technology to study the dynamics and function of the BMI1 protein in DNA lesions. We observed that BMI1 was recruited to UV-damaged chromatin simultaneously with decreased lysine pan-acetylation, followed by the recruitment of heterochromatin protein HP1β to micro-irradiated chromatin. Pronounced recruitment of BMI1 was rapid, contrary to slower recruitment of HP1β. Moreover, histone hyperacetylation, stimulated by HDAC inhibitors TSA or SAHA, suppression of transcription by actinomycin D, and ATP-depletion prevented increased accumulation of BMI1 to γH2AX-positive irradiated chromatin. Taken together, our data indicate that the dynamic recruitment of heterochromatin-related proteins to UV-damaged genomic regions can be influenced by acetylation events.

DNA damage responses in mouse embryonic stem cells

Our subsequent experiments were dedicated towards DNA damage responses (DDRs) in mouse embryonic stem cells (mESCs) that according to the literature display different sensitivities to DNA damage stimuli. For example, mouse ESCs (mESCs) are more sensitive to treatment with UV or γ-ray irradiation than differentiated mouse embryonic fibroblasts MEFs (de Waard et al., 2008). These differences can be ascribed to the more open chromatin configuration in ESCs (de Waard et al., 2008; Giglia-Mari et al.,

2010). Such a high sensitivity of ESCs to genotoxic injury increases the probability of non-repaired DNA lesions, which might lead to genome instability and subsequent severe malformation in developing organisms (de Waard et al., 2008).

Here, we aimed at potential role of Oct4 pluripotency-related gene in DNA lesions (Bártová et al., PLOS One, 2011). It is well known that proper Oct4 transcription is required for the formation of the inner cell mass of blastocysts, while down-regulation of Oct4 is associated with ESC differentiation (Niwa et al., 2000). However, nobody addressed if OCT4 protein participates in DDRs. For such experiments, we used mESCs, stably expressing GFP-OCT4 and we found that OCT4 accumulates in UV-damaged regions immediately after local micro-irradiation in an adenosine triphosphate-dependent manner (Fig. 1). Intriguingly, this event was not accompanied by pronounced NANOG and c-MYC recruitment to the UV-damaged sites. The accumulation of OCT4 to UV-damaged chromatin occurred simultaneously with H3K9 deacetylation and pronounced H2AX phosphorylation (γH2AX) (Fig. 2). Moreover, we observed an ESC-specific nuclear distribution of γH2AX after interference to cellular processes, including histone acetylation, transcription, and cell metabolism. Inhibition of histone deacetylases mostly prevented pronounced OCT4 accumulation at UV-irradiated chromatin. Taken together, our studies demonstrate pluripotency-specific events that accompany DNA damage responses. In this work, we also discuss how ESCs might respond to DNA damage stimuli, including genotoxic injury that might lead to unwanted genomic instability.

Figure 1: Recruitment of OCT4 to UV-damaged chromatin in GOWT1 mESCs. GFP-OCT4 (green) was recruited to UV-damaged chromatin 15-20 s after local irradiation by UV. Irradiated regions (pink frames) were characterized in by increased OCT4 levels (green) and γH2AX positivity appeared (red). It was observed in the cells sensitized by BrdU.

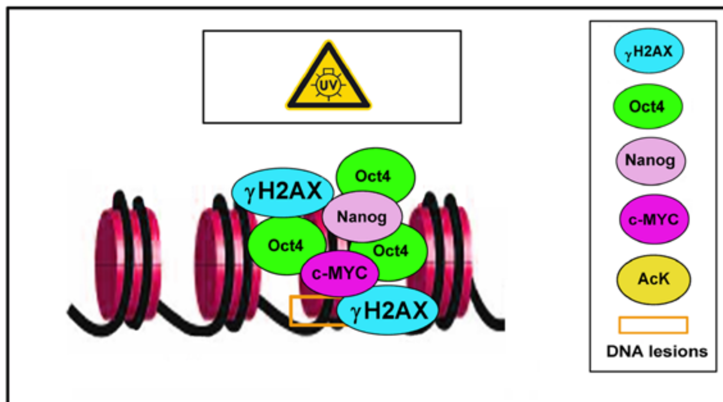

Figure 2: Model of OCT4 accumulation at UV-damaged chromatin. OCT4 was significantly accumulated at chromatin with laser-induced DNA lesions. In these regions, the levels of NANOG and c-MYC were not changed in comparison with entire genome. This event was accompanied by pronounced γH2AX accumulation at DNA lesions.

Group of the Structure, Function and Dynamics of Chromatin

Group Leader
Martin Falk

Senior Scientist
Emilie Lukášová

Scientist
Lenka Štefančíková

Specialist
Alena Bačíková

Higher-order chromatin structure in DNA damage and repair and formation of chromosomal translocations

It was found during the last few years that studies on transcription, replication and DNA repair, focused only on the „biochemical aspects“ of these processes cannot completely describe their mechanisms. Consequently, it was demonstrated that non-random higher-order chromatin structure and its dynamic changes (further referred only as „chromatin structure“) may play an important role. Nowadays, it is obvious that chromatin structure at all its levels of organization represents an exceptionally important regulatory factor, the deregulation of which can initiate (not only) malignant diseases. From the opposite point of view, directed modifications of chromatin structure may sufficiently improve effectiveness of tumor therapy.

This year, we have continued with the topic by studying the influence of chromatin structure on complex cellular response to DNA double-strand breaks (DSBs), the most harmful DNA lesion type. We have focused on the relationship between chromatin structure, its sensitivity to DSB damage, mechanism of DSB repair and formation of chromosomal translocations. In the context of chromatin structure, we have studied also other aspects of cellular response to DSB damage, e.g. cell cycle arrest, apoptosis, cellular senescence or adaptation to DSB damage.

Especially, we have studied in living cells the changes of chromatin structure during DSB repair in euchromatin and heterochromatin. The packaging of chromatin in the nucleus is variable, with two “extremes” represented by an open, gene rich, transcriptionally active euchromatin (EC) and compact, largely inactive heterochromatin (HC). While open chromatin efficiently responds to DNA damage, condensed HC domains require a unique signaling pathway and additional repair factors mediating chromatin relaxation before the DSB processing. This pathway requires ATM-dependent phosphorylation of KAP1 (heterochromatin building factor). In addition, cells can dynamically regulate chromatin architecture by combining of ATM-independent chromatin remodeling with histone modifications. However the exact coordination of heterochromatic DSBs repair with chromatin decondensation is only poorly understood.

We have studied the dynamics of DSB repair proteins in living MCF7 cells (human mammary carcinoma cell line) by the means of high-resolution confocal microscopy. The cells were cotransfected with HP1β-GFP and NBS1-RFP or 53BP1-RFP, respectively. DSBs were induced in selected nuclear subdomains (euchromatin/heterochromatin) by the UVA laser in cells presensitized with BrdU. In condensed chromatin (determined by intense HP1β-GFP fluorescence), induction of DSBs caused an immediate expansion of the irradiated domain, followed by accumulation of NBS1 in its whole volume. The maximal level of NBS1 accumulation was reached simultaneously in both EU and HC; however, its persistence was shorter in EU, indicating more rapid repair of damaged DNA in this region. During the repair process, NBS1 expanded outside the irradiated territory, especially in HC, suggesting decondensation of chromatin structure in DSB surrounding.

These changes were even more pronounced when tracing 53BP. This protein also progressively accumulated at HC periphery but (in contrary to NBS1) did not penetrate into the central area of the domain even in 4 or 5 h post-irradiation. This might reflect extrusion of HC-DSBs into the periphery of condensed chromatin, in order to facilitate the repair. Different penetration of NBS1 and 53BP1 could be explained by their molecular size and/or role in DSB repair. While NBS1 is small and indispensable for repair initiation, 53BP1 is a large dimmer required only for later phases of repair in HC.

The exact mechanisms of chromatin relaxation leading to the relocation of DSBs into the periphery of HC are not known; however, chromatin remodeling complexes with motor ATPases seem to be involved. Some of

these complexes removes nucleosomes from free DSB ends or promote histone exchange. We observed that the intensity of GFP-H2A and GFP-H2B in micro-irradiated regions recovered in about 30 min after photobleaching, however, the intensity of GFP-H3 and GFP-H4 remained unrecovered at least 4 h PI. These results indicate that the interaction of H2A and H2B histones with DNA is weakened after IR, which results to their release from nucleosomes and replacement by newly synthesized molecules of the same or other variants. In contrast, this is not a case of histones H3 and H4. The present results clearly show meaningful changes in chromatin structure flanking DSBs during their repair especially in heterochromatin area.

BRCA1 alternative splicing variants and their impact on DSB repair and mammary tumours development

Another topic (studied in cooperation with Z. Kleibl et al., Dpt. of Experimental Oncology, 1st Faculty of Medicine, Charles University Prague) addresses the role of BRCA1 alternative splicing variants in DSB repair. The BRCA1 gene is frequently mutated in families with hereditary breast/ovarian cancer. For the carriers, BRCA1 mutation poses a high risk of cancer development. In addition, different splicing variants of BRCA1 were also detected in tested woman - the clinical relevance of them is however obscure. Since BRCA1 codes for the nuclear phosphoprotein involved in the DNA repair with a critical role in the DNA double-strand break (DSB) repair, we have studied DSB repair efficiency and kinetics in BRCA1-alternative splicing carriers, together with other important cellular processes. To do this, we have performed functional analysis of selected BRCA1 alternative, in-frame splicing variants affecting the critical regulatory domains of the BRCA1 protein in γ-irradiated MCF7 cells. First, we have focused on variants BRCA1Δ14-15 (shortening the BRCA1 phosphorylation domain) and BRCA1Δ17-19 (disrupting first BRCT domain). We have showed that both variants delay DSB repair as demonstrated by prolonged persistence of gH2AX foci after γ-radiation-induced DNA damage (Fig. 1). Our results thus indicate a possible role of the alternative splicing BRCA1 variants in the regulation of genomic maintenance and deregulation of DSB repair in the mammary tumour-genesis.

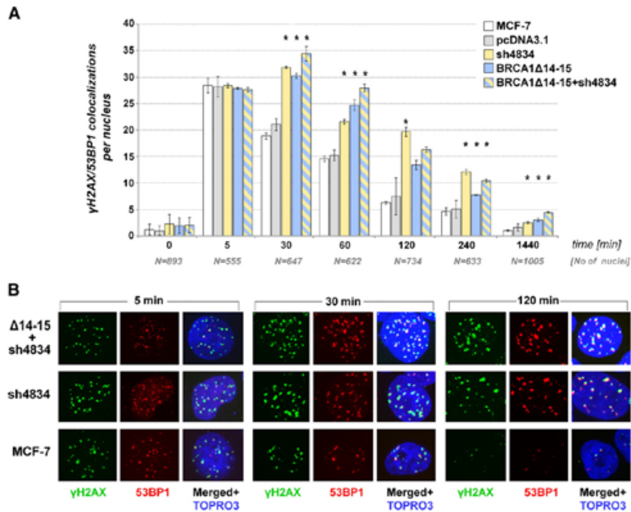

Figure 3: Delayed repair of DNA double strand breaks (DSBs) in MCF7 cells expressing alternative splicing variants of BRCA1 protein. DSB repair kinetics was quantified by measuring the mean number of gH2AX foci (markers of DSB, Green) colocalizing with 53BP1 protein (Red) in different times post-irradiation (PI). High-resolution confocal microscopy was used for image acquisition. Maximal images composed from 40 confocal optical slices taken with a z-step of 0.2 µm are shown. Total nuclear chromatin was counterstained with TOPRO3 (artificially blue). Data are mean +/- StdDev; N = number of nuclei analyzed; *p <0.05, according to the Wilcoxon test.

