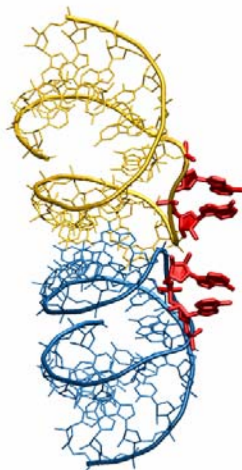
INSTITUTE OF BIOPHYSICS

ACADEMY OF SCIENCES OF THE CZECH REPUBLIC



RESEARCH REPORT 2008

IBP AS CR, BRNO 2008

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Introduction

The year 2008 was the first in which a new legal entity (public research institution) of the Institute of Biophysics AS CR, v.v.i. (IBP) was functioning all year long, including activities of the newly elected Council, the newly appointed Director, and the new Supervisory Board. The new legal form of the Institute enabled its management to improve substantially the tools of evaluation of the departments and to stimulate the best groups. We can conclude that the new legal form provided a higher flexibility, which will lead to a better performance of the IBP.

In 2008, both the Institute and its Research Plan (RP) were evaluated by the Academic Evaluation Committee. The Institute was given the best possible results (A) in both evaluations. The Committee stated that "the IBP is continuously keeping its leading position among the institutes of the 5th section of the AS CR being at a high EU level." "The success of the Institute is due to the appropriate strategy of its management including economic stimulations, reorganization of less successful parts of the Institute etc." "In this direction, the Institute can be an example for other institutes." The Committee appreciated organizational changes that led to creation of highly efficient and equally productive teams. The main recommendations of the Committee were gradual growth of the number of publications in highly impacted journals (IF>5) and keeping equally productive departments.

The institutional evaluation of newly created departments showed that the productivity variations actually decreased substantially: 1.7 times in 2008 as compared to 5 times in 2007. In the evaluation, the departments of V. Brabec, S. Kozubek, J. Sponer, A. Kovarik and B. Vyskot obtained the best results. The departments of V. Brabec, and A. Kovarik achieved an increased cumulative IF; in case of A. Kovařík also the number of citations increased substantially. The department of B. Vyskot achieved a lower cumulative IF compared to 2007, however, this difference was not significant, particularly considering the fact that the scientists of this department participated in preparation of 3 publications with IF>5. Also the department of J. Šponer achieved a lower cumulative IF (IF=24 in 2007 as compared to IF=8 in 2008). We believe that this difference represents just a stochastic fluctuation. Owing to high productivity of the department in the previous years, its position in the evaluation is still very high.

The departments of M. Vorlickova, M. Fojta, A. Lojek, A. Kozubik and the group of J. Fajkus achieved average results (in terms of our Institute). The department of A. Kozubik continuously increases its number of citations. The cumulative IF slightly increased; however, we highly appreciate that the department participated in preparation of a number of publications with IF>5. A higher proportion of publications with a larger number of scientists from the department would be appreciated in the next years. A further improvement was achieved by the department of A. Lojek – the record of its citations increases dramatically and the value of cumulative IF is historically on its maximum value (IF=10). We believe that this tendency of further growth will become a reality. A lower position of the department of M. Fojta in the evaluation in year 2008 was given by a decrease of the number of citations in the period considered, however, it is also due to involvement of grant resources into the evaluation, which does not reflect correctly the reality and requires changes in the algorithm of the evaluation. In case of the department of M. Fojta, we highly appreciate a large number of publications with IF>5. The group of J. Fajkus improved substantially and presented a number of publications for the evaluation with the total impact for the Institute approaching the standard values typical of other teams of similar size (IF=5). This is the highest value of the group in the period of evaluation (2005-2008). The department of M. Vorlickova, if evaluated separately, produces results similar to the other departments.

The groups inside departments are generally highly productive. A lower effectiveness was found with the groups of M. Hofer, M. Stros, B. Brzobohaty and J. Kypr. At the beginning of the year 2008, these groups were joined with the better functioning departments and the form of their further existence should be considered. The group of M. Stros was joined with the group of J. Fajkus, however, its separation and transformation into a new department were not recommended. Additional organizational changes were suggested and performed in case of the groups of B. Brzobohaty and J. Kypr.

The group of B. Brzobohaty was joined with the department of A. Kovarik at the beginning of 2008. However, it did not lead to a sufficiently fast improvement and, therefore, we proposed another solution. On the basis of a mutual agreement, a joint laboratory of the IBP and Mendel University of Agriculture and Forestry in Brno has been founded. The laboratory will be headed by B. Brzobohaty. The laboratory will be located in Mendel University, which will cover the main part of its running costs. The IBP will contribute to the running costs of the laboratory. The group of J. Kypr was

joined with the department of M. Vorlickova at the beginning of 2008. Owing to its low performance in 2008, the group was reduced. Further organizational changes will be considered in 2009 in accordance with the productivity of the department.

At the beginning of 2009, evaluation of research and development in the Czech Republic developed by the Council for Research and Development was performed. The results of this evaluation will be used for calculation of the financial resources for research organizations in 2010. The evaluation used citations (IF) as a quantitative measure of the quality of publications inside various scientific fields, however, among them a rather arbitrary scale was taken that ensures equal results for different disciplines for an „average scientist in the field“. It is obvious that such an approach is not consistent and on the contrary to the requirement of excellent science (journals with a very low IF can be on the top in some fields). In this evaluation, applied results, such as patents or licences, are involved, using relatively high weights, which provide a possibility to get "soft results" for some organizations. In spite of the fact that the evaluation does not correspond to the mission of the IBP, the Institute achieved relatively good results also in this evaluation.

In 2008, 2 conferences were organized by the scientists of the IBP. A satellite symposium to the conference of ESEAC 2008 entitled "Electrochemistry of Nucleic Acids and Proteins" Brno, June 19-22, 2008, was organized by the Centre of Biophysical Chemistry, Bioelectrochemistry and Bioanalysis, which is coordinated by the IBP. The Department of Free Radical Pathophysiology organized the 3rd European Workshop on the "Analysis of Phagocyte Functions", Brno, May 22-23, 2008. Both conferences were very successful and a number of famous scientists took part in the work on these events.

As mentioned in the previous reports, the Institute participates in preparation of the two Major projects, CEITEC and CESLAB, in close cooperation with the central management of the Academy of Sciences of the Czech Republic (AS CR). We expect that both projects will be prepared for the first call in June 2009.

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Cytotoxicity, mutagenicity, cellular uptake, DNA and glutathione interactions of lipophilic trans-platinum complexes tethered to 1-adamantylamine

Cytotoxicity and mutagenicity of *trans,trans,trans*-[$PtCl_2(CH_3COO)_2(NH_3)$(1-adamantylamine)] [trans-adamplatin(IV)] and its reduced analog *trans*-[$PtCl_2(NH_3)$(1-adamantylamine)] [trans-adamplatin(II)] were examined. In addition, the several factors underlying biological effects of these trans-platinum compounds using various biochemical methods were investigated. A notable feature of the growth inhibition studies was the remarkable circumvention of both acquired and intrinsic cisplatin resistance by the two lipophilic trans-compounds. Interestingly, trans-adamplatin(IV) was considerably less mutagenic than cisplatin. Consistent with the lipophilic character of trans-adamplatin complexes, their total accumulation in A2780 cells was considerably greater than that of cisplatin. The results also demonstrate that trans-adamplatin(II) exhibits DNA binding mode markedly different from that of ineffective

transplatin. In addition, the reduced deactivation of trans-adamplatin(II) by glutathione seems to be an important determinant of the cytotoxic effects of the complexes tested in the present work. The factors associated with cytotoxic and mutagenic effects of trans-adamplatin complexes in tumor cell lines examined in the present work are likely to play a significant role in the overall antitumor activity of these complexes.

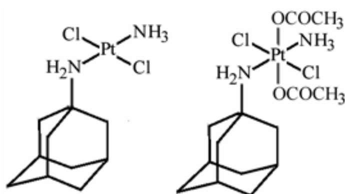

Figure 1: Structures of trans-adamplatin(II) and trans-adamplatin(IV).

Amide-based prodrugs of spermidine-bridged dinuclear platinum. Synthesis, DNA binding, and biological activity

The chemistry and biology of acetyl-protected spermidine-bridged dinuclear platinum complexes [{*trans*-$PtCl(NH_3)_2$]$_2$-*μ*-$NH_2(CH_2)_3N(COR)(CH_2)_4NH_2]X_2$ (R = H, X = Cl (1,1/t,t-spermidine, BBR3571); R =CH_3 , X = Cl (**2**); R = CH_2Cl, X = ClO_4 (**3**); R = CF_3 , X = Cl (**4**)) are compared with their carbamate analogues. The compounds are potential prodrugs for the parent compound **1**, a highly potent antitumor agent. At pH 6–8 hydrolysis of the blocking group with the release of the "parent" protonated species follows the order **4** > **3** >>**2**. For **4**, rate constants for the deprotection increase in this pH range. The DNA binding profile of **4** is similar to the Boc derivative, confirming the central influence of charge on DNA binding properties. The differences in cytotoxicity for the protected compounds in ovarian carcinoma cell lines sensitive and resistant to cisplatin [*cis*-diamminedichloridoplatinum(II)] cannot completely be explained by spontaneous release of 1,1/t,t spermidine at physiological pH. Inherent cytotoxicity and cell line specificity may contribute to the observed behavior. The properties of the compounds present them also as possible "second-generation" analogues of the clinically relevant trinuclear complex [{*trans*-$PtCl(NH_3)_2$}$_2$-*μ*-*trans*-$Pt(NH_3)_2(NH_2(CH_2)_6NH_2)_2](NO_3)_4$, (BBR3464).

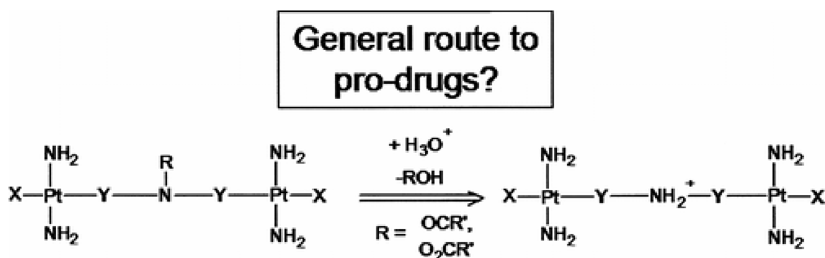

Figure 2: Possible "second-generation" analogues of the clinically relevant trinuclear complex [{*trans*-$PtCl(NH_3)_2$}$_2$-µ-*trans*-$Pt(NH_3)_2(NH_2(CH_2)_6NH_2)_2](NO_3)_4$ (BBR3464).

Unique properties of DNA interstrand cross-links of antitumor oxaliplatin and the effect of chirality of the carrier ligand

The different antitumor and other biological effects of the third generation antitumor platinum drug oxaliplatin [(*1R*,*2R*-diaminocyclo-hexane)oxalatoplatinum(II)] in comparison with those of conventional cisplatin are often explained by the ability of oxaliplatin to form DNA adducts of different conformation and consequently to exhibit different cytotoxic effects. This work describes, for the first time, the structural and biochemical characteristics of the interstrand CLs of oxaliplatin. We find that: 1) DNA bending, unwinding, thermal destabilization, and delocalization of the conformational alteration induced by the CL of oxaliplatin are greater than those observed with the CL of cisplatin; 2) the affinity of high-mobility-group proteins (which are known to mediate the antitumor activity of platinum complexes) for the interstrand CLs of oxaliplatin is markedly lower than for those of cisplatin; and 3) the chirality at the carrier 1,2 diaminocyclohexane ligand can affect some important structural properties of the interstrand CLs of cisplatin analogues. Thus, the information contained in the present work is also useful for a better understanding of how the stereochemistry of the carrier amine ligands of cisplatin analogues can modulate their anticancer and mutagenic properties. The significance of this study is also reinforced by the fact that, in general, interstrand CLs formed by various compounds of biological significance result in greater cytotoxicity than is expected for monofunctional adducts or other intrastrand DNA lesions. Therefore, we suggest that the unique properties of the interstrand CLs of oxaliplatin are at least partly responsible for this drug´s unique antitumor effects.

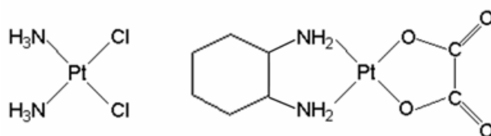

Figure 3: Structures of cisplatin and oxaliplatin.

DNA interactions of monofunctional organometallic osmium(II) antitumor complexes in cell-free media

This work is the first in-depth study of osmium binding to DNA and confirms the pharmacological activity of a new class of anticancer metallodrugs. We investigated the interactions between the potential biological target DNA and four osmium(II) arene complexes, of the type $[(\eta^6\text{-arene})Os(LL)Cl]^{n+}$, where arene = biphenyl or *p*-cymene and LL = ethylenediamine, picolinate, or oxinate in an effort to understand their mechanism of action. Most notably we show that these complexes bind to DNA. DNA adducts of the Os^{II} complexes that exhibit promising cytotoxic effects in ovarian tumor cell lines largely distort its conformation. The data are consistent with DNA binding of the complexes containing biphenyl as the arene ligand that involves combined coordination to guanine residues and noncovalent interactions between the arene ligand and DNA. The results also indicate both a mechanism of action and a detoxification mechanism for Os^{II} arene compounds different from those of cisplatin.

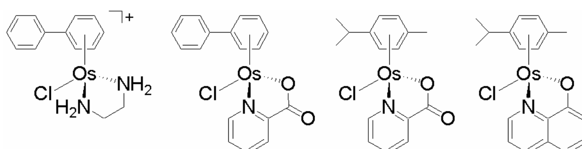

Figure 4: Structures of organometallic Os^{II}-arene antitumor complexes.

DNA binding of dinuclear iron(II) metallosupramolecular cylinders. DNA unwinding and sequence preference

$[Fe_2L_3]^{4+}$ ($L = C_{25}H_{20}N_4$) is a synthetic tetracationic supramolecular cylinder (with a triple helical architecture) that targets the major groove of DNA and can bind to DNA Y-shaped junctions. To explore the DNA-binding mode of $[Fe_2L_3]^{4+}$, we examine herein the interactions of pure enantiomers of this cylinder with DNA by biochemical and molecular biology methods. The results have revealed that, in addition to the previously reported bending of DNA, the enantiomers extensively unwind DNA, with the M enantiomer being the more efficient at unwinding, and exhibit preferential binding to regular alternating purine–pyrimidine sequences, with the M enantiomer showing a greater preference. Also, interestingly, the DNA binding of bulky cylinders $[Fe_2(L\text{-}CF_3)_3]^{4+}$ and $[Fe_2(L\text{-}Ph)_3]^{4+}$ results in no DNA unwinding and also no sequence preference of their DNA binding was observed. The observation of sequence-preference in the binding of these supramolecular cylinders suggests that a concept based on the use of metallosupramolecular cylinders might result in molecular designs that recognize the genetic code in a sequence-dependent manner with a potential ability to affect the processing of the genetic code.

Interaction of dinuclear ruthenium(II) supramolecular cylinders with DNA: Sequence-specific binding, unwinding, and photocleavage

Metallosupramolecular chemistry was used to design a new class of synthetic agents, namely, tetracationic supramolecular cylinders, that bind strongly and noncovalently in the major groove of DNA. To gain additional information on interactions of the cylinders with DNA we explored DNA unwinding and sequence-specific binding properties, as well as DNA photonuclease activity of ruthenium(II) metallosupramolecular cylinder $[Ru_2L_3]^{4+}$, where L is a bis-pyridyl-imine ligand. We found that $[Ru_2L_3]^{4+}$ unwinds negatively supercoiled plasmid DNA and exhibits binding preference to regular alternating purine–pyrimidine sequences in a similar way to the $[Fe_2L_3]^{4+}$ analogue. Photocleavage studies showed that, unlike $[Fe_2L_3]^{4+}$, $[Ru_2L_3]^{4+}$ induces single-strand breaks on irradiation by visible and UVA light and cleaves DNA mainly at guanine residues contained preferentially in regularly alternating purine pyrimidine nucleotides. As $[Ru_2L_3]^{4+}$ binds and cleaves DNA in a sequence-dependent manner, it may provide a useful tool for basic and applied biology, such as for controlled

manipulation of the genome.

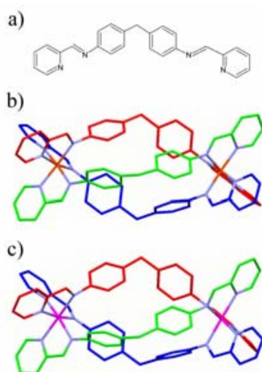

Figure 5: a) Structure of the ligand in the metallosupra-molecular cylinders $[Fe_2L_3]^{4+}$ and $[Ru_2L_3]^{4+}$ ($L=C_{25}H_{20}N_4$). b) Structure of the cylinder $[Fe_2L_3]^{4+}$. c) Structure of the cylinder $[Ru_2L_3]^{4+}$. Structures shown in Figures 4b and 4c illustrate three-dimensional structure of the cylinders determined by X-ray crystallography.