Additional topics include research of the complex cellular response on fractionated irradiation and analysis of molecular mechanisms of pathogenesis of acute promyelocytic leukemia (APL)

Fractionated radiotherapy is frequently used for tumour cells eradication. However, the knowledge of its effects is rather empirical. More detailed knowledge of the cell response at the molecular level is therefore needed. More details are provided in Řezáčová et al. 2011 (see below).

In cooperation with the European Institute of Oncology, Milan, Italy (Prof. P.G. Pelicci, I.G. Dellino) we have also successfully finished our works on the project "New Mechanisms of Oncoprotein Activity in Development of Promyelocytic Leukemia" (GA AS CR, IAA500040802). In the frame of this project, we have revealed a new mechanism of PML/RARa-induced pathogenesis, consisting in formation of large

suppressing chromatin loops that silence transcription of many genes, downregulated in APL. In addition to our (and EIO) work revealing in previous years, we now show that chromatin loops can form even between loci on different chromosomes.

Group of the Analysis of Chromosomal Proteins

Head of the research group
Michal Štros

Senior scientists
Jiří Fajkus, Eva Sýkorová, Miloslava Fojtová, Jana Fulnečková

Scientist
Martina Dvořáčková

PhD. students
Eva Polanská, Marta Hlobilová, Radan Goldmann, Zuzana Kunická, Vratislav Peška

Diploma Student
Pavlína Koščová

Technical assistants
Libuše Jedličková, Kateřina Šipková

Telomere repeats are added onto chromosome ends by telomerase. We have found that knockout of the HMGB1 gene (encoding a chromatin-associated HMGB1 protein acting as a DNA chaperone in transcription, replication, recombination and repair) in mouse embryonic fibroblasts results in reduced telomerase activity, moderate shortening of telomere lengths, chromosomal abnormalities, and enhanced co-localization of γ-H2AX foci at telomeres (Fig. 1), suggesting that HMGB1 protein is required for telomere maintenance.

The research group continued investigation of the structure and function of telomeres. Original results were obtained in functional analysis of the gene coding for the catalytic subunit of telomerase in *Arabidopsis thaliana*, AtTERT, its putative regulatory elements and contribution of individual protein domains to telomerase function and localisation *in vivo* (Fojtova et al., 2011).

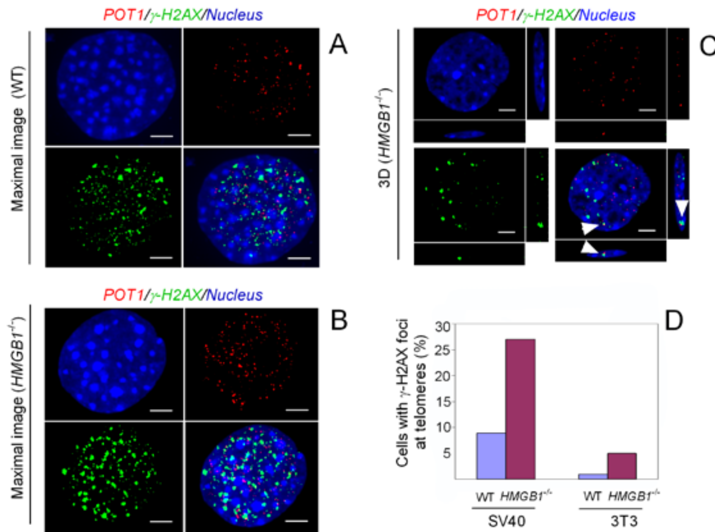

Fig. 4: Knockout of HMGB1 gene in MEFs results in increased DNA damage response at telomeres. Representative images of POT1 (red), γ-H2AX (green), DNA (blue) and merge (POT1/γ-H2AX/DNA, lower right panels in panels A-C) immunofluorescence of the whole nuclei of the wild-type MEFs (A) and the HMGB1$^{-/-}$ MEFs (B). (C) 3D-projections (one focal plane per nucleus) of the HMGB1$^{-/-}$ MEFs. Co-localization of γ-H2AX foci with telomeres (POT1) is indicated in 3D images by arrowheads. Panels A-C show images of MEF cells immortalized by SV40 large T antigen. (D) Percentage of cells with ≥ 5 co-localization events (γ-H2AX foci at telomeres) in MEF cells immortalized by the SV40 large T antigen (SV40) or the 3T3 passage protocol (3T3). Scale bar: 1.5 µm.

In our studies of epigenetic regulation of telomere maintenance, we found that telomeric DNA in tobacco cells, in contrast to that in mammalian cells, is methylated at cytosines of the C-rich telomeric DNA strand. The level of methylation could be decreased due to treatment by hypomethylating drugs (DHPA or zebularine). Although the treatment changed expression levels of telomerase components and levels of telomere (TERRA) transcripts, telomere lengths remained stable (Majerova et al., 2011a,b).

In the field of characterisation of protein components of plant telomeres, novel putative myb-like telomeric proteins were characterised in plants lacking typical telomeres (genus Cestrum, Solanaceae family) which use alternative telomere lengthening (ALT) mechanism to replenish telomeres (Peška et al., 2011). Further, a contribution of HMGB1 proteins to telomere dynamics was described in *A. thaliana* (Prochazkova-Schrumpfova, 2011).

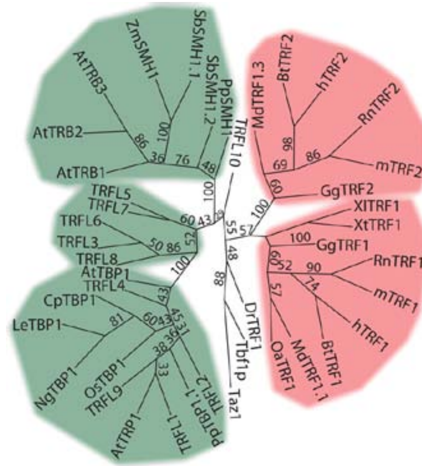

Figure 5: Evolutionary relationships of telobox proteins (Peška et al, 2011): An unrooted consensus phylogenetic tree of amino acid sequences encoding the conserved teloboxes was constructed by the neighbour-joining method using programs from the PHYLIP package. There are five main groups of proteins (a) TRFL family 1 with C-terminal telobox with Myb-extension (b) TRFL family 2 with C-terminal telobox without Myb-extension and ability to bind telomeric DNA *in vitro* (c) SMH1-like proteins with N-terminal telobox (d) C-terminal TRF1-like proteins (e) C-terminal TRF2-like proteins.

Group of Experimental Hematology

Group Leader
Michal Hofer

Senior scientists
Milan Pospíšil, Zuzana Hoferová, Lenka Weiterová

Technical assistant
Květa Láníková

In 2011, research in the Laboratory of Experimental Hematology was aimed predominantly on investigating effects of inhibition of cyclooxygenase-2 on consequences of exposure of experimental mice to deterministic doses of ionizing radiation. We have also continued our studies on mechanisms of hematopoiesis-modulating action of adenosine A_3 receptor agonists.

We have found that pharmacologically induced suppression of prostaglandin production achieved by inhibition of cycloxygenase-2 with a cyclooxygenase-2-selective inhibitor meloxicam promotes the stimulatory action of adenosine A_3 receptor agonist IB-MECA on hematopoiesis in sublethally γ-irradiated mice. Meloxicam was also found to increase survival of mice exposed to lethal radiation doses when given in a single injection early (1 hour) after irradiation. Since meloxicam is a relatively non-toxic drug already used in clinical medicine in other indications, the above results suggest the possibility to extend the spectrum of therapeutical indications for this drug to the treatment of myelosuppression of various etiologies.

Investigations, using the real-time PCR technique, on HL-60 cells, serving as a model for hematopoietic precursor cells, have revealed that the expressions of individual adenosine receptor subtypes vary in different cell cycle phases. These results confirm our earlier considerations about regulatory roles of individual adenosine receptor subtypes and their agonists in modulating hematopoietic processes under normal conditions, as well as under the states of hematopoietic damage of various origin.

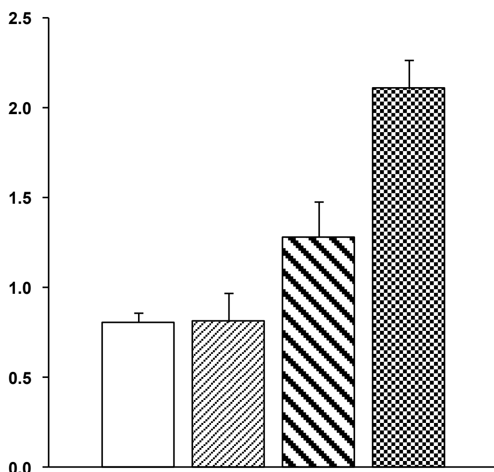

Figure 6: Proliferative granulocytic cells per femur in 4 Gy-irradiated mice. Meloxicam or its solvent (saline) were administered 1 hour after irradiation. IB-MECA or its solvent (2% DMSO in saline) were administered 24 and 48 hours after irradiation. Control - mice treated only with solvents. Data are given as means ± SEM. Five animals per group were used. *,* P < 0.05, P < 0.001, respectively, in comparison with Control. # P < 0.05 in comparison with IB-MECA. +++ P < 0.001 in comparison with meloxicam.**

Granted projects

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

Ministry of Education, Youth and Sports CR, COST LD11020. Principal investigator: E. Bártová, 2011 - 2014

EU Marie Curie, PIRSES-GA-2010-269156. Principal investigator: E. Bártová, 2011 - 2013

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

COST Nano-IBCT, MP1002, European Project, Nano-scale insights in ion beam cancer therapy. Principal investigators: M. Davídková, M. Falk, 2010 - 2014

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 - 2014

GA CR P301/10/0590, Czech Science Foundation, Involvement of HMGB proteins in sensitivity of human cells against anticancer drugs inhibiting topoisomerases II. 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

GA CR P303/11/0128, Role of adenosine A_3 receptor signaling in regulation of hematopoiesis: knowledge obtained from adenosine A_3 receptor knock-out mice. Principal investigator: M. Hofer, 2011 - 2014

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 2011

Vratislav Peška, PhD.: Unusual telomeres in plants

Cytokinetics

Head
Alois Kozubík

Group Leaders
Jiřina Hofmanová, Karel Souček, Jan Vondráček

Scientists
Zdeněk Andrysík, Vítězslav Bryja, Martina Hýžďalová, Pavel Krejčí, Alena Vaculová

Postdocs
Lenka Kočí, Vítězslav Kříž, Jiřina Procházková, Eva Slabáková, Nicol Straková, Kateřina Tmejová, Lenka Umannová

PhD. Students
Barbora Bujoková, Radek Fedr, Iva Jelínková, Jarmila Lauková, Markéta Kabátková, Zuzana Pernicová, Belma Skender, Zuzana Tylichová, Olga Vondálová-Blanářová

Undergraduate Students
Kristýna Adamcová, Stanislav Lerch, Eva Slavíčková, Jana Svobodová, Václav Šeda, Jakub Šenkýř, Šárka Šimečková, Silvie Tománková, Jana Vrbiniaková, Ondřej Zapletal

Technical Assistants
Miluše Andrysíková, Jaromíra Netíková, Martina Urbánková

Labmanager
Kateřina Svobodová

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), Transforming 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)

Dietary n-3 polyunsaturated fatty acids (PUFAs) and short-chain fatty acid butyrate (produced by microbial fibre fermentation) have been proven to exhibit beneficial effects on colon epithelial cell metabolism, signaling, and kinetics, thus preventing colon inflammation and cancer. Moreover, it has been proposed by us and others that PUFAs and butyrate may operate together in the colonic lumen and their interactions may have a substantial impact on colon cell behaviour. It appears that administration of these compounds might be a relatively nontoxic form of supportive therapy improving cancer treatment outcomes and slowing down or preventing recurrence of colorectal cancer (Hofmanová et al., Current Pharmaceutical Biotechnology, review in press).

Our previously published results showed that interaction of sodium butyrate (NaBt) with arachidonic acid (AA, n-6, 20:4) and particularly docosahexaenoic acid (DHA, n-3, 22:6) can significantly affect the behaviour of human colon epithelial cells. Our recent research was focused on a/ molecular mechanisms involved in the effects of NaBt and PUFAs and alterations of lipid composition and metabolism associated with the effects observed; b/ modulation of the effects of therapeutically important endogenous apoptotic inducer - cytokine TRAIL (TNF-related apoptosis-inducing ligand) in colon cancer cells by fatty acids.

i/ The effects and interaction of fatty acids in colon cancer cells
Comparing human colon fetal (FHC) and adenocarcinoma (HCT-116) cells we proved association of composition, accumulation, and metabolism of cellular lipids with their differentiation/apoptotic response after treatment with AA, DHA, NaBt and their combination. Our data from GC/MS and LC/MS/MS analyses showed an effective incorporation and metabolization of supplemented AA or DHA, accompanied by altered content and composition of whole fatty acid spectrum in both FHC and HCT-116 cellular lipids. We provided novel evidence that NaBt when combined with PUFAs modulated AA and DHA cellular levels. NaBt increased, while AA, DHA and their combination with NaBt decreased endogenous fatty acid

synthesis in FHC but not in HCT-116 cells. Altered membrane lipid structure (lipid unpacking), cytoplasmic lipid droplet accumulation, increased reactive oxygen species production, and dissipation of mitochondrial membrane potential were significantly enhanced after combination of AA or DHA with NaBt in comparison with agents used alone. These effects were more pronounced in fetal FHC cells, which correlated well with their strong apoptotic response to this type of treatment. Our results present cooperative effects of PUFAs and butyrate in the colon and show significant association between lipid alterations and differentiation/apoptotic response in colon fetal versus cancer cells (Hofmanová et al., J. Nutr. Biochem., 2011, in press).

ii/ Modulation of TRAIL effects by n-3 PUFA in colon cancer cells

We investigated the mechanisms of sensitizing effect of DHA on apoptosis triggered by Tumor necrosis factor-Related Apoptosis Inducing Ligand (TRAIL) in resistant SW620 epithelial cell line derived from human colon cancer metastasis. We established the role of DHA as an effective sensitizer of epithelial colon cancer cell sensitivity to apoptosis, while no significant cytotoxic effects on normal colon cells were detected. We demonstrated the importance of mitochondrial apoptotic pathway in the combined action of DHA and TRAIL, and suggested several relevant regulators at the level of these organelles, including proapoptotic Bcl-2 family proteins and mitochondrial proapopotic mediators. Additional molecular mechanisms involved in the potentiation of cytotoxic effects of the newly designed combination are under investigation on our laboratory.

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

The resistance of transformed epithelial cells to a detachment-induced apoptosis (anoikis) is known to significantly affect their susceptibility to various types of anticancer therapy. We investigated molecular mechanisms responsible for the different sensitivity of adherent and non-adherent human colon epithelial cells to apoptosis induced by TRAIL, a therapeutically interesting molecule of the Tumor Necrosis Factor (TNF) family. We demonstrated that detachment of colon fetal FHC and cancer HT-29 epithelial cells resulted in the activation of the pro-survival Akt pathway, which was involved in a significant increase of their resistance to TRAIL-induced apoptosis compared to the attached cells. The PI3K/Akt-mediated protection from TRAIL-induced apoptosis was not associated with changes in the cell surface TRAIL death receptor levels, but rather a modulation of downstream intracellular signaling events was suggested to be involved. In addition, these effects were accompanied by significant changes of the

expression/activation of integrin-linked kinase (ILK) and focal adhesive kinase (FAK), the two important molecules involved in modulation of the cell adhesion and apoptosis sensitivity. Our results may have important therapeutic implications in treating cancers at different stages of development, and modulation of the sensitivity/resistance of metastatic cells to therapeutic interventions. These results were described in successfuly defended PhD. thesis of L. Kočí and were published in 2011 (Kočí et al., 2011).

iv) Different sensitivity of colon fetal and cancer cells to photodynamic therapy with hypericin

In cooperation with Faculty of Science, UPJŠ, Košice we continued investigation of mechanisms of the effects of photosensitizer hypericin, which has interesting application in anticancer therapy. We correlated hypericin uptake and cell death response comparing fetal FHC and adenocarcinoma HT-29 cells. We showed that despite the higher accumulation of hypericin in FHC cells, the cytotoxic effects were more pronounced in the HT-29 cells. Our results demonstrated that not only hypericin uptake, but importantly also the cell ability to manage oxidative stress induced by hypericin photodynamic therapy can be important decisive factors finally affecting the cell death response (Mikeš et al., 2011).

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 micro-environment affects precancerous and cancerous cells and apply our findings directly to clinically relevant problems (e.g., dissemination of cancer cells, development of androgen-independent prostate cancer, or resistance to anti-cancer therapy). Production of cytokines belonging to the Transforming Growth Factor-β family (TGF-β) (e.g. Growth/differentiation factor-15) is characteristic for the tissue microenvironment during cancer progression and therapy; however their context-dependent role is not known in all details.

In 2011 we continued with studies focused on molecular mechanisms of TGF-β-induced epithelial-mesenchymal transition (EMT) in epithelial cells,

and mechanisms of neuroendocrine differentiation (NED) of prostate cancer. It was shown that EMT process plays an important role in maintenance and induction of population of stem cells and cancer stem cells, respectively. To confirm this also in both normal prostate and prostate cancer, we successfully introduced methods for detection and isolation of small subpopulations of stem cells and cancer stem cells from both *in vivo* and *in vitro* models. Our results indicate that expression of markers and regulators of EMT is different in stem cell and cancer stem cells subpopulation in comparison with non-stem cells or non-cancer stem cells population, respectively.

Several EMT-associated molecules (i.e., Slug) have been shown to be regulated by the ubiquitin ligase MDM2, whose role in the regulation of cell cycle and cell death in cancer cells has been well established. Our preliminary results suggest that MDM2 could play an unexpected role in the process of EMT, based on differential expression of MDM2 in cells with epithelial and mesenchymal phenotype and on functional experiments proving that modulation of MDM2 expression in cells leads to altered migration capacity. The function of MDM2 in cell motility and cross-regulation between MDM2 and EMT-regulating molecules are currently under investigation.

Anti-androgen therapy is often associated with acquisition of androgen-independence of prostate cancer cells and disease progression. One of the phenomena contributing to this independence is NED of prostate cancer cells. We identified that interestingly not only previously described androgen depletion, but also high cellular density leads to acquisition of neuroendocrine-like phenotype of prostate cancer cells *in vitro* (Pernicová et al., 2011). This is connected with cell cycle arrest and deregulated expression of several cell cycle regulators. Using targeting these regulators in order to modulate cell cycle we found out that deregulation of cell cycle by down-regulation of cyclin-dependent kinase 2 (cdk2) leads to acquisition of NE-like phenotype. This implies that cell cycle arrest is functionally linked with induction of neuroendocrine differentiation. To further elucidate the mechanism by which high cellular density induces NED, we focused on signaling pathway of cyclic adenosine monophosphate (cAMP) and confirmed that activation of cAMP signaling may contribute to induction of NED in our models.

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.

Mechanisms of FGFR signaling (Pavel Krejčí)

The main aim of our work is to understand the mechanisms of signal transduction of Fibroblast Growth Factor Receptor (FGFR) tyrosine kinases as it relates to pathology of human disorders caused by activating mutation in FGFR3 and FGFR2. In 2011, we completed and published the most extensive review of the molecular mechanisms of FGFR3 signaling in skeletal dysplasia to date. We also described, for the first time, a novel human skeletal dysplasia originating from mutation in FGFR2. Finally, we described a novel signaling pathway utilized by FGFR2 and FGFR3 to activate canonical Wnt/beta-catenin signaling in chondrocytes. All these results were finalized and submitted for publication (now in press).

In our work, we also focus on the possibilities of therapeutic targeting of FGFR signaling in both FGFR-related skeletal dysplasias, and tumors caused by FGFR mutations. In 2011, we completed the characterization of a compound named AZD1480 as a novel, potentially therapeutic inhibitor of FGFR3 signaling in Multiple Myeloma (Scuto et al., 2011).

Mechanisms of Wnt signaling (Vítězslav Bryja)

The principal aim of our work is to contribute to understanding of molecular and cellular events associated with the Wnt signal transduction. In the past year, we specifically focused on the role of Amer1 protein in the canonical Wnt signaling, described in detail the role of casein kinases and in collaboration with the group of Jan Vondráček identified the crosstalk of Wnt and Aryl Hydrocarbon Receptor (AhR)-mediated signaling pathways.

We have shown in collaboration with the group of Jürgen Behrens (University of Erlangen, Germany) the key function of Amer1 protein (also know as FAM123B) in the Wnt signal transduction. Specifically, Amer1 was found to be required for the phosphorylation of the key receptor Lrp6 upon ligand binding (Tanneberger et al., 2011a,b). We have also described the set of post-translational modifications in other Wnt receptor, Ror1

(Kaucká et al., 2011). Further, we have identified dual role of casein kinases 1 and 2 in the activation of Dishevelled protein, which is required for function in the Wnt downstream signaling. This finding uncovers intrinsic negative feedback loop in the Wnt machinery at the level of Dishevelled (Bernatík et al., 2011).

Finally, our studies (coordinated by Jan Vondráček) on the crosstalk of Wnt and the Aryl Hydrocarbon Receptor (AhR) signaling machineries have revealed that AhR ligands may modulate components of both canonical and non-canonical Wnt signaling pathways *in vitro* (Procházková et al., 2011; Hrubá et al., 2011) - for more details, see below.

Cellular and molecular toxicology (Jan Vondráček)

The principal aim of our work is to contribute to understanding of molecular and cellular effects of environmental organic pollutants linked to carcinogenesis, reproductive or developmental impairment. In the past year, we specifically focused on physiological signaling pathways affected by environmental toxicants, such as canonical Wnt signaling or signal transduction mechanisms, which are activated by pro-inflammatory mediators.