Binding of mismatch repair protein MutS to mispaired DNA adducts of intercalating ruthenium(II) arene complexes

The present study was performed to examine the affinity of Escherichia coli mismatch repair (MMR) protein MutS for DNA damaged by an intercalating compound. We examined the binding properties of this protein with various DNA substrates containing a single centrally located adduct of ruthenium(II) arene complexes [(η^6-arene)Ru(II)(en)Cl][PF_6] [arene is tetrahydroanthracene (THA) or p-cymene (CYM); en is ethylenediamine]. These two complexes were chosen as representatives of two different classes of monofunctional ruthenium(II) arene compounds which differ in DNA-binding modes: one that involves combined coordination to G N7 along with noncovalent, hydrophobic interactions, such as partial arene intercalation (tricyclic-ring Ru-THA), and the other that binds to DNA only via coordination to G N7 and does not interact with double-helical DNA by intercalation (monoring Ru-CYM). Using electrophoretic mobility shift assays, we examined the binding properties of MutS protein with various DNA duplexes (homoduplexes or mismatched duplexes) containing a single centrally located adduct of Ru^{II} arene compounds. We have shown that presence of the Ru^{II} arene adducts decreases the affinity of MutS for ruthenated DNA duplexes that either have a regular sequence or contain a

mismatch and that intercalation of the arene contributes considerably to this inhibitory effect. Since MutS initiates MMR by recognizing DNA lesions, the results of the present work support the view that DNA damage due to intercalation is removed from DNA by a mechanism(s) other than MMR.

Biophysical studies on the stability of DNA intrastrand CLs of transplatin

Clinically ineffective transplatin [*trans*-diamminedichlorido-platinum(II)] is used in the studies of the structure-pharmacological activity relationship of platinum compounds. In addition, a number of transplatin analogs exhibit promising toxic effects in several tumor cell lines including those resistant to conventional antitumor cisplatin. Moreover, transplatin-modified oligonucleotides have been shown to be effective modulators of gene expression. Owing to these facts and because DNA is also considered the major pharmacological target of platinum complexes, interactions between transplatin and DNA are of great interest. We examined, using biophysical and biochemical methods, the stability of 1,3-GNG intrastrand CLs formed by transplatin in short synthetic oligodeoxyribonucleotide duplexes and natural double-helical DNA. We have found that transplatin forms in double-helical DNA 1,3-GNG intrastrand CLs, but their stability depends on the sequence context. In some sequences the 1,3-GNG intrastrand CLs formed by transplatin in double-helical DNA readily rearrange into interstrand CLs. On the other hand, in a number of other sequences these intrastrand CLs are relatively stable. We show that the stability of 1,3-GNG intrastrand CLs of transplatin correlates with the extent of conformational distortion and thermodynamic destabilization induced in double-helical DNA by this adduct.

Cytotoxicity, cellular uptake, and DNA interactions of new monodentate ruthenium(II) complexes containing terphenyl arenes

We have compared the cancer cell cytotoxicity, cell uptake, and DNA binding properties of the isomeric terphenyl complexes $[(\eta^6\text{-arene})Ru(en)Cl]^+$, where the arene is ortho- (**5**), meta- (**6**), or para-terphenyl (**7**) (o-, m-, or p-terp). Complex **7**, the X-ray crystal structure of which confirms that it has the classical “piano-stool” geometry, has a similar potency to cisplatin but is not cross-resistant and has a much higher activity than **5** or **6**. The extent of Ru uptake into A2780 or A2780cis cells does not

correlate with potency. Complex **7** binds to DNA rapidly and quantitatively, preferentially to guanine residues, and causes significant DNA unwinding. Circular and linear dichroism, competitive binding experiments with ethidium bromide, DNA melting, and surface-enhanced Raman spectroscopic data are consistent with combined intercalative and monofunctional (coordination) binding mode of complex **7**. This unusual DNA binding mode may therefore make a major contribution to the high potency of complex.

Synthesis, biophysical studies, and antiproliferative activity of platinum(II) complexes having 1,2-bis(amino-methyl)carbobicyclic ligands

A selected chemical library of six platinum(II) complexes having 1,2 bis(aminomethyl)carbobicyclic ligands were synthesized after a rational design in order to evaluate their antiproliferative activity and the structure–activity relationships. The cytotoxicity studies were performed using cancer cell lines sensitive (A2780) and resistant (A2780R) to cisplatin. Excellent cytotoxicity was observed for most of complexes, which presented better resistance factors than cisplatin against the A2780R cell line. The interaction of these complexes with DNA, as the target biomolecule, was evaluated by several methods: DNA-platinum binding kinetics, changes in the DNA melting temperature, evaluation of the unwinding angle of supercoiled DNA, evaluation of the interstrand CLs, and replication mapping. The kinetics of the interaction with glutathione was also investigated to better understand the resistant factors observed for the new complexes.

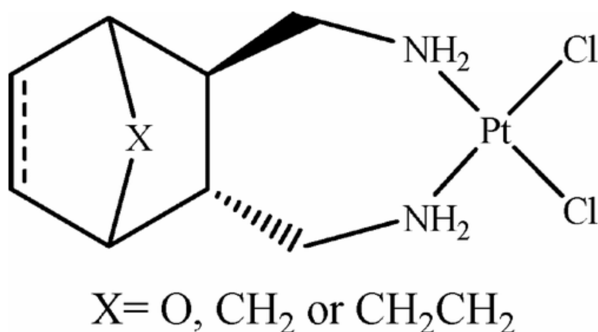

Figure 6: Structure of Pt^{II} complexes having 1,2-bis(aminomethyl)carbo-bicyclic ligands.

The contrasting chemistry and cancer cell cytotoxicity of bipyridine and bipyridinediol ruthenium(II) arene complexes

The synthesis and characterization of ruthenium(II) arene complexes [(η^6-arene)Ru(N,N)Cl]$^{0/+}$, where N,N = 2,2′-bipyridine (bipy), 2,2′-bipyridine-3, 3′-diol (bipy(OH)$_2$) or deprotonated 2,2′-bipyridine-3, 3′-diol (bipy(OH)O) as N,N-chelating ligand, arene = benzene (bz), indan (ind), biphenyl (bip), *p*-terphenyl (*p*-terp), tetrahydronaphthalene (thn), tetrahydroanthracene (tha) or dihydroanthracene (dha), are reported, including the X-ray crystal structures of [(η^6-tha)Ru(bipy)Cl][PF$_6$] (**8**), [(η^6-tha)Ru(bipy(OH)O)Cl] (**9**) and [(η^6 ind)Ru(bipy(OH)$_2$)Cl][PF$_6$] (**10**). Complexes **8** and **9** exhibit CH (arene)/π (bipy or bipy(OH)O) interactions. In the X-ray structure of protonated complex **10**, the pyridine rings are twisted (by 17.31°). In aqueous solution (pH = 2-10), only deprotonated (bipy(OH)O) forms are present. Hydrolysis of the complexes was relatively fast in aqueous solution ($t_{1/2}$ = 4-15 min, 310 K). When the arene is biphenyl, initial aquation of the complexes is followed by partial arene loss. Complexes with arene = tha, thn, dha, ind and *p*-terp, and deprotonated bipyridinediol (bipy(OH)O) as chelating ligands, exhibited significant cytotoxicity toward A2780 human ovarian and A549 human lung cancer cells. Complexes [(η^6-bip)Ru(bipy(OH)O)Cl] (**11**) and [(η^6-bz)Ru(bipy(OH)O)Cl] (**12**) exhibited moderate cytotoxicity toward A2780 cells, but were inactive toward A549 cells. These activity data can be contrasted with those of the parent bipyridine complex [(η^6-tha)Ru(bipy)Cl][PF$_6$] (**8**) which is inactive toward both A2780 ovarian and A549 lung cell lines. DFT calculations suggested that hydroxylation and methylation of the bipy ligand have little effect on the charge on Ru. The active complex [(η^6 tha)Ru(bipy(OH)O)Cl] (**9**) binds strongly to 9-ethyl-guanine (9-EtG). The X-ray crystal structure of the adduct [(η^6-tha)Ru(bipy(OH)O)(9- EtG-*N7*)][PF$_6$] shows intramolecular CH (arene)/π (bipy(OH)O) interactions and DFT calculations suggested that these are more stable than arene/9-EtG π-π interactions. However [(η^6-ind)Ru(bipy(OH)$_2$)Cl][PF$_6$] (**10**) and [(η^6- ind)Ru(bipy)Cl][PF$_6$] (**13**) bind only weakly to DNA. DNA may therefore not be the major target for complexes studied here.

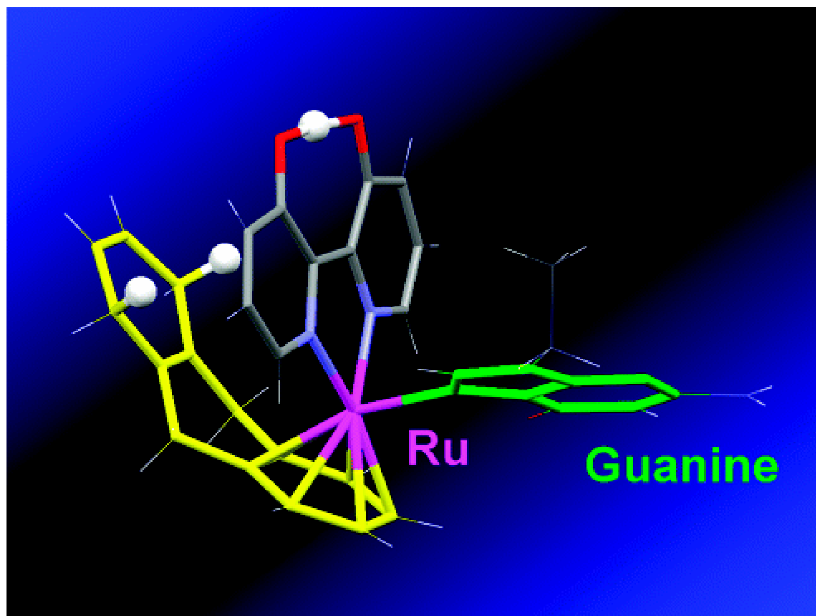

Figure 7: Structure of the complex formed between Ru^{II}-arene compound and guanine.

Granted projects

GA AS CR B400040601, Recognition of DNA modified by antitumor platinum and ruthenium complexes by zinc-finger proteins and topoisomerases. Principal investigator J. Malina, 2006 - 2008

GA CR 203/06/1239, Tolerance and bypass of DNA damage by metal-based anticancer drugs. Principal investigator: O. Nováková, 2006 - 2008

GA AS CR 1QS500040581, Metallodrugs, design and mechanism of action. Principal investigator: O. Vrána, 2005 - 2009

Ministry of Health CR NR8562-4/2005, Inhibition of telomerase by transition metal complexes. A new concept of antitumor drug design. Principal investigator: J. Kašpárková, 2005 - 2008

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

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

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

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

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

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

Publications

Kasparkova, J., Marini, V., Bursova, V., Brabec, V.: *Biophysical studies on the stability of DNA intrastrand cross-links of transplatin*. Biophys. J., 95, 2008, 4361-4371.

Kasparkova, J., Vojtiskova, M., Natile, G., Brabec, V.: *Unique properties of DNA interstrand cross-links of antitumor oxaliplatin and the effect of chirality of the carrier ligand*. Chem. Eur. J., 14, 2008, 1330-1341.

Kostrhunova, H., Florian, J., Novakova, O., Peacock, A.F.A., Sadler, P.J., Brabec, V.: *DNA interactions of monofunctional organometallic osmium(II) antitumor complexes in cell-free media*. J. Med. Chem., 51, 2008, 3635-3643.

Malina, J., Hannon, M.J., Brabec, V.: *Interaction of dinuclear ruthenium(II) supramolecular cylinders with DNA: Sequence-specific binding, unwinding, and photocleavage*. Chem. Eur. J., 14, 2008, 10408-10414.

Malina, J., Hannon, M.J., Brabec, V.: *DNA binding of dinuclear iron(II) metallosupramolecular cylinders. DNA unwinding and sequence preference*. Nucleic Acids Res., 36, 2008, 3630-3638.

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PhD. thesis defended in 2008

Mgr. Anna Halámiková, PhD., Biophysical analysis of mechanism of antitumor effects of anticancer drugs

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Within the DCBMO, two partially autonomous research groups have been established which conducted specifically oriented research. The group **“Analysis of proteins important in biomedicine“** leaded by Prof. Emil Paleček deals 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 toward a new method of electrochemical analysis based on the ability of proteins to catalyze hydrogen evolution on mercury electrodes. Such electrocatalysis is manifested by the so-called peak H, yielded by constant current chronopotentiometric stripping method. Peak H differs from the previously studied electrochemical signals of proteins particularly (i) by its ability to detect proteins down to nanomolar and subnanomolar concentrations and (ii) by its high sensitivity (a) to local and global changes in protein structures and (b) to protein redox states. In 2008 a considerable progress in electrochemical analysis of proteins was achieved. Special attention was paid to proteins important in biomedicine, such α-synuclein playing a critical role in Parkinson’s disease and tumor suppressor p53.

The group **“Physics and Physical Chemistry of Biopolymers“** leaded by Dr. František Jelen is oriented towards (a) interactions of nucleic acids and some proteins with electrically charged surfaces; (b) interactions nucleic acids components with metal ions such as copper; (c) development of electrochemical methods for microanalysis of nucleic acids components, their metabolites and analogues; (c) application of elimination voltammetry (EVLS) in analysis of nucleic acids. It was found that EVLS in connection with the stripping procedure is useful for both qualitative and quantitative microanalysis of purine derivatives, and can reveal details of studied electrode processes. Activities of the group came mainly under the field I.

In 2008 the Department of Biophysical Chemistry and Molecular Oncology pursued research concentrated to three main fields (see below). Despite existence of the above mentioned groups, many results arose from collaboration of scientists through the whole Department.

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

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

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

Research in the Field I included systematic studies of the behavior of synthetic oligonucleotides (ODNs), chemically modified or damaged DNAs at electrodes, studies focused on novel techniques of electrochemical DNA labeling and development of new bioanalytical approaches applicable in practical DNA sensing. In addition, redox properties of metal complexes applicable as electroactive DNA labels were investigated using computational methods.

Electrochemical stripping techniques in analysis of nucleic acids and their constituents

The ability of nucleic acids (NA) and their components to accumulate at electrode surfaces and electrochemical properties of these species are closely related. This review is devoted to electrochemical stripping techniques applied in NA studies. Cathodic or anodic stripping voltammetry have been used for a highly sensitive determination of nucleobases, nucleosides, nucleotides or acid-hydrolyzed NAs, based on formation of sparingly soluble complexes of the NA constituents with electrochemically generated mercury or copper(I) ions. DNAs, RNAs and their synthetic analogues, either unmodified or labeled with electroactive markers, have been analyzed by adsorptive stripping (AdS) techniques with mercury, mercury film, amalgam and carbon-based electrodes. Strong adsorption of NAs at the electrode surfaces has been utilized in adsorptive transfer stripping (AdTS) techniques. In AdTS, a NA-modified electrode is prepared by adsorptive accumulation of the NA at the electrode surface, followed by transfer into background electrolyte not containing any NA. NA-modified electrodes can be used as simple electrochemical NA sensors. Recent applications of AdS and AdTS in NA microanalysis, in detection of DNA damage as well as in studies of DNA hybridization or DNA-protein interactions are reviewed.

Study of copper and purine-copper complexes on modified carbon electrodes by cyclic and elimination voltammetry

Using a paraffin impregnated graphite electrode (PIGE) and mercury-modified pyrolytic graphite electrode with basal orientation (Hg-PGEb) copper(II) and Cu(II)-DNA purine base solutions have been studied by cyclic (CV) and linear sweep voltammetry (LSV) in connection with elimination voltammetry with linear scan (EVLS). In chloride and bromide solutions (pH 6), the redox process of Cu(II) proceeded on PIGE with two cathodic and two anodic potentially separated signals. According to the elimination function E4, the first cathodic peak corresponds to the reduction Cu(II) + e(-) → Cu(I) with the possibility of fast disproportionation 2Cu(I) → Cu(II) + Cu(0). The E4 of the second cathodic peak signalized an electrode process controlled by a surface reaction. The electrode system of Cu(II) on Hg-PGEb in borate buffer (pH 9.2) was characterized by one cathodic and one anodic peak. Anodic stripping voltammetry (ASV) on PIGE and cathodic stripping voltammetry (CSV) on Hg-PGEb were carried out at potentials where the reduction of copper ions took place and Cu(I)-purine complexes were formed. By using ASV and CSV in combination with EVLS, the sensitivity of Cu(I)-purine complex detection was enhanced relative to either ASV or CSV alone, resulting in higher peak currents of more than one order of magnitude. The statistical treatment of CE data was used to determine the reproducibility of measurements. Our results show that EVLS in connection with the stripping procedure is useful for both qualitative and quantitative microanalysis of purine derivatives and can also reveal details of studied electrode processes.

Label-free sequence-specific DNA sensing using copper-enhanced anodic stripping of purine bases at boron-doped diamond electrodes

Stripping voltammetric determination of purine bases in the presence of copper ions at mercury, amalgam or carbon-based electrodes has recently been utilized in analysis of DNA or synthetic oligodeoxynucleotides (ODNs). Here we report on copper-enhanced label-free anodic stripping detection of guanine and adenine bases in acid-hydrolyzed DNA at anodically oxidized boron-doped diamond electrode (AO-BDDE). The AO-BDDE was successfully applied in a three-electrode microcell in which a ~50-μL drop of the analyte solution can be efficiently stirred during the accumulation step by streaming of an inert gas. Accelerated mass transport

due to the solution motion in the presence of copper resulted in enhancement of the guanine oxidation signal by about two orders of magnitude (compared to accumulation of the analyte from still solution not containing copper), allowing an easy detection of ~25 fmol of the ODNs. The proposed technique is shown to be suitable for a determination of purine (particularly guanine) content in DNA samples. Applications of the technique in magnetic beads-based DNA assays (such as hybridization with DNA sequences exhibiting asymmetrical distribution of purine/pyrimidine nucleotides between the complementary strands, or monitoring of amplification of specific DNA fragments in a duplex polymerase chain reaction) are demonstrated.