Our study has revealed that persistent ligands of the AhR negatively modulate the canonical Wnt pathway in liver progenitor cells, which contribute both to liver tissue regeneration and to development of liver neoplasms. At the same time, we found that both signaling pathways (AhR and canonical Wnt signaling) together significantly increase the expression of enzymes participating in bioactivation of pro-mutagens, such as polycyclic aromatic hydrocarbons (PAHs) (Procházková et al., 2011). Long-term AhR activation may thus significantly modulate the function of β-catenin, a key protein participating both in formation of cell-cell junctions and regulation of gene expression (Procházková et al., 2011). In collaboration with the Department of Toxicology, Pharmacology an Immunotherapy (Veterinary Research Institute, Brno), we further participated in the study, which described the impact of AhR ligands on global gene expression in prostate epithelial cells. Among other findings, this study revealed a potentially novel role of AhR in regulation of expression of non-canonical Wnt ligand, Wnt5a, which contributes both to the regulation of the prostate development and to progression of prostate carcinoma (Hrubá et al., 2011).

Furthermore, we have documented that during inflammatory reaction, AhR ligands, such as PAHs, may modulate not only the expression/activity of enzymes participating in bioactivation of PAHs, such as cytochrome P450 1B1. PAHs and inflammatory cytokines together also potentiate the production of inflammatory mediators by alveolar epithelial type II cells *in vitro* (Umannová et al., 2011; Vondráček et al., 2011). These include not only key pro-inflammatory cytokines, such as tumor necrosis factor-α, interleukin-1β or interleukin-6, but also enzymes producing further pro-inflammatory compounds, such as cyclooxygenase-2 or inducible NO synthase. The activation of AhR thus plays a key role in toxic effects of PAHs forming highly complex mixtures of toxicants present in polluted air, which are known to elicit inflammatory response in lung tissue (Andrysík et al., 2011).

The present results contribute to our understanding of the possible mechanisms of toxic actions of various classes of AhR ligands. This information may further shed more light on the role of endogenous AhR signaling within cells, as well as the mode of action of carcinogenic AhR agonists in epithelial cells.

Mechanisms of the effects of platinum-based chemotherapeutic drugs and TRAIL (Alena Hyršlová Vaculová)

Analysis and understanding of the effects of antitumor platinum complexes is one of the essential prerequisites of their successful application in anticancer therapy. We investigated the molecular mechanisms of the action of a novel platinum(IV) complex LA-12 and compared them with those triggered by cisplatin and oxaliplatin in colon and prostate cancer cells. In addition to their individual ability to trigger the cancer cell death, we demonstrated that these drugs can also act (in subtoxic doses) as efficient sensitizers to apoptosis induced by therapeutically interesting cytokine TRAIL. Importantly, we documented a significantly higher anti-tumor efficacy of LA-12 compared to cisplatin and oxaliplatin when applied not only individually, but also in combination with TRAIL, which may potentially reduce undesired side effects and help to minimize therapeutic doses when used in future clinical applications. Molecular mechanisms responsible for these beneficial cytotoxic effects of platinum-based drugs on cancer cells have been investigated in detail in our laboratory.

We described significant differences in regulation of the cell cycle and apoptosis in colon cancer cells treated with LA-12 and cisplatin/oxaliplatin. In contrast to oxaliplatin, p53 and p21 are not essentially required for apoptosis induced by LA-12. Importantly, we were the first to demonstrate the outstanding ability of LA-12 to sensitize human colon and prostate cancer cells to TRAIL-induced apotosis, and suggested the possible intracellular targets for future therapeutic interventions. The cancer cell sensitizing effects of LA-12 to TRAIL-induced apoptosis were compared with those exerted by cisplatin. We showed that both platinum complexes increased the surface and total expression of TRAIL death receptor DR5 in colon cancer cells. Small interfering RNA-mediated DR5 silencing rescued cells from sensitizing effects of platinum drugs on TRAIL-induced caspase activation and apoptosis, showing the functional importance of DR5 in the effects observed. In addition, both cisplatin and LA-12 triggered the relocalization of DR4 and DR5 receptors to lipid rafts and accelerated internalization of TRAIL (Vondálová-Blanářová et al., 2011). We documented that the modulations of the initial steps of the extrinsic apoptotic pathway at the level of DR5 and plasma membrane are important prerequisites for mediating the cytotoxic effects of the drug combinations. Furthermore, we showed that LA-12- or cisplatin-mediated sensitisation to TRAIL-induced apoptosis can occur also in colon and prostate cancer cell lines lacking p53 protein or expressing its mutated form. On the other hand, suggested drug combinations were not significantly harmful to the normal colon cells, which suggest their more selective cytotoxic actions against tumor. These results support the promising application of the drug combinations also in tumors with non-functional mutated p53, which account for at least half of all cancers, and the significantly less harmful toxic effects to the surrounding healthy tissues.

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

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, New mechanisms of platinum-based drug action as a tool for anticancer therapeutic strategies. Principal investigator: A. Kozubík, 2010 - 2014

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GA CR P503/11/1227, Interactions of inflammatory mediators and Ah receptor signaling in toxic effects of polycyclic aromatic hydrocarbons. Principal investigator: J. Vondráček, 2011 - 2013

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PhD. thesis defended in 2011

Mgr. Lenka Kočí, PhD., Changes in adhesive properties and anoikis regulation in colon epithelial cells

Mgr. Eva Slabáková, PhD., Role of TGF-β family proteins in the cytokinetics of prostate and colon cancer cells

FREE RADICAL PATHOPHYSIOLOGY

HEAD

ANTONÍN LOJEK

SCIENTISTS

MILAN ČÍŽ, LUKÁŠ KUBALA, KATEŘINA PEJCHALOVÁ, MICHAELA PEKAROVÁ, JAN VÍTEČEK

TECHNICAL ASSISTANT

LENKA VYSTRČILOVÁ

GRADUATE STUDENTS

GABRIELA AMBROŽOVÁ, LUCIA BINÓ, TOMÁŠ CRHÁK, MARTINA HAŠOVÁ, HANA KOLÁŘOVÁ, EMA RUSZOVÁ, BARBORA ŠAFRÁNKOVÁ, ONDŘEJ VAŠÍČEK, LUCIE VIŠTEJNOVÁ

UNDERGRADUATE STUDENTS

SILVIE GAJDOVÁ, HANA MARTIŠKOVÁ, JANA NAVRÁTILOVÁ, MICHAL RÁJECKÝ, MICHAELA ZAPLETALOVÁ

New role for L-arginine in regulation of inducible nitric-oxide-synthase-derived superoxide anion production in Raw 264.7 macrophages

Our study demonstrate that, besides the regulation of NO production, amino acid L-arginine is able to cause a dose- and time-dependent increase in iNOS-derived O2.- formation in inflammatory macrophages. Our findings have some important implications. We have shown that LPS is able to biphasically induce O2.- production in RAW 264.7 cells. In the first few hours after LPS-stimulation, macrophages produce a relatively small but significant amounts of O2.- which should be considered as being formed by activated NADPH oxidase. In the second phase, LPS causes a massive increase in O2.- production, predominantly due to iNOS uncoupling. More importantly, the second phase of O2.- production is directly controlled by extracellular L-arginine availability. In conclusion, the L-arginine availability seems to play a critical role for the immune state of macrophages and there are now two sides this problematic. One is that

a lack of extracellular L-arginine is responsible for the attenuation of immune functions associated with the decrease in immune cell proliferation and NO production, which can lead to different pathophysiological states. On the other side, supplementation by L-arginine could lead to an increased O2.-, and subsequently an increased ONOO- formation that is critical for host defense, but might also be deleterious for host cells/tissue.

Pathogenic cycle between the endogenous nitric oxide synthase inhibitor asymmetrical dimethylarginine and the leukocyte-derived hemoprotein myeloperoxidase

Leukocyte activation and concomitant release of MPO play a pivotal role in the development of endothelial dysfunction. Because both steps are regulated in an NO-dependent fashion, we investigated the interaction between the endogenous NOS inhibitor ADMA and the leukocyte-derived hemoprotein MPO. The present study provides evidence of the impact of an ADMA-induced pathogenic cycle on increased PMN activation, enhanced MPO release, and subsequent impairment of DDAH activity, which in turn accounts for an increase in ADMA concentrations (Figure 1). Briefly, the salient findings are the following. First, ADMA accumulates in human PMNs and impairs intracellular NOS activity. Second, ADMA accumulation in PMNs leads to PMN degranulation and increases superoxide release, resulting in enhanced PMN adhesion to human umbilical vein endothelial cells ex vivo. Third, the hypothesis of an ADMA/MPO interaction is confirmed in human individuals challenged with a short-term ADMA infusion. Fourth, *in vitro*, MPO via its product HOCl decreases recombinant hDDAH1 activity. Fifth, *in vivo*, the LPS-induced increase in plasma ADMA concentrations is attenuated in MPO-/- mice, and overexpression of the hDDAH1 gene is able to oppose the effects of MPO.

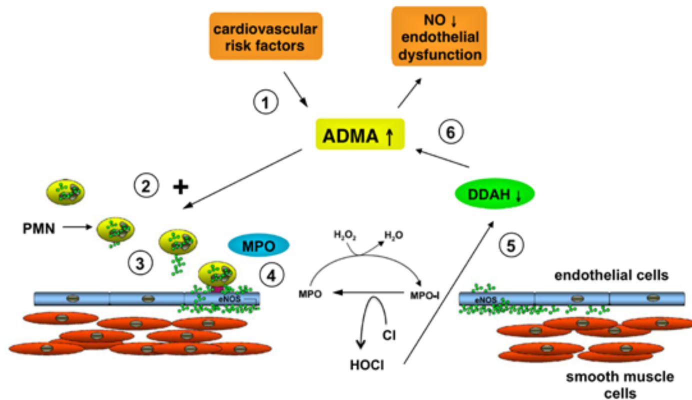

Figure 1: Pathogenic cycle between the nitric oxide (NO) synthase (NOS) inhibitor asymmetrical dimethylarginine (ADMA) and leukocyte-derived myeloperoxidase (MPO). ADMA plasma concentrations are elevated under conditions of increased cardiovascular risk factors (1). Elevated ADMA concentrations accumulate in polymorphonuclear neutrophils (PMNs) and impair intracellular NOS activity (2), resulting in activation, degranulation, and adhesion of PMNs (3). As a consequence, MPO plasma concentrations and local MPO activity are enhanced (4). Subsequently, the MPO product HOCl impairs dimethylarginine dimethylaminohydrolase1 (DDAH) activity (5), resulting in a further increase in ADMA concentrations (6).

Group of Pathophysiology of Free Radicals in Cell Interactions

Head

Milan Číž

Differentiating between intra- and extracellular chemiluminescence in diluted whole-blood samples

The differentiation between extra- and intracellular production of reactive oxygen species (ROS) in whole blood was measured by luminol- and isoluminol-enhanced chemiluminescence (CL).Azide (total CL inhibition), azide + horseradish peroxidase (HRP, restoring extracellular CL), superoxide dismutase + catalase (depleting extracellular ROS) and HRP (enhancing extracellular CL) were used to modulate luminol- and isoluminol-enhanced CL (10-6–10-3 M luminophores) of diluted whole blood which was activated by both calcium ionophore A23187 (Ca-I) and opsonized zymosan particles (OZP) separately. Both activators stimulated intra- and extracellular production of ROS. Luminol-enhanced CL of Ca-I-activated samples detected the intracellular ROS, and with the addition of HRP detected the extracellular CL as well. CL enhanced with isoluminol in concentrations of 10-4 M or less was mostly extracellular. There was a mixture of intra- and extracellular CL in OZP-activated samples, probably because of the ingestion of luminophore molecules. Measurement of Ca-I-activated CL enhanced with 10-4 M luminol is recommended for the detection of intracellular ROS. The addition of HRP leads to the detection of overall ROS production while the OZP-activated system with its addition of HRP can only be used to detect overall ROS production. Ca-I-activated CL enhanced with 10-4 M isoluminol and with addition of HRP is recommended for the detection of extracellular CL.

Effect of serotonin and related tryptamines on the functional activity of murine phagocytes

Serotonin (5-hydroxytryptamine, 5-HT) is a biogenic monoamine. It is produced by the hydroxylation and decarboxylation of the amino acid tryptophan. The highest concentration of 5-HT in the body (about 95%) is found in the cells of the gastrointestinal tract, of which 90% are within

enterochromaffin cells and 10% within enteric neurons. Serotonin is metabolized to N-acetylserotonin (NAS) and then to melatonin (MLT) by the enzymes N-acetyl transferase and 5-hydroxyindole-O-methyltransferase. The aim of the present study was to evaluate the antioxidant properties of 5-HT, NAS and MLT and their effects on functional activity of murine macrophages. Tested tryptamines were used in the concentrations of 10-8M, 10-6M and 10-3M. These cover the concentrations that may occur in the body (10-3M only locally in the areas of inflammation). Non-stimulated murine RAW264.7 macrophages and cells stimulated by lipopolysaccharide (LPS) were cultured with tested tryptamines for 24h and subsequently their chemiluminescence (ROS production) was measured. At the same time, cytokine levels (using commercially available array kit) and NO production (indirectly as the accumulation of nitrites) were measured in the medium medium. iNOS protein expression was measured in lysed cells. Our results showed that 5-HT and NAS were better antioxidants than MLT, which was confirmed by chemiluminescence, TRAP measurements and NO-scavanging evaluation. Production of nitrites and iNOS expression significantly decreased with increasing concentration of tested tryptamines. A significant impact of tested tryptamines on cytokine production in murine macrophages induced by LPS was also proven. It can be concluded that serotonergic system interacts with the immune system, especially with phagocytes. The results suggest that the increase in local concentrations of tryptamines significantly reduces oxidative and nitrative stress.

Group of Free Radicals in Regulation of Cell Physiology

Head

Lukáš Kubala

ABC transporters affect the detection of intracellular oxidants by fluorescent probes

Intracellular production of reactive oxygen species (ROS) plays an important role in the control of cell physiology. For the assessment of intracellular ROS production, a plethora of fluorescent probes is commonly used. Interestingly, chemical structures of these probes imply they could be substrates of plasma membrane efflux pumps, called ABC transporters. ROS production was determined by various methods both employing both fluorescent and chemiluminescent probes in cells differently over-expressing MDR1, MRP1 and BCRP transporters. Interestingly our data show that the determination of intracellular ROS and mitochondrial potential by the most available fluorescent probes is significantly altered by ABC transporter activities. The activity of these transporters must be considered when employing fluorescent probes for the assessment of ROS production or mitochondrial membrane potential.

Hyaluronan minimizes effects of ultraviolet irradiation on human keratinocytes

Exposure to ultraviolet (UV) irradiation has detrimental effects on skin accompanied by the increased metabolism of hyaluronan (HA), a linear polysaccharide important for the normal physiological functions of skin. In this study, the modulation of human keratinocyte response to UVB irradiation by HA (970 kDa) was investigated. The irradiation induced a significant decrease in the gene expression of CD44 and toll-like receptor 2 after irradiation. UVB irradiation induced a significant decrease in the gene expression of HA synthase-2 and hyaluronidase-2 after irradiation. Interestingly, HA treatment did not significantly modulate any of these effects. In contrast, HA significantly suppressed UVB-induced pro-inflammatory cytokine release including interleukin-6 and interleukin-8. Similarly, HA treatment reduced the UVB-mediated production of transforming growth factor β1. HA treatment also significantly reduced

the UV irradiation-mediated release of soluble CD44 into the media. Finally, HA partially, but significantly, suppressed the UVB-induced decrease in cell viability. Data indicate that HA had significant protective effects for HaCaT cells against UVB irradiation.

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

COST - MEYS LD11015, Role of hypoxia and intracellular redox status disbalance in a regulation of cell selfrenewal and differentiation. Principal investigator: L. Kubala, 2011 - 2013

COST - MEYS LD 11010, Effects of histamine receptor H4R antagonists on the production of reactive oxygen and nitrogen metabolites by phagocytes. Principal investigator: A. Lojek, 2011 - 2013

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Structure and Dynamics of Nucleic Acids

Head

Jiří Šponer

Scientists

Kamila Réblová, Naďa Špačková, Judit E. Šponerová

Part time Co-Workers

Pavel Banáš, Daniel Svozil, Michal Otyepka, Petr Jurečka

Technical assistant

Lukáš Posádka

PhD. Students

Ivana Beššeová, Arnošt Mládek, Leona Šerá

Diploma student

Marek Havrila

Bachelor students

Petr Stadlbauer, Miroslav Krepl, Marie Sarázová

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 (QM) calculations and bioinformatics. We also theoretically studied several origin of Life issues by means of state-of-the-art QM computations.

We have applied high-level quantum chemical (QM) calculations in order to investigate the geometry and electronic properties of the arsenate analogue of the DNA backbone. The optimized geometries as well as hyperconjugation effects along the C3'-O3'-X-O5'-C5' linkage (X = P,A s) exhibit a remarkable similarity for both arsenates and phosphates. This suggests that arsenates, if present, might serve as a potential substitute for phosphates in the DNA backbone. In other words our computations show that arsenate anions may behave as a perfect substitute for phosphates in the sugar-phosphate backbone of DNA, both from structural and electronic

points of view. This is not surprising because it is well known that arsenates, when available, readily substitute for phosphates in minerals. On the other hand, among others, the relative abundance of arsenic in the Earth's crust might raise a serious problem to the relevance of an As-based alternative life form because phosphorus is about by three orders of magnitude more frequent than arsenic. However, major obstacle for incorporation of arsenate into DNA could be kinetics.

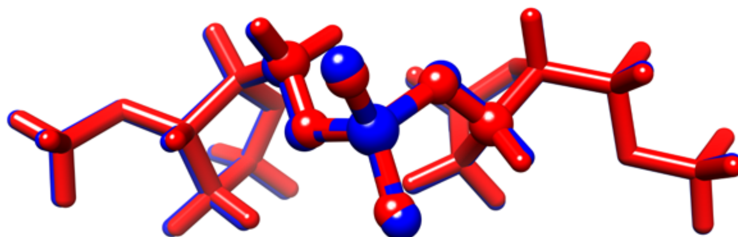

Figure 1: Overlay of the QM optimized geometries of the DNA backbone models with phosphate (red) and arsenate (blue).

We continued our effort in studying electronic properties of As-DNA and revealed that neither steric hindrance nor less polar solvent medium is able to reduce the otherwise high hydrolysis rate of arsenate-esters, i.e., their low kinetics stability. These results from high-level quantum chemical computations question the stability of As-DNA not only in aqueous but also in non-aqueous environments. Thus the existing gap between the activation energies of arsenate- and phosphate- ester hydrolysis does not appear to be influenced by the size of the alkyl-group as long as the hydrolysis proceeds according to the addition–elimination reaction channel, which is the established preferred path when larger alkyl-substituents are considered. This implies, that hydrolytic stability of As-DNA can be reasonably questioned due to the high hydrolysis rates of arsenate-esters and that should hold true for non-aqueous environments, unless very specific solvent effects are operational. In summary, QM calculations do not support ability of As-DNA to stable store genetic information.

We have studied the energetics of the prebiotic glycosylation reaction, which is one of the most controversial questions of the RNA world theory. We have evaluated the thermodynamics of nucleoside formation under prebiotic conditions via the classical reaction route involving ribose and

cytosine as well as via the novel pathway suggested by Powner et al (M. W. Powner, B. Gerland and J. D. Sutherland, Nature 2009, 459, 239-242). Our computations show that in contrast to the classical pathway the route proposed by Powner et al. perfectly satisfies all conditions of a typical metabolic pathway occurring in leaving organisms. In addition, we reveal the reasons that make the reaction of ribose with nucleobases endothermic and thereby prebiotically less plausible. Role of phosphate-catalysis in the Sutherland-reaction has also been examined.

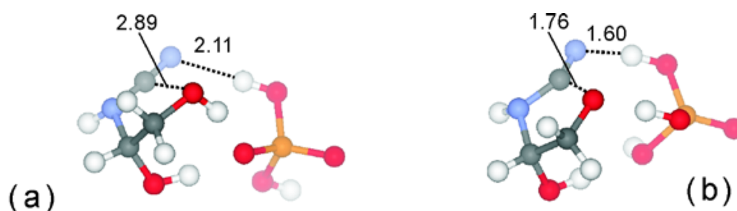

Figure 2: Phosphate-catalysis in the 2-aminooxazole formation, i.e. one of the key steps of the Sutherland synthesis. a) initial state complex, b) transition state complex. The numbers refer to characteristic interatomic distances in Å.

We have investigated the prebiotic stabilization of sugars by silicates. This study is the continuation of our previous work on the analogous complexes of sugars with borates and is based on a recent paper by Lambert et al. (J. B. Lambert, S. A. Gurusamy-Thangavelu, K.B.A. Ma, Science, 2010, 327, 984-986.) which suggests that the silicate-mediated formose reaction facilitates the stabilization of ribose. Using accurate quantum chemical calculations, we have calculated the relative stability of the silicate complexes of arabinose, lyxose, ribose and xylose to determine which would form easier from a formose-like reaction. Five stereoisomers were investigated for each complex. The stereoisomers of 2:1 ribose-silicate complexes are the most stable ones, to the extent that the least stable of these is even more stable than the most stable stereoisomer of any other 2:1 sugar-silicate complexes. Thus, thermodynamically, a formose-like reaction in the presence of silicate minerals should preferentially form the silicate complex of ribose over the silicate complex of arabinose, lyxose and xylose.

We have contributed to a new reparameterization of the glycosidic torsion χ of the Cornell et al. AMBER force field for RNA, named χ_{OL}. The proposed parameters effectively remove destabilization of the anti region found in the

preceding force field and thus prevent formation of spurious ladder-like structural distortions in RNA simulations. They also improve the description of the syn region and the syn-anti balance as well as improve MD simulations of various RNA structures. The new parametrization is based on high-level QM calculations and differs from conventional parametrization approaches in that it incorporates some previously neglected solvation-related effects. Our χ_{OL} force field has been compared with several previous glycosidic torsion parametrizations.

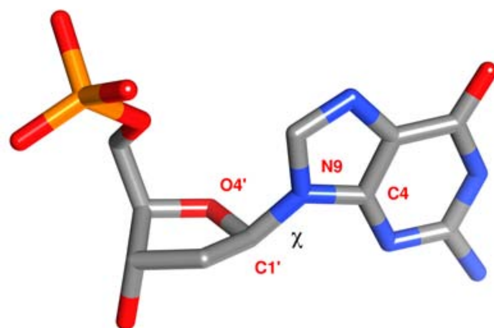

Figure 3: Two low-energy orientations of glycosidic torsion angle χ: anti (left) and syn (right). According to standard nomenclature χ torsion angle is defined as O4′-C1′-N9-C4 and O4′-C1′-N1-C2 for purines (herein) and pyrimidines, respectively. [chi_anti.png (left) and chi_syn.png (right)].