Two-dimensional condensation of pyrimidine oligo-nucleotides during their self-assemblies at mercury based surfaces

For the first time it is shown that homopyrimidine oligodeoxynucleotides (ODNs) adsorbed at mercury or amalgam electrode surface can condensate upon applying negative potentials (around -1.35 V vs. Ag/AgCl/3M KCl). This 2D condensation resulted in formation of capacitance pits on the C-E curves resembling those observed earlier with monomeric nucleic acid bases, nucleosides and nucleotides. Differences in behavior of the condensed layers of dT(30) and dC(30) ODNs, reflecting different physico-chemical and electrochemical properties of thymine and cytosine, were observed. Formation of the ODN condensed film involved reorientation of the oligonucleotide molecules firmly adsorbed at the electrode and took place even in the absence of any ODN in the bulk of solution. Homopurine ODNs did not form these two-dimensional (2D) condensed monolayers under the same conditions. A preliminary thermodynamic analysis of the condensed ODN layers is presented.

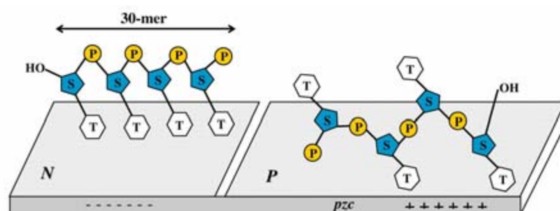

Figure 1: Scheme of negative potential-induced 2D condensation of pyrimidine oligonucleotides at mercury surface.

Sensitive voltammetric detection of DNA damage at carbon electrodes using DNA repair enzymes and an electroactive osmium marker

This paper presents a new approach to electrochemical sensing of DNA damage, using osmium DNA markers and voltammetric detection at the pyrolytic graphite electrode. The technique is based on enzymatic digestion of DNA with a DNA repair enzyme exonuclease III (exoIII), followed by single-strand (ss) selective DNA modification by a complex of osmium tetroxide with 2,2'-bipyridine. In double-stranded DNA possessing free 3'-ends, the exoIII creates ss regions that can accommodate the electroactive osmium marker. Intensity of the marker signal measured at the pyrolytic graphite electrode responded well to the extent of DNA damage. The technique was successfully applied for the detection of (1) single-strand breaks (ssb) introduced in plasmid DNA by deoxyribonuclease I, and (2) apurinic sites generated in chromosomal calf thymus DNA upon treatment with the alkylating agent dimethyl sulfate. The apurinic sites were converted into the ssb by DNA repair endonuclease activity of the exoIII enzyme. We show that the presented technique is capable of detection of one lesion per approximately 10^5 nucleotides in supercoiled plasmid DNA.

Enzyme-linked electrochemical detection of PCR-amplified nucleotide sequences using disposable screen-printed senzor

Electrochemical enzyme-linked techniques for sequence-specific DNA sensing are presented. These techniques are based on attachment of streptavidin-alkaline phosphatase conjugate to biotin tags tethered to DNA immobilized at the surface of disposable screen-printed carbon electrodes (SPCE), followed by production and electrochemical determination of an electroactive indicator, 1-naphthol. Via hybridization of SPCE surface-confined target DNAs with end-biotinylated probes, highly specific discrimination between complementary and non-complementary nucleotide sequences was achieved. The enzyme-linked DNA hybridization assay has been successfully applied in analysis of PCR-amplified real genomic DNA sequences, as well as in monitoring of plant tissue-specific gene expression. In addition, we present an alternative approach involving sequence-specific incorporation of biotin-labeled nucleotides into DNA by primer extension. Introduction of multiple biotin tags per probe primer resulted in considerable enhancement of the signal intensity and improvement of the specificity of detection.

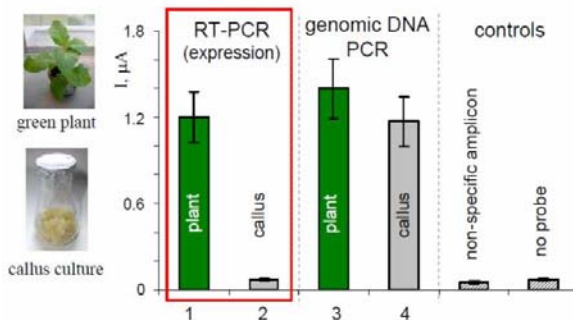

Figure 2: Responses of the enzyme-linked electrochemical sensor to tissue-specific expression of *rbcL* gene in *Nicotina tabacum.*

Cross-coupling reactions of nucleoside triphosphates followed by polymerase incorporation. Construction and applications of base-functionalized nucleic acids

Construction of functionalized nucleic acids (DNA or RNA) via polymerase incorporation of modified nucleoside triphosphates is reviewed and selected applications of the modified nucleic acids are highlighted. The classical multistep approach for the synthesis of modified NTPs by triphosphorylation of modified nucleosides is compared to the novel approach consisting of direct aqueous cross-coupling reactions of unprotected halogenated nucleoside triphosphates. The combination of cross-coupling of NTPs with polymerase incorporation gives an efficient and straightforward two-step synthesis of modified nucleic acids. Primer extension using biotinylated templates followed by separation using streptavidine-coated magnetic beads and DNA duplex denaturation is used for preparation of modified single stranded oligonucleotides. Examples of using this approach for electrochemical DNA labelling and bioanalytical applications are given.

Aminophenyl- and nitrophenyl-labeled nucleoside triphosphates: Synthesis, enzymatic incorporation, and electrochemical detection

Aminophenyl- and Nitrophenyl-Labeled Nucleoside Triphosphates were prepared by cross-coupling reactions and incroporated to oligonucleotides (ON) by primer extension. Both types of modifications serve as excellent electrochemical labels detectable either by oxidation (NH2) or reduction (NO2), allowing a perfect discrimination between the two tags. Redox potentials of both labels differ depending on the nucleobase and respond to incorporation into ON.

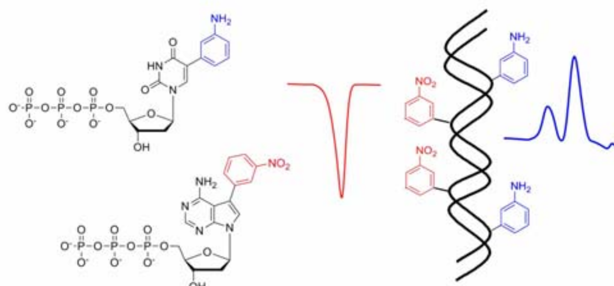

Figure 3: DNA labeling with aminophenyl and nitrophenyl groups and schematic representation of electrochemical response of the labels.

Effect of spin-orbit coupling on reduction potentials of octahedral ruthenium(II/III) and osmium(II/III) complexes: Reduction potentials of several $M^{2+/3+}$ (M = Ru, Os) octahedral complexes, namely, $[M(H_2O)_6]^{2+/3+}$, $[MCl_6]^{4-/3-}$, $[M(NH_3)_6]^{2+/3+}$, $[M(en)_3]^{2+/3+}$ $[M(bipy)_3]^{2+/3+}$, and $[M(CN)_6]^{4-/3-}$, were calculated using the CASSCF/CASPT2/CASSI and MRCI methods including spin-orbit coupling (SOC) by means of first-order quasi-degenerate perturbation theory. It was shown that the effect of SOC accounts for a systematic shift of approximately -70 mV in the reduction potentials of the studied ruthenium (II/III) complexes and an approximately

-300 mV shift for the osmium(II/III) complexes. SOC splits the sixfold-degenerate T-2(2g) ground electronic state (in ideal octahedral symmetry) of the M3+ ions into the E-(5/2)g. Kramers doublet and G((3/2)g) quartet, which were calculated to split by 1354-1573 cm^{-1} in the Ru^{3+} complexes and 4155-5061 cm^{-1} in the Os^{3+} complexes. It was demonstrated that this splitting represents the main contribution to the stabilization of the M^{3+} ground state with respect to the closed-shell (1)A(1g) ground state in M^{2+} systems. Moreover, it was shown that the accuracy of the calculated reduction potentials depends on the calculated solvation energies of both the oxidized and reduced forms. For smaller ligands, it involves explicit inclusion of the second solvation sphere into the calculations, whereas implicit solvation models yield results of sufficient accuracy for complexes with larger ligands. In such cases (e.g., $[M(bipy)_3]^{2+/3+}$ and its derivatives), very good agreement between the calculated (SOC-corrected) values of the reduction potentials and the available experimental values was obtained. These results led us to the conclusion that especially for $Os^{2+/3+}$ complexes, inclusion of SOC is necessary to avoid systematic errors of ~ 300 mV in the calculated reduction potentials.

In the Field II the work included basic studies of electrochemical behavior of peptides and proteins. Adsorption proteins at titanium surfaces was studied to contribute to the development of materials for dental implantates. Efficient, highly sensitive electrochemical techniques suitable for monitoring protein denaturation, aggregation, determination of redox state, as well as for studies of DNA-protein interactions, were being developed. Osmium tetroxide complexes were introduced as new tools for studies of protein structure and interactions.

Sensing of human plasma fibrinogen on polished, chemically etched and carbon treated titanium surfaces by diffractive optical element based sensor

Adsorption of human plasma fibrinogen (HPF) on 6 differently treated titanium samples (polished, polished and etched, and 4 titanium carbide coatings samples produced by using plasma-enhanced chemical vapour deposition (PECVD) method) is investigated by using diffractive optical element (DOE) sensor. Permittivity (susceptibility) change and fluctuation in optical roughness (R-opt) of treated titanium surface in the presence of background electrolyte without and with HPF molecules are sensed by using DOE sensor and optical ellipsometry. Correlation between transmitted

light and thickness of molecule layer was found. The findings allow to sense temporal organization and severity of adsorption of nano-scale HPF molecules on polished, on polished and etched, and on titanium carbide surface.

Constant current chronopotentiometry and voltammetry of native and denatured serum albumin at mercury and carbon electrodes

Constant current chronopotentiometric peak H at mercury electrodes was recently shown as a sensitive tool for global and local changes in protein conformation. Large differences between the heights of peak H of native (hBSAnat) and denatured BSA (hBSAden) were observed. The ratio hBSAden/hBSAnat increased with more negative stripping current suggesting that the rate of potential change is important for discrimination between native and denatured BSA. Voltammetric peaks of BSA were less well developed and BSAden/BSAnat was much smaller. It was not possible to discriminate BSAden and BSAnat using carbon electrodes.

Native, denatured and reduced BSA - Enhancement of chronopotentiometric peak H by guanidinium chloride

In proteomics and biomedicine fast techniques applicable for preliminary tests of the protein properties and structural changes are sought. Methods of electrochemical analysis have been little utilized in these fields. We show that using constant current chronopotentiometric stripping peak H, minute amounts of denatured and reduced bovine serum albumin (BSA) can be easily discriminated from native BSA. Peak H, which is due to catalytic hydrogen evolution, is greatly enhanced in the presence of non-denaturing concentrations of guanidinium chloride. The course of BSA reduction and denaturation can be followed and traces of the damaged protein can be detected in native BSA samples.

Osmium tetroxide, 2,2 '-bipyridine: Electroactive marker for probing accessibility of tryptophan residues in proteins

A complex of osmium tetroxide with 2,2′-bipyridine (Os,bipy) has been applied as a chemical probe of DNA structure as well as an electroactive DNA label. The Os,bipy has been known to form covalent adducts with

pyrimidine DNA bases. Besides the pyrimidines, electrochemically active covalent adducts with Os,bipy are formed also by tryptophan (W) residues in peptides and proteins. In this paper we show that Os,bipy-treated proteins possessing W residues (such as avidin, streptavidin, or lysozyme) yield at the pyrolytic graphite electrode (PGE) a specific signal (peak rW) the potential of which differs from the potentials of signals produced by free Os,bipy or by Os,bipy-modified DNA. No such signal is observed with proteins lacking W (such as ribonuclease A or r-synuclein). Subpicomole amounts of W-containing proteins modified with Os,bipy can easily be detected using adsorptive transfer stripping voltammetry with the PGE. Binding of biotin to avidin interferes with Os,bipy modification of the protein, in agreement with the location of W residues within the biotin-binding site of avidin. These Ws are accessible for modification in the absence of biotin but hidden (protected from modification) in the avidin-biotin complex. The Os,bipy-modified avidin is unable to bind biotin, and its quarternary structure is disrupted. Analogous effects were observed with another biotin-binding protein, streptavidin. Our results demonstrate that modification of proteins with Os,bipy under conditions close to physiological, followed by a simple electrochemical analysis, can be applied in the microanalysis of protein structure and interactions.

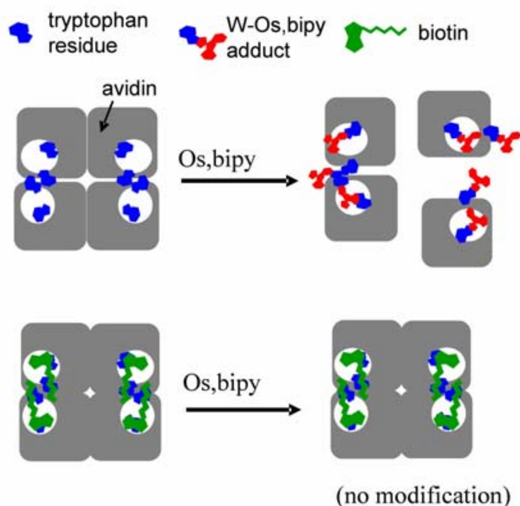

Figure 4: Scheme of modification of tryptophan residues in biotin-binding pockets of (strept)avidin with Os,bipy.

In the Field III, the studies on structure and interactions of the p53-family proteinss were continued. Effects of DNA topology on p53-DNA binding within target sites capable of forming cruciform structures were described. In addition, the DCBMO contributed to the research of cell type-specific activities of the p73 protein.

DNA topology influences p53 sequence-specific DNA binding through structural transitions within the target sites

Tumor suppressor protein p53 is one of the most important factors regulating cell proliferation, differentiation and programmed cell death in response to a variety of cellular stress signals. The p53 is a nuclear phosphoprotein, and its biochemical function is closely associated with its ability to bind DNA in a sequence-specific manner and operate as a transcription factor. Using a competition assay, we investigated the effect of DNA topology on the DNA binding of human wild type p53 protein. We prepared sets of topoisomers of plasmid DNA with and without p53 target sequences differing in their internal symmetry. Binding of p53 to any DNA increased with increasing negative superhelix density ($-\sigma$). At $-\sigma \leq 0.03$, the relative effect of DNA supercoiling on protein-DNA binding was similar for DNAs bearing both symmetrical and non-symmetrical target sites. On the other hand, at higher $-\sigma$ target sites with a perfect inverted repeat sequence exhibited a more significant enhancement of p53 binding due to increasing levels of negative DNA supercoiling. For $-\sigma = 0.07$, about a three-fold additional increase of binding was observed for a symmetrical target site, compared to a non-symmetrical target. The p53 target sequences possessing the inverted–repeat symmetry were shown to form cruciform structure in sufficiently negative scDNA. We show that formation of the cruciforms in DNA topoisomers at $-\sigma \geq 0.05$ correlates with the extra enhancement of p53-DNA binding.

The cell type-specific effect of TAp73 isoforms on the cell cycle and apoptosis

p73, a member of the p53 family, exhibits activities similar to those of p53 including the ability to induce growth arrest and apoptosis. p73 influences chemotherapeutic responses in human cancer patients, in association with p53. Alternative splicing of the *TP73* gene produces many p73 C- and N-terminal isoforms, which vary in their transcriptional activity towards p53-

responsive promoters. In this paper, we show that the C-terminal spliced isoforms of the p73 protein differ in their DNA-binding capacity, but this is not an accurate predictor of transcriptional activity. In different p53-null cell lines, p73β induces either mitochondrial-associated or death receptor-mediated apoptosis, and these differences are reflected in different gene expression profiles. In addition, p73 induces cell cycle arrest and p21WAF1 expression in H1299 cells, but not in Saos-2. This data shows that TAp73 isoforms act differently depending on the tumour cell background, and have important implications for p73-mediated therapeutic responses in individual human cancer patients.