RNA kink-turn (Kt) is a recurrent RNA building block often utilized by evolution. Our structural search of several Kt motifs (16S rRNA Kt-23, 23S rRNA Kt-7, 23S rRNA Kt-15, 23S rRNA Kt-38, 23S rRNA Kt-46, Kt-C/D box and snRNA Kt-U4) pointed to the fact that these recurrent RNA patterns contained two types of A-minor interaction (types 0 and I). The A-minor interaction is a triplet containing one Watson-Crick and at least one non-Watson-Crick base pair. The performed molecular dynamics simulations showed that only certain orientations of the canonical C=G base pairs within these two types of the A-minor interactions were allowed. The type 0 pattern was considered to be the less stable one and tended to shift toward the type I pattern. These facts were consistent with the covariation patterns seen during the evolution and with quantum mechanical calculations evaluating stabilities of the triplets. The obtained data

suggested that the formation of A-minor interaction type I was likely supported by the surrounding protein and RNA molecules, while A-minor interaction type 0 might also be stabilized by additional kink-turn nucleotides not belonging to the kink-turn consensus sequence.

We further studied reverse kink-turn that is an elbow-like RNA building block occurring in the ribosome and in the group I intron. The unrestrained, explicit solvent molecular dynamics simulations of these RNA motifs (54 simulations with 7.4 μs of data in total) reported their directional intrinsic flexibility being pertinent to their folded functional geometries. The reverse kink-turns appeared to be the most flexible RNA motifs studied so far by explicit solvent simulations. These molecules were capable at the present simulation time scale of spontaneous and reversible sampling of wide range of geometries, i.e., from tightly kinked ones through flexible intermediates up to extended, unkinked structures. In addition, the tests of several variants of the Cornell et al. AMBER force field, ion conditions and two water models showed necessity of using our latest χ_{OL} variant to obtain stable trajectories of reverse kink-turns. We revealed considerably larger impact of used type of explicit water model on simulations than applied concentration and the type of ions.

We have carried out series of large-scale molecular dynamics simulations of quadruplex DNA (G-DNA) molecules. The computations have highlighted the very basic rules which govern the intrinsic preference of the orientation of strands in monomolecular G-DNA molecules depending on the number of their quartets. The computations also contributed to our knowledge of the interplay between the loop lengths and the overall topologies of G-DNA molecules. Finally, the simulations provided unique insights into the structural dynamics of cation binding to G-DNA, including the very first observation of a complete exchange of an ion between G-DNA stem and the bulk solvent.

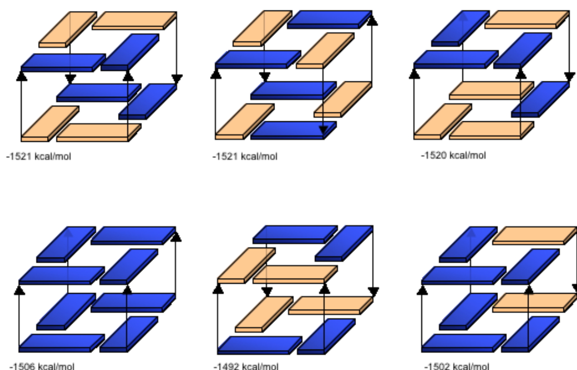

Figure 4: Total free energies of the six 2-quartet G-DNA stem models were estimated using simulation-based free energy computations. All models in the first row are composed of four syn-anti 5'-GpG-3' steps, with difference in strand orientations. The three models in the second row are composed of four anti-anti steps, four anti-syn steps and three anti-anti + one syn-syn steps, respectively. Through comparing the total free energy estimates of these simplified models, relative stabilities of the four glycosidic steps and basic rules for strand orientation in G-stems were obtained. Salmon is for syn and blue is for anti glycosidic bond orientations. The channel cation (K^+) in each model is not shown.

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

Publications

Cang, X.H., Sponer, J., Cheatham, T.E.: *Insight into G-DNA structural polymorphism and folding from sequence and loop connectivity through free energy analysis*. Journal Of The American Chemical Society, 133, 2011, 14270-14279.

Fiala, R., Spackova, N., Foldynova-Trantirkova, S., Sponer, J., Sklenar, V., Trantirek, L.: NMR *Cross-correlated relaxation rates reveal ion coordination sites in DNA*. Journal Of The American Chemical Society, 133, 2011, 13790-13793.

Reshetnikov, R.V., Sponer, J., Rassokhina, O.I., Kopylov, A.M., Tsvetkov, P.O., Makarov, A.A., Golovin, A.V.: *Cation binding to 15-TBA quadruplex DNA is a multiple-pathway cation-dependent process*. Nucleic Acids Research, 39, 2011, 9789-9802.

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Reblova, K., Sponer, J.E., Spackova, N., Besseova, I., Sponer, J.: *A-minor tertiary interactions in RNA kink-turns. molecular dynamics and quantum chemical analysis.* Journal Of Physical Chemistry B, 115, 2011, 13897-13910.

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Mladek, A., Sponer, J., Sumpter, B.G., Fuentes-Cabrera, M., Sponer, J.E.: *On the geometry and electronic structure of the as-DNA backbone*. Journal Of Physical Chemistry Letters, 2, 2011, 389-392.

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

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

Sklenovsky, P., Florova, P., Banas, P., Reblova, K., Lankas, F., Otyepka, M., Sponer, J.: *Understanding RNA flexibility using explicit solvent simulations: the ribosomal and group I intron reverse kink-turn motifs*. Journal Of Chemical Theory And Computation, 7, 2011, 2963-2980.

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CD Spectroscopy of Nucleic Acids

Head

Michaela Vorlíčková

Scientist

Iva Kejnovská, Daniel Renčiuk

Graduate students

Klára Bednářová, Petra Školáková, Martin Tomaško, Zuzana Dvořáková, Helena Konvalinová

Bachelor

Martin Chvátal

Group Leader

Jaroslav Kypr

Scientist

Karel Nejedlý

Conformational properties of CGG repeats associated with X chromosome fragility

We continued in the effort to identify the structure responsible for expansion of the CGG repeats in the human genome associated with the syndrome of the X chromosome fragility. In our previous reports we demonstrated that the structure responsible for expansion is not a quadruplex as was suggested by others. In this work we show 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.

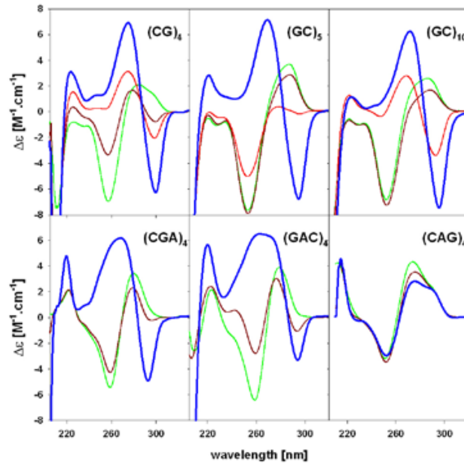

Figure 1: CD spectra of CG rich repetitive sequences measured in 10 mM sodium phosphate (pH 7), 0.3 mM EDTA with 300 mM NaCl (green spectra), 2 M NaCl (dark red spectra) and 4 M NaCl (red spectra). $NiCl_2$ was added to 4M NaCl up to 0.4 mM in the case of $(CG)_4$, $(GC)_{10}$, and $(GAC)_4$ and up to 0.6 mM in the case of $(CGA)_4$ and $(CAG)_4$. Spectra with the negative band around 295 nm correspond to Z-DNA. The figure shows that the 5´CG 3´step is crucial for Z-DNA formation.

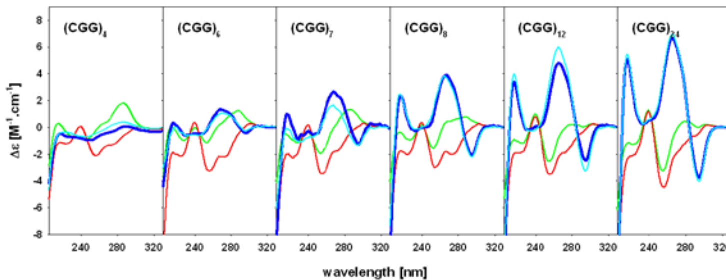

Figure 2: CD spectra of $(CGG)_n$ sequences measured in 1 mM sodium phosphate (pH 7), 0.3 mM EDTA (green), and in 10 mM sodium phosphate (pH 7) plus 5M NaCl (red). The following spectra were taken 2 days after 20 mM $NiCl_2$ addition (blue) and 6 days after 30 mM $NiCl_2$ addition (cyan). The figure shows that the likelihood of the Z-DNA formation increases with increasing number of the (CGG) repeats.

8-Oxoguanine in a quadruplex of the human telomere DNA sequence

8-Oxoguanine or 8-oxoG is a ubiquitous oxidative base lesion. We report in the paper the effect of this lesion on the structure and stability of quadruplexes formed by the human telomeric DNA sequence, $G_3(TTAG_3)_3$, in NaCl and KCl. CD, PAGE and absorption-based thermodynamic stability data show that replacement of any of the tetrad-forming guanine base by 8-oxoG does not hinder the formation of monomolecular, antiparallel quadruplexes in NaCl. The modified quadruplexes are, however, destabilized in both salts, the extent of which is depending on the position of the lesion.

These results and results of previous studies on guanine-to-adenine exchanges and guanine abasic lesions in the same quadruplex show a noticeable trend: it is not the type of the lesion but the position of the modification that determines the effect on the conformation and stability of the quadruplex. Type of lesion only governs the extent of changes, such as of destabilization. Most sensitive sites were found in the middle tetrad of the 3-tetrad quadruplex, and the smallest alterations were observed if guanines of the terminal tetrad with the diagonal TTA loop were substituted, although even these substitutions brought about unfavorable enthalpic changes. Interestingly, majority of these base-modified quadruplexes did not adopt the folding rearrangement induced in the unmodified $G_3(TTAG_3)_3$ by K^+ cations, an observation that could implicate biological relevance of the results.

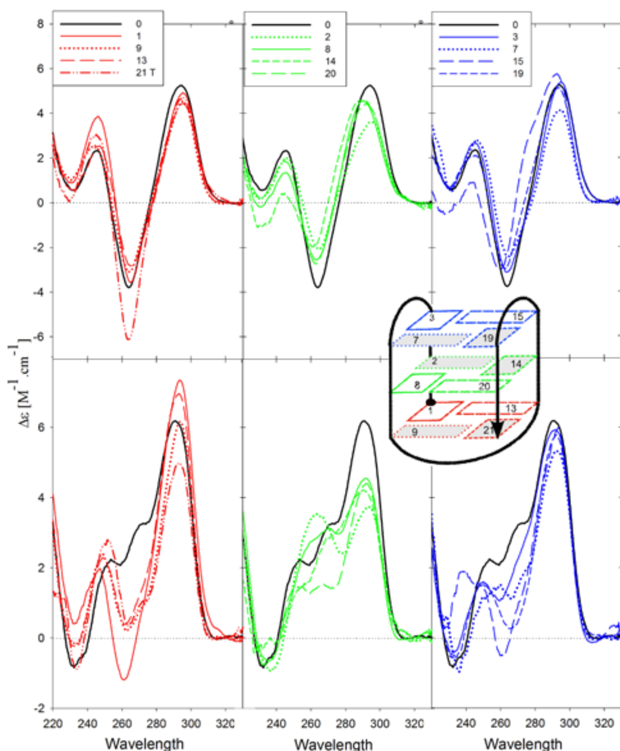

Figure 3: CD spectra of $G_3(TTAG_3)_3$ and its 8-oxoG analogs. CD spectra were measured in the R-B buffer, pH 7 and (upper panels) 0.1 M NaCl or (bottom panels) 0.1M KCl at 2°C. Black spectra correspond to unmodified $G_3(TTAG_3)_3$. The other spectra are drawn by the same line types as the respective substituted guanines in the antiparallel $G_3(TTAG_3)_3$ quadruplex shown in the insert. The figure shows that the presence of the 8-oxoG does not distinctly change conformation of the $G_3(TTAG_3)_3$ quadruplex in the presence of Na^+ ion. However the 8-oxo modified quadruplexes are unable to adopt quadruplex folding adopted by the unmodified $G_3(TTAG_3)_3$ in physiological K^+ solution.

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

Publications

Renciuk, D., Kypr, J., Vorlickova, M.: *CGG repeats associated with fragile X chromosome form left-handed Z-DNA structure.* Biopolymers, 95, 2011, 174-181.

Vorlickova, M., Tomasko, M., Sagi, A., Bednarova, K., Sagi, J.: *8-Oxoguanine in a quadruplex of the human telomere DNA sequence.* FEBS Journal, 279, 2012, 29-39.

Kypr, J., Kejnovska, I., Bednarova, K., Vorlickova, M.: *Circular dichroism spectroscopy of nucleic acids. In: Comprehensive Chiroptical Spectroscopy, Volume 2, 2011: Applications in Stereochemical Analysis of Synthetic Compounds, Natural Products, and Biomolecules, 1st Edition, N. Berova, P. L. Polavarapu, K. Nakanishi, and R. W. Woody, Eds. John Wiley & Sons, Inc.* pp. 573 - 584.

Plant Developmental Genetics

Head

Boris Vyskot

Research Fellows

Roman Hobza, Bohuslav Janoušek, Eduard Kejnovský, Zdeněk Kubát, Jiří Široký

Technicians

Magda Soukupová, Veronika Šlancarová

Graduate Students

Jiří Baloun, Hana Blavet-Kubeková, Radim Čegan, Tomáš Čermák, Vojtěch Hudzieczek, Jana Kandalcová, Viera Kováčová, Tereza Králová, Monika Michalovová, Lucie Najdekrová, Eva Nevrtalová, Pavlína Šteflová, Martina Taliánová

A large scale analysis of the nuclear genome of the model dioecious plant *Silene latifolia* was realised in collaboration with the laboratory of Dr. Jiří Macas from the Biological Center in České Budějovice. *Silene latifolia* (family Caryophyllaceae) is a dioceous plant possessing heteromorphic sex chromosomes and a sex determination system similar to the human type, with heterogametic male (XY) and homogametic female (XX) individuals. It has an intermediate genome size, which differs between males (5.85 Gb/2C) and females (5.73 Gb/2C) due to the unequal size of the X and Y chromosomes. A substantial fraction of the *S. latifolia* genome is composed of repetitive DNA and this type of genomic sequence is also supposed to be involved in the differentiation of its sex chromosomes. Indeed, several repeats with specific localization patterns have been identified on the sex chromosomes, including satellite and simple sequence repeats and plastid DNA. To date, the most complex analysis of repetitive DNA was performed by sequencing 379 clones from a *S. latifolia* shortinsert genomic library and subsequent FISH analysis of selected clones. In addition to identification of the chromosome Y-specific tandem repeat STAR-Y, this study revealed depletion of Ogre-like retrotransposon sequences from the non-recombining part of the Y chromosome. Despite this progress, there remains a lack of detailed sequence and quantitative information about the global repeat composition of the *S. latifolia* genome, similar to that available for

extensively sequenced model species. The introduction of next generation sequencing (NGS) technologies, based on the fast and cost-efficient parallel processing of millions of templates, has revolutionized many areas of the current life sciences. The power of NGS, which can generate up to gigabases of sequence data in a single run, has presented new opportunities for the investigation of highly complex populations of repetitive elements in plant genomes. The procedure is cloning-free, thus avoiding the potential bias caused by difficulties in propagating some repeat types in bacteria, and provides random sequence sampling across the genome.

We performed low-pass 454 sequencing followed by similarity-based clustering of 454 reads in order to identify and characterize sequences of all major groups of *S. latifolia* repeats. Illumina sequencing data from male and female genomes were also generated and employed to quantify the genomic proportions of individual repeat families. The majority of identified repeats belonged to LTR-retrotransposons, constituting about 50% of genomic DNA, with Ty3/gypsy elements being more frequent than Ty1/copia. While there were differences between the male and female genome in the abundance of several repeat families, their overall repeat composition was highly similar. Specific localization patterns on sex chromosomes were found for several satellite repeats using *in situ* hybridization with probes based on k-mer frequency analysis of Illumina sequencing data. This study provides comprehensive information about the sequence composition and abundance of repeats representing over 60% of the *S. latifolia* genome. The results revealed generally low divergence in repeat composition between the sex chromosomes, which is consistent with their relatively recent origin. In addition, the study generated various data resources that are available for future exploration of the *S. latifolia* genome (Figure 1).

We also studied nuclear horizontal gene transfer (HGT) in flowering plants with an emphasis on tobacco *(Nicotiana tabacum)*, its close *Nicotiana* relatives, and other Solanaceae. There are several reasons why we focused our attention on the representatives of this family. Although the frequency of HGT in eukaryotes has been assumed to be very low, there are several reports of foreign genes from various donors (bacteria and viruses) being incorporated in the genome of solanaceous species. Moreover, in some species, foreign insertions have been experimentally investigated in detail, providing valuable clues about the mechanisms of HGT. So far, the detection of numerous natural nuclear HGT events within one taxonomic group is rather exceptional (in contrast to HGT of mitochondrial genes).

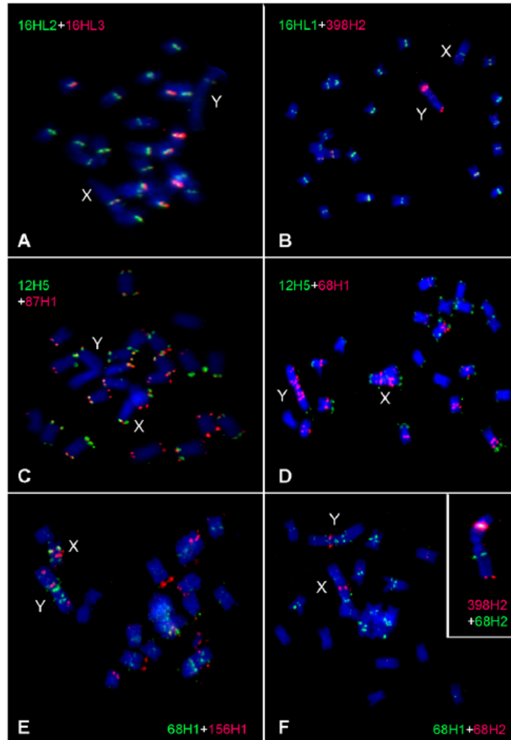

Figure 1: Localization of satellite repeats on metaphase chromosomes of *S. latifolia*. FISH experiments were performed simultaneously with two probes labeled by different fluorochromes (red or green, as indicated) in order to investigate the co-localization of sequence variants of the STAR repeats (A–B), 15Ssp and X43.1 (C) and TRAYC-like repeats (D–F). (A) Co-localization of the CL16/STAR-C repeat consensus (16HL2) and its sequence variant (16HL3). (B) The probes for a different region of the CL16/STAR-C consensus (16HL1) and for the chromosome Y-enriched subfamily CL398/STAR-Y (398H2). (C) Consensus probes for CL87/15Ssp (87H1) and X43.1 (12H5) satellites. Co-localization of the CL68/TRAYC-like consensus (68H1) with X43.1 (12H5) (D), CL156 (156H1) (E) and with the CL68 subfamily (68H2) (F). The inset in (F) is an example of the Y chromosome hybridized to 68H2 and 398H2, showing the localization of their interstitial signals on different chromosome arms. The chromosomes were counterstained with DAPI (blue). Sex chromosomes are indicated with X and Y.

The greater number of examples in solanaceous species may be largely due to the fact that many of these species are economically important and more attention has been focused on their investigation. In addition, in-depth

experimental studies related to various HGT issues have been performed on tobacco, which is also one of the best model species in transgenic research. We summarize the studies concerning cases of natural HGT involving various donors (bacteria and viruses) as well as experiments on HGT mechanisms using artifi cially transformed tobacco. The ability to detect and study horizontal gene transfer events is of significant importance to our understanding of its effect on the evolution of eukaryotic genes and genomes. We performed phylogenetic analysis of a published anti-bacterial protein AP1 from potato *(Solanum tuberosum)*. One domain encodes a phosphoesterase with high similarity to an acid phosphatase of Ralstonia solanacearum and closely related Betaproteobacteria. The second domain encodes an UspAlike domain similar to those present in plants. Our phylogenetic analyses suggest that both domains evolved along different evolutionary pathways until they merged into a single gene. We propose that the phosphoesterase domain was acquired by HGT. Our results support claims in favor of HGT detection at the protein domain level. The case of antibacterial protein AP1 in potato highlights the significance of gene fusion/protein domain fusion as an important feature of horizontal gene transfer which may contribute substantially to the adaptive abilities of eukaryotic organisms (Figure 2).

Sexual dimorphism (the systematic difference in form or other trait(s) not present in sexual organsbetween individuals of different sex in the same species) is a widely studiephenomenon inanimal models and in humans. Much less is known about sexual dimorphism in vascular dioeciousplants. Among vascular plants displaying sexual dimorphism, *Silene latifolia* is (together with *Fragaria virginiana*) the most studied species. The first study on sexually dimorphic traits in *S. latifolia* was performed already in the 19th century, and since that time many sexually dimorphic traits have been described. However, the only known genes involved in sexual dimorphism are those involved in the control of flower development. During flower development, sexual dimorphism starts to occur very early. At the morphological level, the central zone of the floral meristem is significantly smaller in males than in females. This is caused by cell division arrest in male tissues. The difference between male and female flower bud morphology is preceded by differences at the gene expression level. Developmental pathways involved in the switch of male or female flower program have been also identified. We re-tested the expression patterns of 22 available *S. latifolia* ESTs previously described according to Northern blots or Virtual Northerns as preferentially expressed in male flowers and/or early stamen.