Granted projects

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

GA AS CR A500040513, Wild type and mutant tumor suppressor protein p53. Intermolecular interactions, confromational changes and novel micromethods of its analysis. Principal investigator: E. Paleček, 2005 - 2008

GA AS CR A100040602, New approaches in electrochemical analysis of nucleic acids and oligonucleotides aimed at an ultrasensitive microdetection of DNA and DNA hybridization. Principal investigator: F. Jelen, 2006 - 2008

GA AS CR A400040611, Application of electrochemical methods in studies of oligonucleotides as models of unusual DNA structures. Principal investigator: L. Havran, 2006 - 2008

GA AS CR KAN400310651, Nanotechnologies for protein and gene diagnostics. Principal investigator: F. Foret (IACH Brno), Co-investigator: E. Paleček, 2006 - 2010

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

GA AS CR 1QS500040581, Metallodrugs, design and mechansim of action Principal investigator: O. Vrána, Co-investigator: M. Fojta, 2005 - 2009

GA CR 203/07/1195, Analysis of DNA structure and interactions using electrochemical techniques and chemical probes. Novel techniques and sensors for DNA damage detection. Principal investigator: M. Fojta, 2007 - 2009

GA CR 204/07/P476, Interactions of p73 protein and its isoforms with DNA. Influences of DNA supercoiling, conformation and anticancer drugs. Principal investigator: H. Pivoňková, 2007 - 2009

GA CR 301/07/P160, Study of posttranslation modification of the tumor suppressor protein p53, its homologues and interacting proteins in human cancer cell lines. Principal investigator: E. Brázdová Jagelská, 2007 - 2009

GA CR 202/07/P497, Interactions of proteins with surfaces. New biophysical methods of analysis of tumor suppressor p53. Principal investigator: V. Ostatná, 2007 - 2009

GA CR 203/06/1685, Microfluidic instrumentation for analyses of biopolymers modified by structural probes. Principal investigator: F. Foret (IACH Brno), Co-investigator: E. Paleček, 2006 - 2008

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

Ministry of Education, Youth and Sports of the CR - 1 M0528, Stomatological Research Centre. Principal investigator: J. Vaněk (MU Brno), Co-investigator and Guarantor at IBP: V. Vetterl, 2005 - 2009

6FP EU Integrated Project No. 502983, Mutant p53 as a target for improved cancer treatment. Coordinator: K. Wiman (Karolinska Institute, Stockholm, Sweden); Co-investigator: E. Paleček, 2004 - 2008

Marie Currie TOK No. 42708, Interactions of Nucleic Acids and Proteins at Interfaces - Fundamentals and Applications. Principal investigator: J. Radecki (Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences in Olsztyn, Poland), Co-investigator: E. Paleček, 2007 - 2010

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PhD. thesis defended in 2008

Ing. Mojmír Trefulka, PhD., Reactions of osmium complexes with biomacromolecules and electrochemistry of their products. New tools for molecular biology

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Rapid chromosome evolution in recently formed polyploids in tragopogon (Asteraceae), (*Roman Matyášek)*

Polyploidy, frequently termed "whole genome duplication", is a major force in the evolution of many eukaryotes. Indeed, most angiosperm species have undergone at least one round of polyploidy in their evolutionary history. Despite enormous progress in our understanding of many aspects of polyploidy, we essentially have no information about the role of chromosome divergence in the establishment of young polyploid populations. Here we investigate synthetic lines and natural populations of two recently and recurrently formed allotetraploids *Tragopogon mirus* and *T. miscellus* (formed within the past 80 years) to assess the role of aberrant meiosis in generating chromosomal/genomic diversity. That diversity is likely important in the formation, establishment and survival of polyploid populations and species.

Applications of fluorescence *in situ* hybridisation (FISH) to natural populations of *T. mirus* and *T. miscellus* suggest that chromosomal rearrangements and other chromosomal changes are common in both allotetraploids. We detected extensive chromosomal polymorphism between individuals and populations, including (i) plants monosomic and trisomic for particular chromosomes (perhaps indicating compensatory trisomy), (ii) intergenomic translocations and (iii) variable sizes and expression patterns of individual ribosomal DNA (rDNA) loci. We even observed karyotypic variation among sibling plants. Significantly, translocations, chromosome loss, and meiotic irregularities, including quadrivalent formation, were observed in synthetic (S_0 and S_1 generations) polyploid lines. Our results not only provide a mechanism for chromosomal variation in natural populations, but also indicate that chromosomal changes occur rapidly following polyploidisation.

These data shed new light on previous analyses of genome and transcriptome structures in *de novo* and established polyploids. Crucially our results highlight the necessity of studying karyotypes in young (<150 years old) polyploid species and synthetic polyploids that resemble natural species. The data also provide insight into the mechanisms that perturb inheritance patterns of genetic markers in synthetic polyploids and populations of young natural polyploid species.

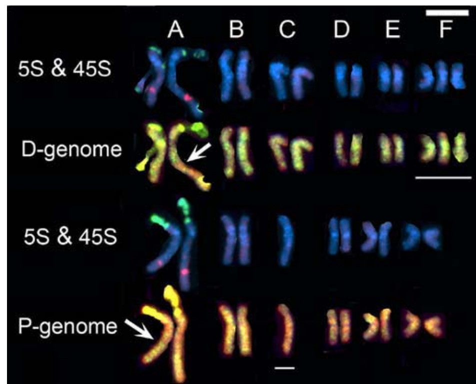

Figure 1: Asymmetrical karyotype of a *T. miscellus* individual from population 2604. The karyotype is shown with DAPI staining, simultaneously labelled for 45S rDNA (yellow fluorescence) or 5S rDNA (red fluorescence), and after GISH with *T. dubius* (green fluorescence) and *T. pratensis* (red fluorescence) total genomic DNA probes. Monosomic and trisomic chromosomes are underlined. Note that there is a large intergenomic translocation, chromosome $A^{du/pr}$ (arrow). Scale bar (top right) is 10 µm.

Faithful inheritance of cytosine methylation patterns in repeated sequences of the allotetraploid tobacco correlates with the expression of DNA methyltransferase gene families from both parental genomes, (*Jaroslav Fulneček)*

In plants, polyploidy (chromosome multiplication) often occurs in association with interspecific hybridization leading to formation of new allopolyploid species. Epigenetic processes including DNA methylation are thought to pay important role in harmonizing gene expression and maintenance of genome integrity in early allopolyploid nucleus.
In this project we addressed the questions of inheritance of DNA methyltransferase genes and their transcription activity in a model allopolyploid plant *Nicotiana tabacum* (tobacco) thought to form less than 200 thousand years ago by interspecific hybridization of diploid progenitors close to modern species *Nicotiana sylvestris* and *Nicotiana tomentosiformis*.. From the parental species we cloned cDNA sequences corresponding to all three plant DNA methyltransferase families, *MET1*, *CMT3* and *DRM*.. Bioinformatic analysis of gene-space sequence read data library allowed us to reconstruct exon-intron gene structures. A microsatellite sequence was found in the eighth intron of *MET1* genes. This feature seems to be unique for Nicotiana species. Using molecular methods we identified all parental DNA methyltransferase genes in tobacco genome. At the epigenetic level, we showed additive expression of inherited genes in different plant organs: leaf, roots, flowers and non-differentiated callus. These data indicate Mendelian inheritance of DNA methyltransferase loci and absence of their structural and epigenetic changes during evolution of allopolyploid genome. We further demonstrated inheritance of cytosine methylation patterns in repetitive sequences known to be major substrates for DNA methylation reactions. On the basis of these results we proposed the hypothesis that additive expression of parental DNA methyltransferase genes may be needed for maintaining high level of methylcytosine in large heterochromatic genome of tobacco.

Granted projects

GA AS CR IAA600040611, Mendelian and non-mendelian interactions of the plant transgenes. Principal investigator: M. Fojtová, 2006 - 2008

GA CR 204/06/1432, Nicotiana sylvestris DNA-(cytosine-5)-methyltransferases. Principal investigator: J. Fulneček, 2006 - 2008

GA CR 521/07/0116, Dynamics of repetitive DNA sequences in polyploid genomes. Principal investigator: A. Kovařík, 2007 - 2009

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PhD. thesis defended in 2008

Mgr Jana Lunerová, PhD., Epigenetic regulation of expression in plant transgenes

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Functional analysis of the aglycone-binding site of the maize β-glucosidase Zm-p60.1

β-Glucosidases such as Zm-p60.1 (*Zea mays*) and Bgl4:1 (*Brassica napus*) have implicated roles in regulating plant development by releasing biologically active cytokinins from O-glucosides. A key determinant of substrate specificity in Zm-p60.1 is the F193-F200-W373-F461 cluster. However, despite sharing the same substrates, amino acids in the active sites of Zm-p60.1 and Bgl4:1 differ dramatically. In members of the Brassicaceae we found a group of β-glucosidases sharing both high similarity to Bgl4:1 and a consensus motif A-K-K-L corresponding to the F193-F200-W373-F461 cluster.

To study the mechanism of substrate specificity further, we generated and analyzed four single (F193A, F200K, W373K and F461L) and one quadruple (F193A-F200K-W373K-F461L) mutants of Zm-p60.1. The F193A mutant showed a specific increase in affinity for a small polar aglycone, and a deep drop in k_{cat} compared with the wild-type. Formation of a cavity with decreased hydrophobicity, and significant consequent alterations in ratios of reactive and non-reactive complexes revealed by computer modeling may explain the observed changes in kinetic parameters of the F193 mutant.

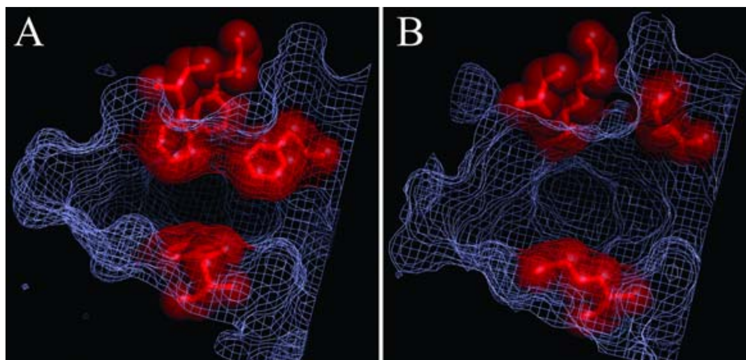

Figure 1: (A) The main hydrophobic amino acid cluster (from the left: F193, F200 and F461, with W373 below) superimposed on the active site cavity of Zm-p60.1 β-glucosidase. (B) Model of the putative arrangement of amino acid alterations (from the left F193A, F200K, F461L, with W373K below) in the active site cavity of Zm-p60.1 β-glucosidase. In each case the protein surface is represented by a wire. Rotamer positions were calculated using the scoring function in Swiss-PdbViewer v3.7 and results were visualized using PyMol v0.97.

The large decrease in the k_{cat} of the W373K mutant was unexpected, but the findings are consistent with the F193-aglycone-W373 interaction playing a dual role in the enzyme's catalytic action; influencing both substrate specificity, and the catalytic rate by fixing the glucosidic bond in a favorable orientation for attack by the catalytic pair. Investigation of the combined effects of all of the mutations in the quadruple mutant of Zm-p60.1 was precluded by extensive alterations in its structure and almost complete abolition of its enzymatic activity.

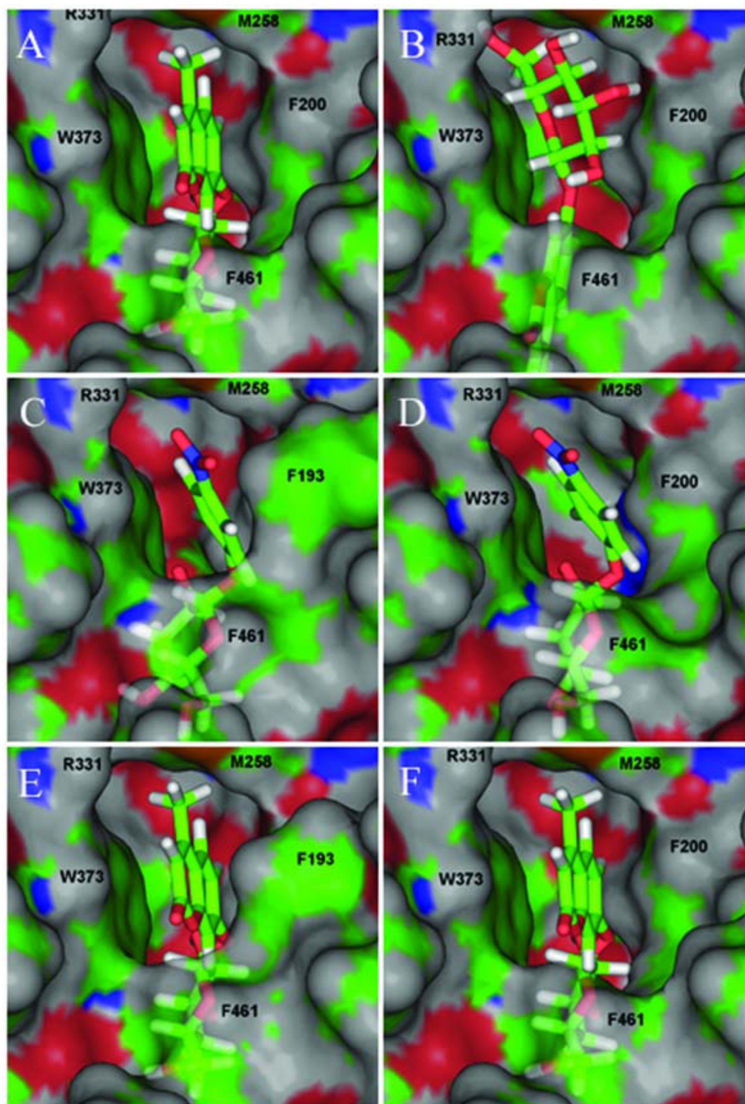

Figure 2: Modelled enzyme-substrate complexes viewed from the aglycone-binding site. Models of 4-methylumbeliferyl β-glucoside (A-D) and *p*-nitrophenyl β-glucoside (E-F) docked into the aglycone-binding site of wild-type type Zm-p60.1 β-glucosidase (C, E) and the F193A mutant (A, B, D, F). Reactive complexes – A, C, D, E, F; non-reactive complex – B.

Cytokinin-induced photomorphogenesis in dark-grown Arabidopsis: a proteomic analysis

High concentrations of cytokinins (CKs) in cultivation media can induce partial photomorphogenesis in dark-grown *Arabidopsis* seedlings. However, no significant increases in endogenous CK levels have been found in de-etiolated mutants, suggesting either that parallel pathways are involved in the light and CK responses, or changes in the sensitivity to CKs occur during photomorphogenesis. Here we show that even modest increases in endogenous CK levels induced by transgenic expression of the CK biosynthetic gene, *ipt*, can lead to many typical features of light-induced de-etiolation, including inhibition of hypocotyl elongation and partial cotyledon opening. In addition, significant changes in expression of 37 proteins (mostly related to chloroplast biogenesis, a major element of light-induced photomorphognesis) were detected by image and mass spectrometric analysis of two-dimensionally separated proteins. The identified chloroplast proteins were all upregulated in response to increased CKs, and more than half are upregulated at the transcript level during light-induced photomorphogenesis according to previously published transcriptomic data. Four of the upregulated chloroplast proteins we identified have also been shown to be upregulated during light-induced photomorphogenesis in previous proteomic analyses. In contrast, all differentially regulated mitochondrial proteins (the second largest group of differentially expressed proteins) were downregulated. Changes in the levels of several tubulins are consistent with the observed morphological alterations. Further, 10 out of the 37 differentially expressed proteins we detected have not been linked to either photomorphogenesis or CK action in light grown *Arabidopsis* seedlings in previously published transcriptomic or proteomic analyses.

Granted projects

GA AS CR IAA600040701, Proteome dynamics in response to increased cytokinin levels in Arabidopsis. Principal investigator: B. Brzobohatý, Principal co-investigator: J. Bobáľová, Principal co-investigator: P. Bouchal, 2007 - 2010

GA AS CR IAA600040612, Functional analysis of *Shooting* gene in Arabidopsis and tobacco. Principal investigator: B. Brzobohatý, Principal co-investigator: R. Vaňková, 2006 - 2008

GA CR 522/06/0979, Mechanisms of plant resistance against ROS induced in photosynthetic apparatus by a photoinhibitory stress. Principal investigator: M. Barták, Principal co-investigator: B. Brzobohatý, Principal co-investigator: P. Ilík, 2006 - 2008

Publications

Dopitová, R., Mazura, P., Janda, L., Chaloupková, R., Jeřábek, P., Damborský, J., Filipi, T., Kiran, N.S., Brzobohatý, B.: *Functional analysis of the aglycone-binding site of the maize β-glucosidase Zm-p60.1*. FEBS J., 275, 2008, 6123-6135.

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PhD. thesis defended in 2008

Mgr. Pavel Mazura, PhD., Molecular determination of substrate specificity in plant beta-glucosidases

Mgr. Přemysl Souček, PhD., Hormonal regulation of *KNOX* gene expression in plants

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Chromatin structure and epigenetics in human embryonic stem cells

Laboratory is focused on the studies on nuclear architecture in the view of gene expression. We tried to find correlations between structural features and changes in epigenetic profiles such as histone acetylation and methylation. Our analyses were focused on nuclear location of markers responsible for human embryonic stem cells (hESCs) pluripotency and differentiation. ESCs are characterized by specific genomic, epigenomic, and proteomic features that differ from those of terminally differentiated cells (Allegrucci and Young, 2007). Since the establishment of the first hESC lines (Thomson et al., 1998), much progress has been made in hESC biology. The potential of hESCs to differentiate via various pathways makes

these cells promising tools in regenerative medicine. However, the clinical application of hESCs has been hampered by cultivation conditions, which cause genetic and epigenetic instability following long-term cultivation (Brimble et al., 2004; Cowan et al., 2004; Mitalipova et al., 2005; Allegrucci and Young, 2007). Despite the effects of variable culture conditions, majority of hESC lines have a similar morphology and they share similar expression of pluripotency markers such as *Oct3/4*, *Nanog*, *Nodal* and *GDF3* (summarized by Allegrucci and Young, 2007). However, not all hESC cell lines express identical level of these pluripotency markers, and epigenetic variation exists among distinct hESCs due to X chromosome inactivation (Sperger et al., 2003; Hoffman et al., 2005; Enver et al., 2005, Bártová et al., 2008a).

ESCs also display unique epigenetic patterns (Spivakov and Fisher, 2007; Bártová et al., 2008a). For example, Bernstein et al. (2006) identified specific bivalent domains of histone modifications that contained large regions of chromatin repressive mark (e.g., H3K27 methylation), which harbor smaller region of trascriptionally active chromatin mark such as H3K4 methylation. These domains are though to regulate developmentally important gene expression in pluripotent ESCs upon induction of their differentiation. (Bernstein et al., 2006; Jørgensen et al., 2006). We performed ChIP-on-chip analysis to investigate genome-wide H3K9 acetylation, a mark of transcriptionally active chromatin, in pluripotent and endoderm-like differentiated hESCs. Our results support the idea that global transcription is a hallmark of mESC pluripotency (Efroni et al., 2008) and suggest that analysis of genome-wide histone modification can enable discrimination between pluripotent and highly differentiated stages of hESCs. Higher level of H3K9 acetylation, generally associated with gene transcriptional activation, was observed in pluripotent hESCs, in comparison with their differentiated counterpart (Krejčí et al., 2009). These studies showed that individual chromosomes of pluripotent and differentiated hESCs contain specific H3K9 acetylated and deacetylated gene clusters, which are likely responsible either for hESC pluripotency or differentiation. As example, endoderm-like differentiation was characterized by pronounced changes in H3K9 acetylation especially at chromosomes 11, 12, 17 and 19. However, it is evident that H3K9 acetylation is not the only regulatory process but acts in combination with other histone-specific epigenetic modifications.

We also analyzed differentiation-associated changes in the nuclear radial arrangement of select genetic elements such as Oct3/4, Nanog, c-myc gene and the nuclear patterns of several epigenetic histone modifications. Our

data reveal that differentiation of hESCs is associated with distinct perinucleolar H3K9 trimethylation foci, along with centromere rearrangement and peripheral repositioning of the inactive X chromosome. Moreover, epigenetic variation between two hES cell lines was observed not only for inactivation of X chromosome but also for select pluripotency and differentiation markers. Positioning of transcriptionally active Oct3/4 gene on pronouncedly extended chromatin loops was also documented by our experiments (Bártová et al., 2008b).

Deficiency of histone methyltransferase Suv39h1 influences chromatin structure

Contemporary knowledge on histone code show that acetylated, methylated, ubiquitinated and/or phosphorylated N-terminal histone tails are very important epigenetic marks responsible for induction of functionally distinct nuclear sub-domains (Jenuwein and Allis, 2001). Moreover, knowledge on effects of histone modifying enzymes such as histone acetyltrasferase (HATs), histone deacetylases (HDACs), histone methyltrasferases (HMTs) and newly described histone demethylases (Shi et al., 2004) lead to discovery of mechanisms responsible for stabilization of histone code. It is well known that H3K9 methylation is mediated by HMTase called by Suv39h, which was firstly described by Rea et al. (2000) as heterochromatin-enriched enzyme, transiently accumulating around centromeres during mitosis (Aagaard et al., 2000) and at pericentromeric heterochromatin of interphase nuclei (Peters et al., 2001). The fact that Suv39h1 and Suv39h2 HMTases govern H3K9 methylation in heterochromatic regions is supported by the observation of abnormally long telomeres in primary cells of Suv39h double null mouse cells (Suv39h-/-) (García-Cao et al., 2004). Furthermore, Suv39h-mediated H3K9 methylation creates a binding site for heterochromatin protein 1 (HP1) (Bannister et al., 2001; Lachner et al., 2001; Nakayama et al., 2001).

In the present study we tested the hypothesis if orchestrated epigenetic regulation of chromatin by specific histone code is highly responsible for nuclear radial arrangement of chromatin domains and whole chromosome territories. In our experimental system with Suv39h1 deficient cells, it seems to be evident that one histone epigenetic mark can substitute another one, similarly as subtypes of heterochromatin protein 1(HP1) play an important role in maintaining genome integrity. As example we have analyzed nuclear pattern and fluorescence intensity of H3K9 monomethylation (me1) and HP1α, HP1β proteins in chromocentres of Suv39h1 deficient cells (Fig.1). Currently, we are testing the hypothesis on

how Suv39h1 HMTs can regulate epigenome of nucleoli-associated chromocentres as well as nucleoli, which seems to be a very important compartment of interphase nuclei owing to the regulation of rRNA synthesis.

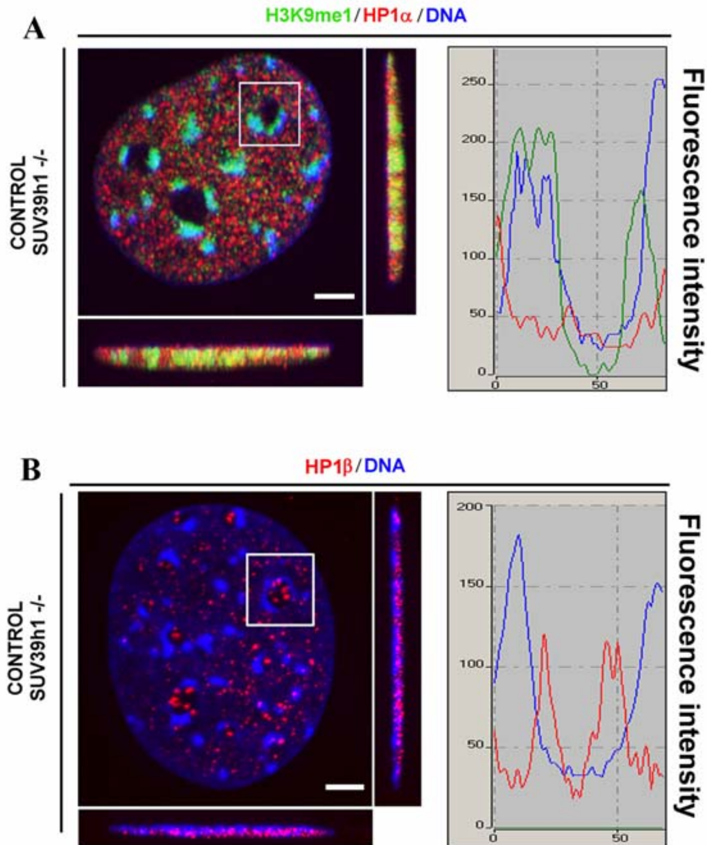

Figure 1: 3D-projection of Suv39h1 deficient mouse embryonic fibroblasts. (A) By the use of immunofluorescence, H3K9me1 (green) and HP1α (red) were visualized within interphase nucleus (blue). (B) The presence of HP1β (red) in chromocentres (blue) was also determined by immunofluorescence, combined with Nipkow disc-based confocal microscopy.

Chromatin structure and epigenetic in multiple myeloma cells

Multiple myeloma (MM) is a clonal cell malignancy of terminally differentiated B-cells that are characterized by their expansion in the bone marrow to form osteolytic bone lesions. MM is usually, but not always, generated from pre-malignant tumour stage called the monoclonal gamopathy of undetermined significance (MGUS) (summarized by Kuehl and Bergsagel 2002; Hideshima et al., 2004). Both MGUS and MM cells secret the monoclonal imunoglobulin, however, MGUS is defined by a lower intramedullary tumour cell content, osteolytic bone lesions and low level of tumour mass. Several stages of MM such as smouldering myeloma, intramedulary and extramedulary myeloma have been histologically clearly described (Kuehl and Bergsagel 2002); however, specific cytogenetic marks typical for all multiple myeloma stages have not been found yet.

Karyotypic instability is very common epiphenomenon for majority of tumour cells and multiple myeloma is not an exception (Pratt 2002; Fonseca et al., 2002). Detection of an abnormal karyotype, especially hypodiploidy, strongly correlates with poor multiple myeloma prognosis (Smadja et al., 2001). The using of chromatin immunoprecipitation (ChIP) with antibodies against specifically modified histones and combination of this technique with array technology (ChIP-on-chips) has ability to reveal the genome-wide incidence of histone modifications. This high-grade technology enables us to better understand the dynamics and correlations between epigenome and chromatin structure. Certain step in the understanding of epigenetics in tumour cell transformation was provided by Nguyen et al. (2001) showing relationship between DNA methylation, chromatin structure and transcription of cancer-related genes. Therefore, we focused on chromatin arrangement and epigenetic patterns of cytogenetically important loci of MM such as c-myc, IgH, CCND1 and TP53 genes. In CD138- (ARH-77) and CD138+ (MOLP-8) MM cell lines and in patients with diagnosed MM, the effects of select cytostatics were analysed. We tried to provide comprehensive information on how bortezomib, dexamethasone, gamma-radiation and melphalan influence the cell growth, cell cycle progression, the nuclear arrangement of loci studied, their epigenetic patters and protein levels in MM. Changes in the histone H3K9 acetylation and H3K9 dimethylation, induced by cytostatic treatment, were determined by ChIP-PCR and by ChIP-on-chip analyses for selected promoter regions. In this case we have found that melphalan has ability to increase genome-wide acetylation.

Granted projects

GA CR 204/06/0978, Post-translation modification of histones after cell treatment by inhibitors of histon deacetylases and during cell differentiation. Principal investigator: E. Bártová, 2006 - 2008

LC06027, Center of basic research for monoclonal gamapathy of multiple myeloma. Principal investigator: R. Hájek, Principal co-investigator: E. Bártová, 2006 - 2010

Publications

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PhD. thesis defended in 2008

Mgr. Andrea Harničarová, PhD., Nuclear topography and transcription activity of the c-myc gene during enterocytic differentiation

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Květa Láníková

In 2008, we made further progress in describing the hematopoiesis-modulating effects and in understanding their mechanisms connected with both pharmacological approaches tested currently in our laboratory .

An inhibitor of cyclooxygenase-2, meloxicam, has been shown to increase the production of a principal hematopoietic growth factor, granulocyte colony-stimulating factor (G-CSF), both in normal mice and in mice irradiated with sublethal or lethal doses of gamma-rays. Meloxicam, administered post-irradiation, has been observed to potentiate stimulatory effects of exogenous G-CSF on the growth of hematopoietic progenitor cells for granulocytes and macrophages. Furthemore, meloxicam, given 1 hour pre-irradiation, has been found to elevate numbers of bone marrow GM-CFC and peripheral blood granulocytes, as well as to increase 30-day survival in lethally irradiated animals. These observations may have an impact in possible clinical use of this drug in indications of myelosuppression of various etiology.

Two adenosine receptor agonists, IB-MECA and CPA, which selectively activate adenosine A_3 and A_1 receptors, were tested for their ability to influence proliferation of granulocytic and erythroid cells in femoral bone marrow of mice. The results obtained indicate opposite effects of the two adenosine receptor agonists on proliferation of hematopoietic cells (IB-MECA is a stimulator whereas CPA an inhibitor of cell proliferation). Moreover, the findings suggest the plasticity and homeostatic role of the adenosine receptor expression.

Granted projects

GA CR 305/06/0015, Interactions of stable adenosine receptor agonists and granulocyte colony-stimulating factor (G-CSF) in hematopoiesis. Principal investigator: M. Hofer, 2006 - 2008

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

Publications

Hofer, M., Pospíšil, M., Znojil, V., Holá, J., Vacek, A., Štreitová, D.: *Meloxicam, a cyclooxygenase-2 inhibitor, increases the level of serum G-CSF and might be usable as an auxiliary means in G-CSF therapy.* Physiol. Res., 57, 2008, 307-310.

Hofer, M., Pospíšil, M., Znojil, V., Holá, J., Štreitová, D., Vacek, A.: *Homeostatic action of adenosine* A_3 *and* A_1 *receptor agonists on proliferation of hematopoetic precursor cells.* Exp. Biol. Med., 233, 2008, 897-900.

Hofer, M., Pospíšil, M., Holá, J., Vacek, A., Štreitová, D., Znojil, V.: *Inhibition of cyclooxygenase 2 in mice increases production of G-CSF and induces radioprotection.* Radiat. Res., 170, 2008, 566-571.

Group of Structure, Function and Dynamics of Chromatin

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Significance of higher order chromatin structure remodeling during granulocyte differentiation for function of mature cells

Our earlier results show the large remodeling of higher order chromatin structure during differentiation of blood stem cells into granulocytes. The changes consist in elimination of HP1 proteins and tighter chromatin condensation manifested by inaccessibility of dimethylated histone H3 on the lysine 9 (H3K9) to immunodetection in mature, terminally differentiated granulocytes. Due to profound changes in chromatin structure accompanied by the stable repression of many genes, differentiated cells dramatically change their metabolic activity and responsiveness to signals. We observed, that terminally differentiated granulocytes do not respond on induction of double-strand breaks (DSB) by ionizing radiation. They do not phosphorylate H2AX in chromatin flanking DSB and do not form foci known as IRIF (ionizing radiation-induced foci) that are generally used for the detection of DSB. In addition, they do not express any of proteins accumulating in IRIFs and participating in the breaks repair. DSBs present the most serious damage to genome integrity that could lead to the cell death if not repaired. Therefore, all cells analyzed until now, try to rapidly repair this damage. However, as it follows from our results, DSBs do not present the danger for human granulocytes because these cells do not repair them. This non expected change in mature granulocyte behavior is probably related with the specific role of granulocyte chromatin in the basic function of these cells consisting in trapping of infectious microorganisms in the body. In this process, the tightly condensed chromatin of granulocytes must be rapidly relaxed into the fibers to kill the infection. This conformational transition of chromatin does not require, likely, the integrity of genome and

therefore DSBs are not repaired. The results show that the extensive changes in higher order chromatin structure during cell differentiation should ensure specific function of terminally differentiated granulocytes. Granulocytic differentiation is damaged in myeloid leukemia. We show that contrary to terminally differentiated granulocytes, incompletely differentiated neutrophils of AML patients expressing HP1β and γ express also proteins participating on DSBs repair. These cells are not mature and can not, therefore exert their function correctly. The overall haemopoiesis in AML is damaged and it should be restored by the treatment. We show that the treatment does not lead always to the maturation of granulocytes, even if it restores the level of total blood cells including neutrophils to the normal values. The detection of HP1 expression in granulocytes of AML patients after the treatment could serve as new the more sensitive prognostic indicator. Existing methods used for evaluation of healing are not able to detect the immature granulocytes. The presence of large fraction of immature neutrophiles in blood after the treatment might lead to the soon relapse of the disease. The role of immature granulocytes in the peripheral blood after the leukemia treatment on progress of the disease is not known and should be followed.

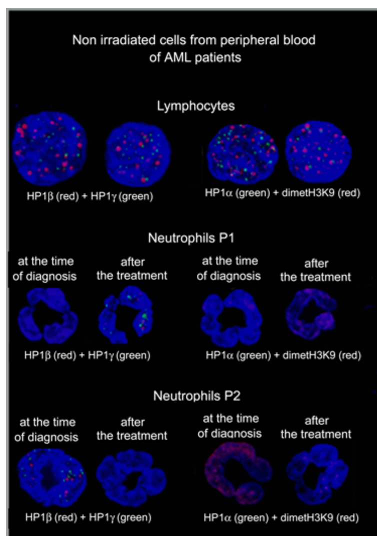

Figure 1: HP1α, β, γ and dimethylated histone H3K9 immunodetected in lymphocytes and neutrophils isolated from the peripheral blood of AML patients P1 and P2. The proteins were detected at the time of leukemia diagnosis and after the disease treatment.

Induction and repair of double-strand breaks in human fibroblasts with experimentally increased chromatin condensation

We tried to find whether the simple changes in chromatin condensation without participation of additional heterochromatin proteins could influence the induction of double-strand breaks (DSBs) by ionizing radiation and efficiency of their repair. These processes were studied in human fibroblasts with temporary changed chromatin condensation obtained by short time (<10 min) exposure of cells to the media with different osmolarity. Inhibitory effects of hyperosmotic environment (HOM) on the growth of cells and increase of their radiosensitivity were already described. We were therefore interested whether cell death induced by this environment is caused by chromatin hypercondensation (<10 min) provoked by this medium or by increased sensitivity of cells to DSB induction and/or decreased efficiency of their repair. Our results show that the short time exposure (<10 min) of cells to HOM results in completely reversible changes in chromatin structure after the transfer of cells to isotonic medium. In addition, this short time expose to HOM did not lead to the increase of DSBs and did not change the efficiency of their repair in comparison with cells growing in isotonic conditions. It follows from these results that contrary to physiological heterochromatin, the hypercondensed chromatin (HCC) does not protect DNA against its damage by ionizing radiation. The explanation for the different protective effect of these two types of condensed chromatin could reside in their dissimilar way of compaction and consequently their distinct structure. Physiological chromatin is formed with assistance of chromatin binding proteins (e.g. HP1) and high density of histone H1. Chromatin binding proteins form the principal protective barrier against ionizing radiation. HCC is probably formed by the contraction of relaxed chromatin, due to the decrease in the negative charge of the DNA in the high concentration of salts, without participation of other proteins. Longer exposure (>10 min) of non-irradiated cells to HOM resulted in DSB induction, inhibition of their repair and the cell death.