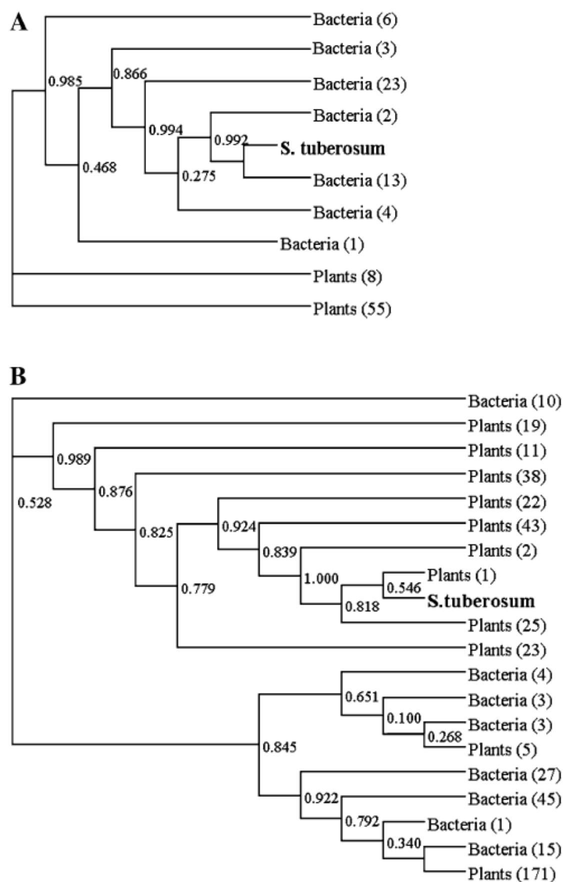

Figure 2: Reduced FastTree phylogenetic tree of (A) the phosphoesterase domain, based on alignment of plant (angiosperms, gymnosperms, bryophytes) and bacterial (order Burkholderiales: *Ralstonia solanacearum, R. metallidurans, R. pickettii, Burkholdia xenovorans, B. graminis*, and *B. glumae*; and *Neisseriales*: *Chromobacterium violaceum*) phosphoesterase domain family members, and (B) the Usp domain based on alignment of selected plant (sequences from completed genomes: *Arabidopsis thaliana, Medicago truncatula, Oryza sativa, Populus trichocarpa, Vitis vinifera, Zea mays*, and *Ricinus communis*) and bacterial (sequences from order Burkholderiales: *R. solanacearum, R. metallidurans, R. pickettii, B. xenovorans, B. graminis*, and *B. glumae*; and *Neisseriales*: *C. violaceum*) Usp domain family members. Numbers in parentheses represent the number of sequences that have been reduced to create the final branch.

Fewer genes than previously claimed have expression limited to male flower buds suggesting the importance of RT-PCR analyses in this case. Only six out of 15 genes previously described as male flower bud specific were expressed in male flower buds only and not in the leaves or in female flower buds. Two genes, originally described as male flower bud specific, were expressed in male flower buds earlier than in female flower buds. Twelve genes were expressed in all samples tested. We also found one EST (Men-470) expressed exclusively in male flower buds and leaves and one EST (CCLS79.1) expressed exclusively in female flower buds and leaves. We also serendipitously found one new 550 bp long EST as a "by-product" of PCR amplification of Men-262 and named it Serendip2. Serendip2 was expressed exclusively in male flower buds and leaves. To elucidate the discrepancy between the expression patterns found here and those presented in previous research, we performed a homology search of the studied ESTs followed by a search for putative orthologous sequences in *Arabidopsis thaliana* and their expression patterns according to Genevestigator V3. We found that the gene expression patterns described in A. thaliana were not in a contradiction to our results. We present RT-PCR-based evidence that in *S. latifolia*, sexual dimorphism in gene expression is present long before the initiation of flowering. We describe three ESTs that show sex-specific (two male specific and one female specific) transcription at the rosette stage before the first flowering season. To our knowledge, this study provides the first molecular evidence of early pre-flowering sexual dimorphism in angiosperms (Figure 3).

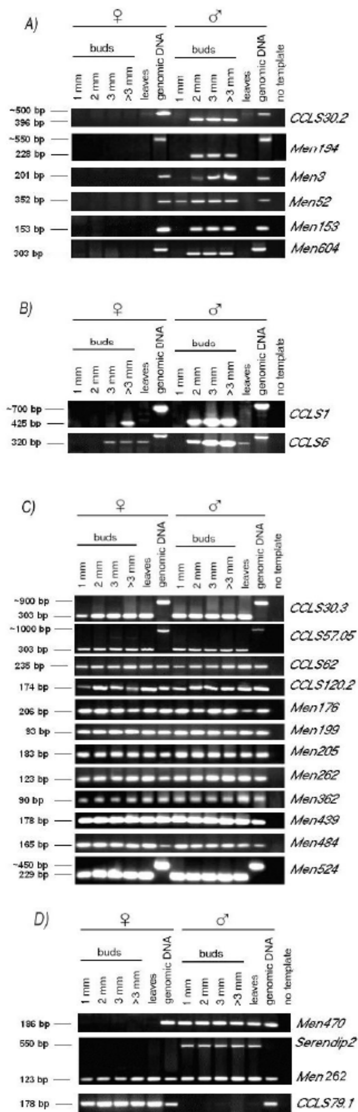

Figure 3: RT-PCR analyses of all the studied ESTs. Expression was investigated by RT-PCR analysis on the tissues indicated above each lane. The ESTs amplified are indicated on the right. Male buds of two mm length represent the stage when meiosis starts in anthers. Female meiosis starts in eight mm long female buds. (A) ESTs expressed exclusively in male flower buds. (B) ESTs starting to be expressed in male flower buds

earlier than in female flower buds. (C) ESTs expressed in leaves and flower buds of both sexes. (D) ESTs showing sex specific expression in all the studied tissues. Two of them (Serendip2, and Men-470) are showing male specific expression while CCLS79.1 gene shows female specific expression. Men-262 is included to illustrate that Serendip2 is amplified with the same pair of primers and it serves also as a proof of the sufficient quality of templates.

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 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

Cioffi, M.B, Kejnovsky, E, Bertollo, L.A.C.: *The chromosomal distribution of microsatellite repeats in the wolf fish genome Hoplias malabaricus, focusing on the sex chromosomes*. Cytogenetics And Genome Research, 132, 2011, 289-296.

Neumann, P., Navrátilová, A., Koblížková, A., Kejnovský, E., Hřibová, E., Hobza, R., Widmer, A., Doležel, J., Macas, J.: *Plant centromeric retro-transposons: A structural and cytogenetic perspective*. Mobile DNA 2, 2011, e4.

Talianová, M., Janoušek, B.: *What can we learn from tobacco and other Solanaceae about horizontal DNA transfer?* American Journal Of Botany 98, 2011, 231-1242.

Talianová, M., Vyskot, B., Janoušek, B.: *Interkingdom protein domain fusion: the case of an antimicrobial protein in potato (Solanum tuberosum).* Plant Systematics And Evolution, 297, 2011, 129-139.

Pokorná, M., Kratochvíl, L., Kejnovský, E.: *Microsatellite distribution on sex chromosomes at different stages of heteromorphism and heterochromatinization in two lizard species (Squamata: Eublepharidae: Coleonyx elegans and Lacertidae: Eremias velox)*. BMC Genetics, 12, 2011, e90.

Macas, J., Kejnovský, E., Neumann, P., Novák, P., Koblížková, S., Vyskot, B.: *Next-generation sequencing resources for the model dioceous plant Silene latifolia*. PLoS ONE 6, 2011, e27335.

Čermák, T., Doyle, E.L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, J.A., Somia, N.V., Bogdanove, J.A., Voytas, D.F.: *Efficient design and assembly of custom TALEN and other TAL effector based constructs for DNA targeting.* Nucleic Acids Research, 39, 2011, e82.

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: Photobiology

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

Jiřina Hofmanová, Alois Kozubík: Health risks

Jiřina Hofmanová, Alois Kozubík: Genotoxicity and carcinogenesis

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: Special methods of microorganisms analysis I.

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, Karel Souček, Jan Vondráček: Modern methods of cell biology

Lukáš Kubala, Antonín Lojek, Milan Číž: Special physiology of blood

Antonín Lojek, Milan Číž, Lukáš Kubala: Immunology

Arnošt Mládek: Introduction to Biophysics

Arnošt Mládek: Basics of molecular biophysics

Arnošt Mládek: Physics for chemists II, seminar

Olga Nováková: Selected themes of application biophysics

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

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

Karel Souček: Journal club - Cancer biology I, II

Karel Souček, Eva Lincová, Alena Vaculová: Analytical cytometry - practical course

Jiří Šponer: Basics of molecular biophysics

Jiří Šponer: Molecular interactions in biology and chemistry

Jiří Šponer: Structure and dynamics of nucleic acids

Alena Hyršlová Vaculová: Mechanisms of cell death, function, methods

Alena Hyršlová Vaculová: Molecular biology and genetics

Alena Hyršlová Vaculová: Scientific work methodics

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: English 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 I

Boris Vyskot: Genetic engineering II

University of Veterinary and Pharmaceutical Sciences Brno

Marie Brázdová: Biochemistry (Lectures in Czech)

Marie Brázdová: Biochemistry (Lectures in English)

Eduard Kejnovský, Roman Hobza: Evolutionary biology

Comenius University in Bratislava

Veronika Ostatná: Medical biophysics

<u>University of South Bohemia in České Budějovice</u>

Roman Hobza, Eduard Kejnovský: Evolutionary genomics

<u>University of Ostrava</u>

Boris Vyskot, Jiří Široký: Developmental biology

<u>Brno University of Technology</u>

Miroslav Fojta: Molecular biology

Aleš Kovařík: Basic bioinformatics

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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 data update, development and maintenance of computer hardware and software jointly used by all laboratories (servers and 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 2011 there was installed a new fileserver with disk array and tape library. The fileserver is used as the secure data storage available to all scientist of the IBP. Disk array is connected redundantly to a pair of fiber

channel switches and so it is accessible to all servers connected to fiber channel network. A backup software was installed and user data are regularly copied to tapes in tape library connected by fiber channel.

There was resolved project “Implementation of IPv6 protocol in networks of institutes of the AS CR Brno” (FR CESNET-proj.no. 368R1/2010). IBP now has its IPv6 address space - 2001:67c:1222:0000::/56. Main network services of IBP (DNS, mail, web and samba in LAN) are now accessible over IPv6 protocol, the IPv6 to IPv4 tunneling is under control. IPv6 network activities are monitored in the same way as IPv4 activities by NetFlow.

VMware virtualization software was upgraded to vSphere 5.

Conferences and Workshops Organized and Co-Organized by the Institute

Conference **"Analysis of DNA Damage by Metal-Based Compounds"**, COST Working Group Meeting of D39/04/06 in Brno at the Institute of Biophysics, Academy of Sciences of the Czech Republic, v.v.i. (May 13-14, 2011).
The participants (22) presented their latest results of the studies focused on molecular mechanisms of new anticancer metallodrugs, had critical discussions and planned further publications.

COST D39 Final Whole Action Meeting **„Metallo-Drug Design and Action"**, Dublin, Royal College of Surgeons in Ireland (July 5-6, 2011).
The participants (~150) presented overwiev of their research in the last five years focused on problems of medicinal inorganic chemistry, which offers real possibilities to pharmaceutical industries for the discovery of truly novel drugs with new mechanisms of action.

LEICA Confocal and De-Convolution Workshop, Brno, (November 8, 2011)
Participants (30) presented their results in the field of contemporary confocal microscopy and superresolution.

Workshop on Animal Physiology and Immunology, Brno (June 23-24, 2011)
The meeting was organized as a conference of PhD students with scientists' supervision. The participants (23) presented their results related to biology and physiology of phagocytes, comparative innate immunity, cell and molecular physiology, and modulation of physiological and immune processes.

Institute of Biophysics of the Academy of Sciences of the Czech Republic, v.v.i.

Královopolská 135
612 65 Brno
Czech Republic

Phone: +420-541 517 111
Fax: +420-541 211 293
www.ibp.cz