Granted projects

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

GA CR 204/06/P349, Dynamic structure and function of the cell nucleus associated with DNA breaks. Principal investigator: M. Falk, 2006 - 2008

GA AS CR 1QS500040508, Methylation of histone H3 as a prognostic marker of chronic myeloid leukemia remission. Principal investigator: S. Kozubek, 2005 - 2009

ME CR LC535, Chromosome dynamics and organization during the cell cycle in normal and pathologic conditions. Principal investigator: I. Raška, Principal co-investigator: S. Kozubek, 2005 - 2009

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GROUP OF ANALYSIS OF CHROMOSOMAL PROTEINS

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Modulation of activity and cellular expression of DNA topoisomerase IIα by HMGB1/2 proteins

DNA topoisomerase IIα (topo IIα) is an essential nuclear enzyme and its unique decatenation activity has been implicated in many aspects of chromosome dynamics such as chromosome replication and segregation during mitosis. We have for the first time demonstrated that chromatin-associated protein HMGB1 (a member of the large family of HMG-box proteins with possible functions in DNA replication, transcription, recombination, and DNA repair) could interact with topo IIα and stimulace the activity of the enzyme. We have also demonstrated that HMGB1 could up-regulate the activity of the *topo IIα* promoter in human cells that lack functional retinoblastoma protein pRb. Transient over-expression of pRb in pRb-negative Saos-2 cells inhibited the ability of HMGB1 to activate the *topo IIα* promoter. Consequently, up-regulation of the *topo IIα* promoter by HMGB1 was low in cells with functional pRb. The involvement of HMGB1 and its close relative, HMGB2, in modulation of activity of the *topo IIα* gene was further supported by knock-down of HMGB1/2, as evidenced by significantly decreased levels of *topo IIα* mRNA and protein. Our

experiments also suggested a possible mechanism of up-regulation of cellular expression of topo IIα by modulation of binding of transcription factor NF-Y by HMGB1/2 in pRb-negative cells. Our results indicated a possible link the previously observed pRb-inactivation, and increased levels of HMGB1 and topo IIα in tumors.

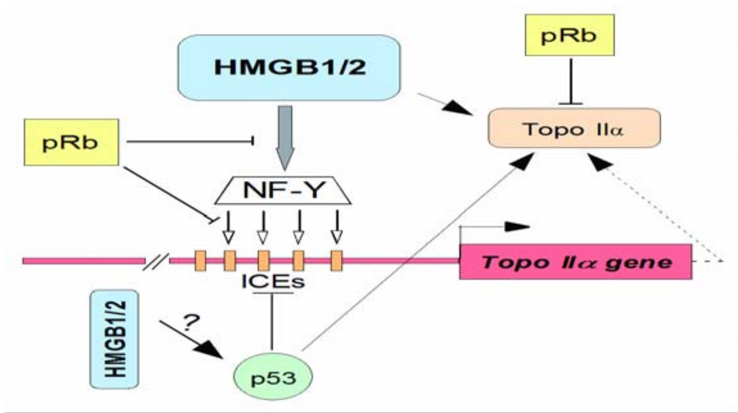

Figure 1: Modulation of activity of human *topo IIα* gene and topoisomerase IIα by pRb and HMGB1/2 (a hypothesis). HMGB1 and HMGB2 proteins up-regulate the expression of the human *topo IIα* gene in pRb-negative cells by enhancement of binding of crucial transcription factors, such as NF-Y, to their specific DNA binding sites (ICEs) within the *topo IIα* gene promoter. Binding of NF-Y to the ICEs is facilitated by pre-bending the DNA sequences by HMGB1/2. pRb can inhibit the activity of the *topo IIα* gene, as well as the ability of HMGB1 to up-regulate the gene, possibly by reducing binding of NF-Y to specific ICEs. HMGB1/2 promote cellular expression of topoisomerase IIα (this paper), and the proteins have also the potential of enhancing catalytic activity of the enzyme, as previously demonstrated *in vitro*.

Modulation of telomerase activity by HMGB1 in mammalian cells

Genomic instability can occur through a variety of mechanisms, one of them includes the dysfunction of telomeres, the specific nucleoprotein structures forming chromosome ends and protecting them from recombination and degradation. To counteract this “replicative shortening”, cells can activate telomerase. In humans, telomerase is ubiquitously expressed only during the first weeks of embryogenesis, and is subsequently down-regulated in most cell types. Previously we have demonstrated that knock-outed mouse embryonic fibroblasts (MEFs) had significantly lower

telomerase activity and shorter telomere lengths. Our latest experiments suggested that HMGB1 either acted as a chaperone and/or it could modulate the expression of cellular chaperons enabling correct folding of telomerase subunits into a functional enzyme. Our results may explain a role of HMGB1 protein in maintaining of genomic stability.

Role of alternative telomere lengthening in plant development

While a typical mechanism of maintenance of chromosome ends (telomeres) in plants is the enzyme complex of telomerase, it has been found that in its absence, an alternative telomere lengthening mechanism (ALT) becomes activated. Activation occurs on critical telomere shortening. Based on a simple experiment, in which changes in telomere length were monitored in plants which underwent a different number of cell divisions per seed-to-seed generation, we demonstrated what so far has only been speculated, that in the absence of telomerase, the number of cell divisions within one generation influences the control of telomere length. Fast and efficient activation of ALT in response to the loss of telomerase activity also implies that ALT is probably involved also in normal plant development.

Two faces of Solanaceae telomeres: a comparison between *Nicotiana* and *Cestrum* telomeres and telomere-binding proteins

While most Solanaceae genera (e.g. *Solanum*, *Nicotiana*) possess *Arabidopsis*-type telomeres of (TTTAGGG)n maintained by telomerase, the genera *Cestrum*, *Vestia* and *Sessea* (*Cestrum* group) lack these telomeres. Here we show that in the *Cestrum*-group the activity of telomerase has been lost. Nevertheless, proteins binding the single-stranded G-rich strand of the Arabidopsis-type and related human-type (TTAGGG)n telomeric sequences are present in nuclear extracts of both *Nicotiana* and *Cestrum* species. These proteins may have a role in telomere function or other cellular activities. In addition to characterizing DNA binding specificity and molecular weights of these proteins, we searched in both *N. tabacum* (tobacco) and *C. parqui* for the presence of POT1-like proteins, involved in telomere capping and telomerase regulation. Analysis of POT1-like proteins available on public databases and cloned by us from *C. parqui*, revealed the N-terminal OB folds typical for this protein family and a novel, plant-specific conserved C-terminal OB-fold domain (CTOB). We propose that CTOB is involved in protein-protein interactions.

Telomerase activity in head and neck cancer

Telomerase activity is associated with many malignancies, including head and neck cancer. The use of telomerase activity. as a diagnostic and prognostic marker of head and neck cancer development was examined and compared with standard histological analysis. Telomerase activity was determined using quantitative Dual-Colour-Real-Time TRAP (telomeric repeat amplification protocol). In each of 58 patients, a sample of tumour tissue, adjacent mucosa and normal muscle was collected. Telomerase activation was observed in 88% of tumour tissues and 34% of tumour-adjacent mucosa samples. No telomerase activity was detected in normal muscle tissues. Telomerase activity correlated with tumour grade, showing an average of 4.6 telomerase units (T.U.) in well-differentiated, 8.3 T.U. in mid-differentiated and 20 T.U. in poorly-differentiated tumours. Relapse occurred in 13 patients and no telomerase activity was detected in 3 recurrent tumours. Telomerase activity may be used as an objective parameter inversely-related to tumour differentiation. Prognosis in telomerase-negative tumours is worse than that of the telomerase-positive group.

Granted projects

ME LC06004, Integration of research activities to study the plant genome, Principal investigator: B. Vyskot, 2006 - 2010

GA AS CR IAA600040505, Telomerase-independent mechanismus of telomere synthesis and loss. Principal investigator: J. Fajkus, 2005 - 2009

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

GA CR 204/08/1530, Understanding of HMGB1 protein involvement in maintaining genome stability. Principal investigator: M. Štros, 2008 - 2010

GA AS CR IAA400040702, Hemicatenated DNA loops: their occurence in humen genome and recognition by tumor suppressor protein p53. Principal investigator: M. Štros, 2007 - 2010

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PhD. thesis defended in 2008

Mgr. Gabriela Rotková, PhD., Characterisation of nucleoprotein complexes in Asparagales plants

Ing. Zuzana Kunická, PhD., Use of telomerase as diagnostic and prognostic tumour marker

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The attention is focused on the research of cellular signaling and physiology/pathophysiology relevant to cancer. A potential role of lipid membrane elements and their derivatives in these processes is investigated. In particular, the effects of environmental substances, such as lipid nutrition components (essential polyunsaturated fatty acids and butyrate) and xenobiotics (cytostatics and environmental organic pollutants) on regulation

of cytokinetics, i. e. cell proliferation, differentiation and apoptosis are studied. Using both tumor and non-tumorigenic cells, new types of interaction of lipid dietary components, anticancer drugs (non-steroidal anti-inflammatory drugs-NSAIDs, cytostatics) or selected environmental pollutants (polycyclic aromatic hydrocarbons, PCBs, dioxins) with physiological regulators of cytokinetics are being investigated. Attention is being paid especially to tumor necrosis factor (TNF) family, tumor growth factor (TGF) family, fibroblast growth factor (FGF) and Wnt/beta-catenin pathway signaling. The results are exploited in cancer prevention/therapy and in ecotoxicology.

Cellular and molecular physiology of lipids (Jiřina Hofmanová)

Processes of colon cell differentiation and apoptosis are attended by changes of biophysical properties of cell membranes and by modulation of constitution, structure, symmetry and metabolism of cell lipids. Dietary polyunsaturated fatty acids (PUFAs) and their metabolites play a significant role in maintainance of homeostasis in self-renewing tissue of the colon. Moreover, their interaction with other compounds such as short-chain fatty acid butyrate (produced by intestinal microbial fermentation of dietary fibre) and endogenous regulatory molecules such as cytokiness affects colon cytokinetics. In our studies we examined:

i/ Differentiation and apoptotic response of the cell lines derived from "normal" human fetal colon (FHC), well-differentiated (HT-29) and poorly-differentiated (HCT-116) colon adenocarcinoma cells to treatment with sodium butyrate (NaBt) and ω-3 (docosahexaenoic acid - DHA, 22:6) or ω-6 (arachidonic acid - AA, 20:4) PUFAs alone or in combination.
FHC and HT-29 cells, but not HCT-116 cells were able to differentiate after treatment with NaBt as detected by ALP activity. This effect was significantly suppressed by PUFAs, especially DHA. In all cell lines, NaBt induced certain level of apoptosis (the highest in HCT-116 cells), but only in FHC cells PUFAs significantly increased the percentage of apoptotic cells. Changes in cytokinetics induced by fatty acids were accompanied by membrane lipid unpacking, reactive oxygen species (ROS) production, and decrease in mitochondrial membrane potential (MMP). Detection of caspase-3 activation and dynamic modulation of Mcl-1 protein expression imply their possible role in both cell differentiation and apoptotic response. Our results go along with the idea that the effects of fermentable fiber or butyrate in the colon depend on the presence of type and amount of

unsaturated fatty acids and that these compounds may have diverse impact in normal and neoplastic epithelium (*Hofmanová et al. 2008*).

ii/ Interaction of fatty acids with apoptotic inducers of TNF family in colon cells.

Omega-3 DHA, present especially in fish oil, may alter membrane properties, influence receptor activities, modulate intracellular signaling, oxidative metabolism and gene expression. Tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) is known to selectively induce apoptosis in cancer cells, while sparing normal cells. Despite it, some cancer cell type including colon are resistant to its effect. Supposing modulation of TRAIL apoptotic signaling by DHA, we investigated the effects of DHA pre-treatment and subsequent TRAIL treatment on cell cycle, viability and apoptosis of two human colon cancer cell lines sensitive (HCT-116) or relatively resistant (HT-29) to TRAIL-induced apoptosis. Our results showed that cell growth is inhibited and percentage of floating and apoptotic cell is increased after pretreatment with DHA in HT-29 cells but not in HCT-116 cells. These findings indicate that DHA can increase sensitivity to the apoptotic effect of TRAIL in resistant colon cancer cells.

The mechanisms of TRAIL effects were further investigated using previously established models of non-adherent cultivation of colon epithelial cells. We found an increase in TRAIL-induced apoptosis during non-adherent cultivation (in comparison with adherent conditions) in adenocarcinoma HT-29 cells but not in fetal FHC cells. Evaluation of expression of TRAIL receptors (DR4, DR5, Dc) on the cell surface revealed no differences between either cell lines or both types of cultivation. Since we found increased activation (phosphorylation) of focal adhesion kinase (FAK) and pro-survival PI3/Akt kinase during non-adherent cell cultivation compared to adherent one, we have proposed the role of these pathways in decreased sensitivity of the colon cells to the TRAIL action during non-adherent cultivation. Significantly increased TRAIL-induced apoptosis after inhibition of PI3/Akt pathway confirmed involvement of this pathway in cellular sensitivity to TRAIL.

Investigation of interaction of NaBt with TNF-α showed suppressed differentiation and potentiated cell death induced by NaBt in both colon adenocarcinoma HT-29 and fetal colon FHC cells. Since TNF-α is a typical activator of NF-κB pathway, we studied the role of activation of this transcription factor in cell differentiation and death during the TNF-α and NaBt co-treatment. TNF-α induced rapid NF-κB activation in both cell lines but this effect was modulated differently by NaBt in fetal and cancer cells.

Moreover, we also detected a different response of HT-29 and FHC cells to the pre-treatment with the NF-κB activation inhibitor parthenolide. Our results indicated that NaBt-mediated differentiation and apoptosis of HT-29 and FHC cells are similarly modulated by TNF-α. However, there are significant differences in the mechanism of these effects suggesting that the NF-κB pathway is more effectively involved in these processes in cancer cells (*Hýžďalová et al. 2008*).

Growth factors in cancer cell signaling (Karel Souček)

Pathophysiological conditions reflected in deregulation of differentiation, proliferation and apoptosis modify homeostasis and function of prostate epithelia and can lead to diseases such as benign hyperplasia and cancer. Transforming growth factor-β (TGF-β), IL-6 and Wnt family members represent highly biologically active molecules, secreted to the tissue microenvironment, where they can induce different signaling pathways in paracrine and/or autocrine manner. These autocrine/paracrine factors have been shown to change microenvironment in prostate gland and modulate growth and surviving of cancer cells. Defects in functions of components of these pathways have been observed in various human cancers.

In 2008 we continued with studies focused on (1) signal transduction and functional role of TGF-β family in various type of cells, (2) mechanisms of interaction between IL-6 and TGF-β signaling pathways, (3) neuroendocrine trans-differentiation of prostate cancer cells.

Epithelial–mesenchymal transition (EMT), in which epithelial cells loose their polarity and become motile mesenchymal cells, occurs during development and is viewed as an essential early step in tumour metastasis. Transcription factors and EMT regulators of the ZEB family are thought to be involved in tumour progression, thus having potential clinical interest. miR-200 family and miR-205, which regulate expression of ZEB transcription factors, have been found downregulated during EMT, suggesting an important role in inhibition of EMT. In our study, EMT of BPH-1 cell line was induced by (TGF-β treatment and assessed by cell morphology and expression of epithelial (E-cadherin) and mesenchymal markers (N-cadherin, vimentin), ZEB1, ZEB2, miR-200 family and miR-205. The early changes of ZEB proteins transcripts observed suggest a possible role of miR-200 family and ZEB proteins in early stages of the EMT process of nontumorigenic prostate epithelial cell line.

IL-6 and TGF-β1 treatment caused activation of signaling molecules STAT3 and SMAD2 in BPH-1 cell line. TGF-β1 has antiproliferative effects as we determined by measuring the cell numbers and cell cycle, whereas it seems that IL-6 has opposite effects, respectively IL-6 does not decrease the cell numbers and percentage of cells in S-phase. IL-6 treatment did not affect TGF-β1 induced epithelial-mesenchymal transition of BPH-1 cells. However, long-term treatment of cells with TGF-β1 resulted in abolition of IL-6 induced STAT3 phosphorylation. Our data demonstrate that there is a crosstalk between IL-6 and TGF-β1 pathways and TGF-β1 modulates proinflammatory microenvironment in prostate tissue by downregulation of IL-6 signaling.

Neuroendocrine differentiation of epithelial prostate cancer cells is phenomena clearly associated with cancer progression. However, mechanisms controlling differentiation of prostate epithelial cells have remained poorly characterized. We found out that expression of NED markers in LNCaP cells increased also with cell density. This observation was confirmed in mouse cell line TRAMP-C1. NED is associated with cell cycle arrest and downregulation of cyclin D1. Using RNA interference we showed that level of cyclin D1 does not reflect changes in expression of NED markers. Senescence associated with permanent cell cycle arrest is well known in primary cells, but it was observed also in cancer cells. Using staining for conventional marker of senescence - senescence associated β-galactosidase (SA-β-gal), we showed, that cells trans-differentiated by androgen depletion express this marker. Acquisition of senescent phenotype was confirmed by increased level of another marker of senescence $p16^{INK4A}$. We can summarize that cyclin D1 is not the key regulator of NED and that NED is associated with induction of senescence in prostate adenocarcinoma.

Molecular mechanisms of Wnt and fibroblast growth factor 3 (FGFR3) signaling (Vítězslav Bryja, Pavel Krejčí)

We contributed to the discovery of novel mechanism regulating proliferation of embryonic stem cells and identified unique, lineage-dependent, composition of cyclin-CDK-p27 containing complexes, which form in differentiating embryonal carcinoma and stem cells.

We studied biochemical and biological properties of non-canonical Wnt signaling. We have identified crucial importance of beta-arrestin and casein

kinase 1 and 2 in the regulation of specificity in the non-canonical Wnt signaling pathway. Phosphorylation of Dishevelled, crucial mediator of the Wnt signaling cascade, by CK1 and CK2 acts as a switch which shuts off the pathway towards Rac1, which is mediated by beta-arrestin. CK1/2 and beta-arrestin acting at the level of Dvl thus have an opposing role in the non-canonical Wnt signaling. Moreover, in cooperation with dr. M. Andäng (Karolinska Institutet, Sweden) we have contributed to the finding, which shows that Wnt5a, typical non-canonical Wnt ligand, regulates morphogenesis in the midbrain (*Bryja et al. 2008, Andäng et al. 2008*).

Our analysis of fibroblast growth factor (FGF) signaling in the proliferating chondrocytes demonstrated that STATs are not crucial mediators of FGFR3-driven actions in these cells. Activation of the FGF pathway can inhibit STAT signaling. In cooperation with W. R. Wilcox (Medical Genetics Inst. California) we show that STAT1 and STAT3 do not participate in the FGF-mediated growth arrest in chondrocytes, which represents the major phenotype of pathological FGFR3 signaling in skeletal dysplasia. Moreover, only some mutations of FGFR3 receptor associated with skeletal dysplasias result in the phosphorylation of STATs, whereas all lead to the activation of ERK signaling cascade. Among six FGFR3 mutants associated with skeletal dysplasia and cancer, only K650E and K650M-FGFR3 activate STAT1 or STAT5, thus undermining the central role of STATs in pathological FGFR3 signaling in skeletal dysplasia (*Krejci et al 2008*).

Cellular and molecular toxicology (Jan Vondráček)

The principal aim of our studies is to contribute to understanding of effects of environmental organic pollutants at molecular and cellular level, which might be linked to carcinogenesis, reproductive or developmental impairment. Therefore, we investigate interactions between signaling pathways activated by toxicants and major signaling pathways involved in maintenance of cellular homeostasis, such as those controlling cell proliferation, differentiation and apoptosis. In 2008, we further concentrated on the mechanisms underlying disruption of cell proliferation and cell-to-cell communication control by toxic compounds, including polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and dioxins. We characterized the impact of a series of monomethylated derivatives toxic events in rat liver cells (*Machala et al., 2007*). We found that position of methyl group is a strong determinant of AhR activation or

inhibition of gap-junctional intercellular communication (GJIC). Thus, several of these compounds might significantly contribute to the overall AhR-mediated activity of environmental mixtures, an important toxic mode of action of environmental organic pollutants. Second, we have evaluated impact of model environmental pollutants (methylated anthracenes, non-dioxin-like PCBs) on lipid signaling in liver epithelial cells. We described that NDL-PCBs may induce release of arachidonic acid (AA) in target cells, which is partly mediated by activity of cytosolic phospholipase A_2 and ERK1/2 signaling (*Umannová et al., 2008*). Similar to that, 1-methylanthracene was shown to activate AA release and further disruption of lipid signaling in liver epithelial cells, which was associated with GJIC inhibition (*Upham et al., 2008*). Moreover, in collaboration with the research group of C. Dietrich (Institute of Toxicology, JGU, Mainz, Germany), it was shown that AhR ligands induce disruption of contact inhibition in both liver and kidney epithelial cells through activation of an AhR-dependent mechanism, which involves JunD and cyclin A (*Weiss et al., 2008*). This mechanism might contribute to known tumor promoting effects of dioxins in liver. Finally, we collaborated on a study of in vivo estrogenic effects of PAHs, which confirmed our previous in vitro results suggesting that some PAHs may activate estrogen receptors and induce ER-dependent cell proliferation (*Kummer et al.,* 2008). Taken together, these results contribute to our understanding of multifaceted toxic effects of PAHs in PCBs in vertebrates.

The effects of cytostatics (Alois Kozubík)

LA-12 is a platinum(IV) compound with bulky hydrophobic ligand adamantylamine. In our previous studies, LA-12 was shown to be able to overcome acquired and intrinsic resistance to widely used platinum antitumor drugs cisplatin in ovarian cancer cell lines. It was also more efficacious than cisplatin and oxaliplatin in decreasing proliferation rate and induction of the cell death in colorectal cancer cell lines HCT-116 and HT-29.

We continued our investigation of mechanisms of LA-12 cytotoxicity on a model of colorectal cancer cell line HCT-116, insensitive to cisplatin and oxaliplatin. We focused on LA-12 induced cell cycle modulation and its cross connection with induction of programmed cell death apoptosis. It was found that LA-12 caused accumulation of the cells in G2/M phase of the cell cycle independently on p21 or p53 protein. In spite of this event, there

were found no decline in the level of key G2/M regulator cyclin B1 and activity of Cdc2 kinase and unaltered level of mitotic cells. Moreover, early apoptotic cells were found predominantly in G1 phase of the cell cycle.

Resistance of tumor cells to therapy is a substantial impediment in the treatment of cancer. Various combinations of drugs with different mechanisms of action are tested to overpower the prosurvival processes in the tumor cells. Platinum drugs were described to reinforce the ability of TRAIL (TNF-related apoptosis-inducing ligand) to induce apoptosis. In our experiments, incubation of PC-3 prostate cancer cells and HCT-116 colon cancer cells with cisplatin or LA-12 caused an increase in cellular and membrane expression of TRAIL receptor DR5, which transduces proapoptotic signals. Preincubation with cisplatin or LA-12 and consecutive incubation with TRAIL led to enhanced cleavage of caspase 8 and 3 and apoptosis commitment.

Hypericin photocytotoxicity mediated by photodynamic therapy (PDT)

Our previous study in cooperation with UPJS Košice, Slovak Republic, proved that colon cancer cells HT-29 pre-treated with specific 5-lipoxygenase inhibitor MK-886 became more susceptible to photodynamic therapy (PDT) with hypericin and we also found that this mutual combination induced cell cycle arrest and stimulated onset of apoptosis. To further explain events associated with MK-886 mediated sensitization of tumor cells toward PDT with hypericin, more detailed study of signaling pathways leading to increase in apoptosis as well as cell cycle perturbations was performed and is presented herein. Intensive accumulation of HT-29 cells in G0/G1 phase of cell cycle led to expression analyses of several G0/G1 checkpoint molecules (cyclin A, cyclin E, cdk-2, pRb). Similarly, accumulation of apoptotic cells invoked analyses of key molecules involved in apoptotic signaling (caspase-3, -8, -9; PARP; Lamin B; Mcl-1; Bax) by Western blotting and caspase activity assay. Long term survival of cells was examined by clonogenicity test. As the effect of PDT is mediated by ROS production, levels of hydrogen peroxides and superoxide anion were monitored by flow cytometric analyses. In addition, an impact of MK-886 on LTB4 production and expression of 5-LOX was monitored. Massive G0/G1 arrest in the cell cycle accompanied by increase in cyclin E level and decrease/absention of cyclin A, cdk-2 and pRb expression indicated incapability for G1/S transition. The clonogenicity test revealed disruption of colony formation after mutual combination of both agents as compared to

MK-886 or PDT alone. Pre-treatment with MK-886 modulated distribution of ROS production in mutual combination with PDT. In conclusion, we presume that stimulation of apoptosis in our experimental model was accomplished preferentially through the mitochondrial pathway, although caspase-8 activation was also noticed (*Kleban et al. 2008*).

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

GA CR 204/07/0834, Role of transforming growth factor-beta in regulation of proliferation, differentiation and apoptosis in prostate and colon cancer. Principal investigator: K. Souček, 2007 - 2009

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

GA AS CR 1QS500040507, Lipid nutrition compounds-modulation of their effects and possibilities of practical application. Principal investigator: A. Kozubík, 2005 - 2009

GA CR 524/06/0517, Mechanisms of disruption of cell-to-cell communication and regulation of cell proliferation in liver cells. Principal investigator: J. Vondráček, 2006 - 2008

GA CR 524/06/P345, Activity of inflammatory regulator NF-kappaB modulated by alteration of arachidonic acid metabolism. Principal investigator: J. Procházková, 2006 - 2008

Programme KONTAKT, International Scientific and Technological Cooperation (Czech Republic - Hungary), Role of lipid rafts in regulation of cell signalling leading to modulation of cytokinetics of cancer cells. Principal investigator: A. Kozubík, 2007 - 2008

PLIVA-LACHEMA, a. s. - Contract (LA-12 programme). Principal investigator: A. Kozubík, 2006 - 2008

MU Rektor's Programme for Students´ Creative Activity Support MUNI/31/C0008/2008, Differences of adhesive properties and anoikis regulation in colon cells during interaction of fatty acids with cytokines of TNF family. Principal investigator: L. Kočí, 2008 - 2009

MU Rektor's Programme for Students´ Creative Activity Support MUNI/31/C0005/2008, Role of TGF beta family proteins in the cytokinetics of prostate and colon cancer cells. Principal investigator: E. Lincová, 2008 - 2009

MU Rektor's Programme for Students' Creative Activity Support MUNI/31/A0004/2008, Modulation possibilities of cytokine IL-6 signal transduction in prostate epithelial cells. Principal investigator: A. Staršíchová, 2008 - 2009

MU Rektor's Programme for Students′ Creative Activity Support MUNI/31/A0003/2008, Possibilities in modulation of neuroendocrine differentiation of prostate cancer cells. Principal investigator: Z. Pernicová, 2008 - 2009

FRVS/666/2008, Innovation of lecture modern methods of cell biology. Principal investigator: M. Dušková, Principal co-investigator: J. Hofmanová, 2008

FRVS/58/2008, Analytic cytometry. Principal investigator: A. Kozubík, Principal co-investigator: K. Souček, 2008

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Weiss, C., Faust, D., Schreck, I., Ruff, A., Farwerck, T., Melenberg, A., Schneider, S., Oesch-Bartlomowicz, B., Zatloukalová, J., Vondráček, J., Oesch, F., Dietrich, C.: *TCDD deregulates contact inhibition in rat liver oval cells via Ah receptor, JunD and cyclin A.* Oncogene, 27, 2008, 2198-2207.

PhD. thesis defended in 2008

Mgr. Jiřina Procházková-Zatloukalová, PhD., Deregulation of cell proliferation and apoptosis by xenobiotics and cytostatics

Free Radical Pathophysiology

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Continuous electrochemical monitoring of nitric oxide production in murine macrophage cell line RAW 264.7

We realized the continual and long-term electrochemical detection of NO production by stimulated macrophages using modified porphyrinic microsensor. The NO release from RAW 264.7 cells stimulated by lipopolysaccharide started 5 hours after the lipopolysaccharide administration. After reaching its maximum at 6th hour, the stable level of NO production was observed between 7-12th hour of the experiment. This phase was followed by a gradual decline in NO production. A close correlation between the NO signal detected with micro-electrode and nitrite accumulation in supernatants of stimulated cells was observed. The presence of iNOS enzyme, which constitutes a main requirement for NO production by stimulated macrophages, was confirmed by Western blot analysis of iNOS protein expression at key time-points of corresponding electrochemical experiment. The capability of our microsensor to instantaneously monitor the changes in the NO production by stimulated RAW 264.7 cells was demonstrated by the immediate decrease in the signal due to NO as a response to the addition of iNOS inhibitor into the cell culture medium.

Effect of polyunsaturated fatty acids on the reactive oxygen species (ROS) and reactive nitrogen species production by RAW 264.7 macrophages

Polyunsaturated fatty acids (PUFAs) can affect various functions of immune systems including inflammatory responses in biological systems. An oxidative burst of phagocytes accompanied by reactive oxygen species (ROS) and reactive nitrogen species (RNS) formation is one of the phagocyte functions which could be modulated by PUFAs. We investigated the effects of ω-3 (α-linolenic, docosahexaenoic, eicosapentaenoic) and ω-6 (arachidonic, linoleic) PUFAs on lipopolysaccharide stimulated ROS and RNS production by the murine macrophage cell line RAW 264.7. Cells were stimulated with LPS (0.1 µg/ml) and treated with 0.1 - 100 µM ω-3 or ω-6 PUFAs for either 8 or 20 h. Cytotoxicity of PUFAs was evaluated by an ATP test. Changes in ROS production were determined by luminol-enhanced chemiluminescence, while the production of RNS was determined as the concentration of nitrites in cell supernatants (Griess reaction). Changes in inducible nitric oxide synthase (iNOS) expression were evaluated by Western blot analysis. Antioxidant properties of PUFAs were tested by TRAP (total peroxyl radical-trapping antioxidant parameter) assay. All PUFAs in 100 µM concentration except eicosapentaenoic acid decreased ROS production. The effect was the most significant when docosahexaenoic acid was used. Arachidonic acid decreased phorbol myristate acetate activated ROS production even in 1 and 10 µM concentrations. On the other hand, 10 and 100 µM eicosapentaenoic acid potentiated activated ROS production. As concerns RNS production, all the fatty acids that were tested in a concentration of 100 µM decreased iNOS expression and nitrite accumulation. Fatty acids had no significant effect on the viability and proliferation of RAW 264.7 cells. The TRAP assay confirmed that none of the tested PUFAs exerted any significant antioxidant properties. In conclusion, high concentrations of polyunsaturated fatty acids of both ω-3 and ω-6 groups can inhibit ROS and RNS formation by stimulated macrophages. The expression of iNOS can also be inhibited. This effect together with the absence of antioxidant activity and cytotoxic properties indicates that PUFAs influence enzymes responsible for the reactive species production.

Group of Pathophysiology of Free Radicals in Cell Interactions

Group Leader

Milan Číž

Oxidatively modified collagens type I and type IV lost their activating properties towards platelet aggregation and oxidative burst of phagocytes

Human collagen type IV and rat collagen type I samples were subjected to the oxidative modification by incubation of collagen solutions with various oxidants: hydrogen peroxide (100 mM, 300 mM), hydroxyl radical (100 microM $FeSO_4$/2 mM H_2O_2; 50 microM $FeSO_4$/5 mM H_2O_2), peroxyl radical (obtained by the thermal decomposition of 200 mM ABAP), and sodium hypochlorite (5% NaOCl).

It was observed that various chemical systems generating individual reactive oxygen species caused oxidative modification of human collagen type IV, characterized by a decrease in its denaturation temperature (Table 1). The denaturation temperature of non-modified human collagen type IV (T_d = 45.3°C) was higher when compared to animal collagens type I (T_d = 36.3°C) studied previously.

collagen modification	T_{d1} (°C)	T_{d2} (°C)
non-modified	-	45.3 ± 0.1
peroxyl radical	32.8 ± 0.1	-
hydrogen peroxide	32.1 ± 0.1	46.4 ± 0.1
hydroxyl radical	33.8 ± 0.1	45.1 ± 0.1
sodium hypochlorite	33.7 ± 0.1	45.7 ± 0.1

Table 1: Denaturation temperature of human collagen type IV.

The effect of collagen samples on platelet aggregation was measured in isolated human platelets obtained after differential centrifugation. Non-modified human collagen type IV, similarly to collagen type I, induced platelet aggregation comparable with that induced by thrombine. All oxidatively modified collagen samples, independently of the oxidation treatment applied, lost their platelet-aggregating activity. Platelet aggregation in a presence of non-modified collage type IV was accompanied by a release of serotonin and ATP.

Both non-modified and oxidatively modified rat collagen type I non-significantly decreased nitric oxide production by murine peritoneal macrophages RAW 264.7. There were no differences observed either between non-modified and oxidatively modified collagens or among samples of collagen modified with various reactive oxygen species. Similarly, no changes in inducible nitric oxide synthase expression by RAW 264.7 cells were observed in the presence of either non-modified or oxidatively modified collagens.

A production of reactive oxygen species by phagocytes in human whole blood was evaluated by luminol-enhanced chemiluminescence. Spontaneous chemiluminescence and chemiluminescence of phagocytes activated with one of activators - opsonized zymosan particles, calcium ionophore A23187, phorbol-12-myristate-13-acetate or N-formyl-Met-Leu-Phe was determined. Non-modified human collagen type IV itself had a capacity to induce an oxidative burst of phagocytes in whole blood. Oxidatively modified collagen samples lost their capacity to induce production of reactive oxygen species by phagocytes.
It can be concluded that reactive oxygen species produced by activated professional phagocytes are able to modify collagen, a major constituent of the extracellular matrix. On the other hand, both non-modified and oxidatively modified collagens can differentially modulate the activity of professional phagocytes either directly or indirectly via mediators released from activated platelets.

GROUP OF FREE RADICALS IN REGULATION OF CELL PHYSIOLOGY

GROUP LEADER

LUKÁŠ KUBALA

Modulation of neuronal differentiation of P19 mouse embryonal carcinoma cells by changes in redox intracellular enviroment

Contribution of reactive oxygen species (ROS) and components of extracellular matrix to regulation of differentiation of pluripotent mouse embryonal carcinoma P19 cells was studied. It was shown that neuronal differentiation of P19 cells induced by retinoic acid (RA) and a potentiation of this process by leukemia inhibitory factor were abolished by inhibition of the JAK2 → STAT3 signalling pathway. Interestingly, RA increased intracellular ROS production that was accompanied by a decrease in thiol groups in cells. Application of the ROS scavengers and flavoprotein inhibitor reduced RA-induced ROS production, RA-induced activity of RARE, and it decreased the RA-induced differentiation determined by expression of N-cadherin and III-beta tubulin. These data outline a role of ROS as important molecules in the transduction of an intracellular signal during the neuronal differentiation of ES cells.

Characterization of mesenchymal stem cells (MSC) obtained from bone marrow collection sets and modulation of their differentiation to chondrocytes by hyaluronic acid

Differentiation potential of MSCs wash out from used bone marrow collection sets was evaluated. Collected cells revealed all characteristics given for pluripotent MSC cells including positivity to all essential MSC surface molecules and negativity for most haematopoietic and endothelial cell markers. The cells were capable of differentiation along adipogenic, osteogenic and chondrogenic pathways. Thus, these MSCs are highly ethical source of MSCs for research purposes and may be utilized also in clinical applications. Further, effects of hyaluronan (HA), an abundant component of extracellular matrix often used as a fundamental constituent in cartilage tissue substitutes, on chondrogenesis was tested. Interestingly, either low or high molecular weight HA did not significantly modulate early

phases of chodrogenesis as was evaluated based on changes in expressions of characteristic genes (transcription factor Sox-9 and extracellular matrix proteins collagen type II and XI, aggrecan, and COMP) and by histological analysis. Thus, data suggest that HA of any tested molecular weight does not significantly modulate chondrogenesis of MSCs in pellet system.

The effect of uric acid on homocysteine-induced endothelial dysfunction

Elevated plasma uric acid is known to indicate an increased risk of cardiovascular diseases associated with endothelial dysfunction. However, the role of uric acid in the pathogenesis of endothelial dysfunction is still a matter of debate. Its potential harmful effect is in contrast with the fact that uric acid is one of the most important antioxidants in body fluids. It is not clear, whether uric acid is a real causative risk factor, an inert marker or even protective molecule with respect to its antioxidant properties We have studied the effect of uric acid on intact endothelial cells as well as on the cells with homocysteine-induced endothelial dysfunction. Bovine aortic endothelial cells (BAEC) were treated with uric acid (0.1 – 0.6 mM) and homocysteine (0.1 mM), or uric acid only for 24 h. Then the cells were stimulated with 0.001mg/ml of calcium ionophore A23187 and nitric oxide (NO) production was measured electrochemically with the use of NO-sensitive microelectrode. Expression of endothelial nitric oxide synthase (eNOS) and eNOS phosphorylation at Ser1179 was estimated with the use of Western blotting. Interaction between NO and uric acid was measured with NO electrode. Superoxide generation was measured with the use of fluorescence dye MitoSox Red. 24h incubation of the cells with 0.1 mM homocysteine strongly diminished A23187-induced NO release. Concomitant treatment with 0.1 mM uric acid slightly restored NO production. Higher uric acid concentrations were ineffective. In the cells treated with uric acid only, a dose-dependent decrease in A23187-induced NO release was observed. Uric acid did not scavenge NO and did not change eNOS expression or phosphorylation at Ser1179, but dose-dependently increased superoxide production in A23187-stimulated cells. In conclusion, uric acid decreased nitric oxide bioavailability and enhanced superoxide generation in A23187-stimulated BAEC.

Granted projects

NATO CBP.EAP.CLG.982048, Collagen, platelet and neutrophil interactions with respect to wound healing. Principal investigator: M. Číž, 2006 - 2008

GA CR 524/07/1511, Interactions between collagen, platelets and neutrophils with respect to wound healing. Principal investigator: M. Číž, 2007 - 2009

GA CR 524/06/1197, Role of free radicals in the regulation of lung inflammation induced by acute and chronic exposure to endotoxin. Principal investigator: L. Kubala, 2006 - 2008

GA CR 525/06/1196, The use of terrestrial luminescent bacteria in ecotoxicology. Principal investigator: A. Lojek, 2006 - 2008

GA CR 204/07/P539, The role of uric acid in endothelial dysfunction. Principal investigator: I. Papežíková, 2007 - 2009

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 - 20012

GA CR 305/08/1704, Role of hyaluronan of different molecular weight in the course of inflammation. Principal investigator: L. Kubala, 2008 - 20010

MEYS - Kontakt MEB 0808106, The role of platelets, neutrophils and components of extracellular matrix in inflammation. Principal investigator: A. Lojek, 2008 - 2009

GA AS CR – DAAD Germany: D2-CZ14/07-08, Trombocytes, an extra-leukocyte pool for Myeloperoxidase. Principal investigator: L. Kubala, 2007 - 2008

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PhD. thesis defended in 2008

Mgr. Veronika Hájková, PhD., Potential of glucomannan from *Candida utilis* to activate human innate immunity and skin resistance

Structure and Dynamics of Nucleic Acids

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Arnošt Mládek

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

We continued in our Molecular Dynamics (MD) studies of RNA segments from the ribosome and other RNA molecules. Our preceding study of RNA kissing loop complexes from human immunodeficiency virus utilizing AMBER force field was complemented by study employing CHARMM force field. The kissing loop complex is a tertiary interaction formed by base pairing between the single-stranded residues of two hairpin loops with complementary sequences. X-ray and NMR kissing loop complexes of subtype A, B and F from dimerization initiation site were studied using explicit solvent MD simulations. The kissing complexes consist of unpaired (flanking) purine bases that are important for folding and stabilization, and also for mediating of tertiary contacts with distant molecules. The utilized X-ray structures show bulged-out flanking bases, either four base stack or two separate stacks depending on crystal packing.

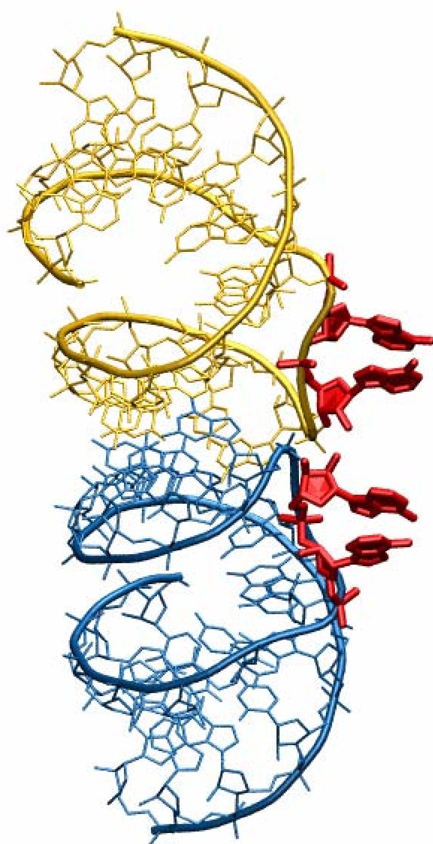

Figure 1: 3D structure of X-ray kissing loop complex consisting of two loops (in orange and in blue) showing bulged-out flanking purine bases (in red).

NMR studies suggest bulged-in positions of bases which, however, are mutually inconsistent. Performed simulations revealed conformational variability of the flanking bases. In particular, the flanking bases, which in the starting X-ray structures are bulged-out and stack in pairs, formed a consecutive stack of four bulged-out adenines at the beginning of several simulations. This conformation is seen in the crystal structure of DIS Subtype F with no interference from crystal packing, and was frequently

reported in our preceding MD studies performed with the AMBER force field. However, as CHARMM simulations progressed, the four continuously stacked adenines showed conformational transitions from the bulged-out into the bulged-in geometries. Although such an arrangement has not been seen in any X-ray structure, it has been suggested by a recent NMR investigation. In CHARMM simulations, in the longer time scale, the flanking bases display the tendency to move to bulged-in conformations. This is in contrast with the AMBER simulations, which indicate a modest prevalence for bulged-out flanking base positions in line with the X-ray data.

Recent views on the origin of the "RNA world" suggest that complexation with borate minerals had an indispensable role at stabilizing the cyclic form of aldopentoses that are the potential building blocks of RNA.

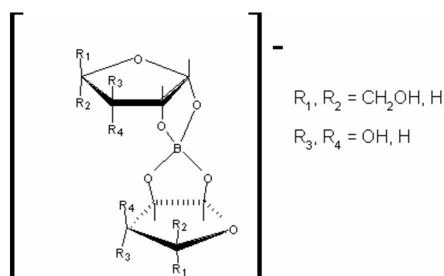

Figure 2: Schematic model of the studied pentose-borate complexes.

Experimental investigations have shown that stability of the 2:1 complexes formed between ribose and borate is superior to those of the analogous compounds of the other three aldopentoses (xylose, arabinose and lyxose). The distinct stability of the ribose-borate 2:1 complexes was thought to be one of the basic reasons why evolution selected ribose (out of the four aldopentoses) to build up RNA molecules. We carried out electronic structure calculations to predict the structure and determine the relative stability of the 2:1 aldopentose-borate complexes of ribose, arabinose, lyxose and xylose. Our results, in agreement with the experiment, clearly show that solution stability of the ribose-borate 2:1 complex is superior to those of other aldopentoses. This is attributable to the favorable configuration of the $–CH_2OH$ group complemented by a H-bonding contact

between the 3-OH and one of the borate oxygens. Thus, we believe that the fortuitous interplay of intra- and intermolecular H-bonding, electrostatic and steric interactions present in the hydrated ribose-borate 2:1 complexes is responsible for the fact that, among the four aldopentoses, ribose had the greatest potential to survive in pre-biotic conditions and be used as a building unit of the first RNA-architectures.

We continued in our studies of the catalytic Hepatitis Delta Virus (HDV) ribozyme. The HDV ribozyme (Fig. 3a,b) is a representative of a group of naturally occurring, small, non-protein coding RNAs that catalyze site-specific self-cleavage of their own backbones. The HDV ribozyme was the first catalytic RNA motif for which structural and biochemical data suggested participation of a specific side chain, cytosine 75 (C75), in reaction chemistry. There are two proposed mechanisms of the self-cleavage reaction of the HDV ribozyme. In the first model, C75 acts as the general base that deprotonates the cleavage site 2’-OH (Fig. 3c). In the second model, C75 acts as the general acid which protonates the 5’-oxygen leaving group (Fig. 3d). In both models, a hydrated Mg^{2+} ion is proposed to provide complementary general acid and base functionality, respectively.

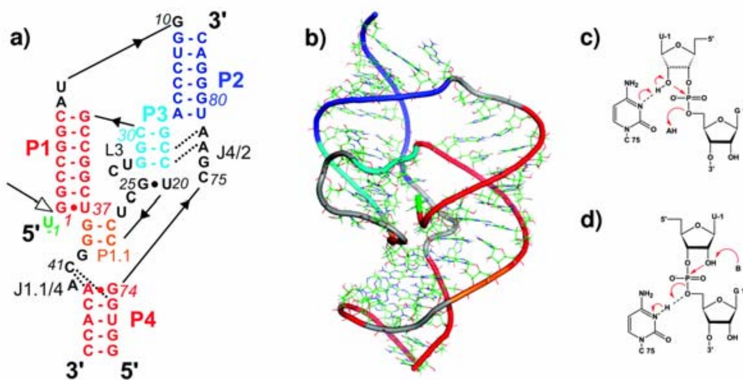

Figure 3: Structure of the precursor form of the HDV ribozyme and two proposed mechanisms of the self-cleavage reaction. a) secondary structure and b) 3D structure of the HDV ribozyme, c) general base mechanism, d) general acid mechanism.

We used combined quantum chemical/molecular mechanical (QM/MM) calculations to study the possibility that C75 may act as the general base in the HDV ribozyme self-cleavage reaction. In this model, C75(N3) accepts

a proton from the U-1(O'2-H'2) nucleophile. The available structure for the wild type HDV genomic ribozyme was used as a starting point. Several starting configurations differing in Mg^{2+} ion placement were considered and both one-dimensional and two-dimensional potential energy surface scans were used to explore plausible reaction pathways. QM/MM calculations show that C75 can act as the general base, in concert with the hydrated Mg^{2+} ion as the general acid. We also identified a most likely position for the Mg^{2+} ion. However, we do not conclude that the general base mechanism is the dominant mode of HDV ribozyme self-cleavage. Results based on the available X-ray structures indicate that this mechanism is readily available, while the general acid mechanism is much less structurally consistent.

Granted projects

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

GA AS CR 1QS500040581, Metallodrugs, design and mechanism of action. Principal investigator: O. Vrána, Principal co-investigator: J. Šponer, 2005 - 2009

GA AS CR IAA400550701, Structure and dynamics in complexes of solvated biomolecules. Principal investigator: J.E. Šponerová, 2007 - 2009

ME CR LC06030, Biomolecular Center. Principal investigator: V. Sklenář, Principal co-investigator: J. Šponer, 2006 - 2010

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CD Spectroscopy of Nucleic Acids

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Guanine quadruplex formation by RNA/DNA hybrid analogs of telomere $G_4T_4G_4$ DNA fragment

Using circular dichroism spectroscopy, gel electrophoresis, and ultraviolet absorption spectroscopy, we have studied quadruplex folding of RNA/DNA analogs of the Oxytricha telomere fragment, $G_4T_4G_4$, which forms a bimolecular, basket-type, antiparallel quadruplex (Fig. 1). We have substituted riboguanosines (rG) for deoxyriboguanines (G) in the positions G1, G4, G9, or G12, i.e. in terminal quadruplex tetrads. We have shown that substitution of a single sugar was able to change quadruplex topology. With the exception of the hybrid with rG substituted for G12, all the RNA/DNA hybrid analogs formed parallel quadruplexes in concentrated (~10 mM nucleoside) solution at low salt (Fig. 1). In dilute (~0.1 mM) solutions, the RNA/DNA hybrids substituted at positions 4 or 12 preferably adopted antiparallel quadruplexes (figure). The hybrids substituted at positions 1 or 9 preferably formed parallel quadruplexes, which were more stable than the unmodified $G_4T_4G_4$ quadruplex in K^+ solutions. The hybrid substituted at both 1 and 9 positions formed parallel quadruplex even in the presence of Na^+ ions (Fig. 1).

We have further studied the influence of DNA for RNA and vice versa loop substitution on the stability and topology of DNA $G_4T_4G_4$ and RNA $r(G_4U_4G_4)$ quadruplexes. We found that the T_4 loop destabilized the tetramolecular parallel quadruplex adopted by RNA tetrads. In the presence of K^+ ions, the DNA loop even permitted RNA strands to fold into an antiparallel quadruplex arrangement. On the other hand, r(U4) loop forced DNA tetrads into the same parallel quadruplex conformation as adopted by $r(G_4U_4G_4)$.

These findings may be useful for engineering the particular quadruplex folding in different quadruplex-forming sequences.

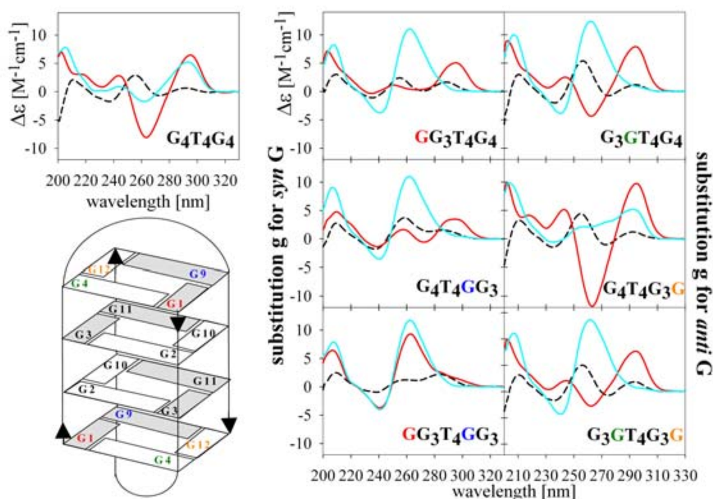

Figure 1: $G_4T_4G_4$ forms an antiparallel quadruplex of the basket type (left bottom, guanosines in syn geometry are shaded). The G's that were replaced by r(G)'s in the RNA/DNA hybrids studied are highlighted in colors in the sketch. Left top: CD spectrum of 0.1 mM $G_4T_4G_4$ in 100mM NaCl (red, CD spectrum with positive band at 290 and negative band at 260 nm is characteristic of antiparallel quadruplex), and in 1mM Na phosphate, pH 7 at 0.1mM (dashed black) and 10mM (cyan) DNA concentration. The CD spectra in the right half of the figure correspond to RNA/DNA hybrids measured under the same conditions as $G_4T_4G_4$. They show that riboguanosine substitution in position 12 stabilizes the antiparallel quadruplex in the presence of NaCl (red). The other substitutions destabilize it, which holds especially for syn G substitutions. The hybrid substituted in positions G1 and G9 even generates a parallel quadruplex (positive CD maximum at 265 nm) under the same conditions. With the exception of the hybrid substituted at the very 3´end (position 12), all the other RNA/DNA hybrids studied form parallel quadruplexes at high DNA concentrations (cyan).

Interruptions of FRAXA $(CGG)_n$ repeats by AGG triplets promote guanine quadruplex formation

The (CGG) repeats associated with X-chromosome fragility are generally believed to form quadruplexes. This notion has persisted although we had shown that only very short $(CGG)_n$ sequences form quadruplexes and that this quadruplex formation occurs in conditions far from physiological. We have now studied quadruplex formation of $(CGG)_n$ (n= 4, 7, 8, or 16) and their analogs interrupted by (AGG) triplets under various solvent conditions. In healthy individuals, (AGG) triplets are interspersed throughout the (CGG) repeat regions and appear to hinder $(CGG)_n$ motif expansion. We have shown that the presence of (AGG) triplets markedly stabilizes quadruplex formation (Fig. 2). Quadruplexes may thus hinder rather than support $(CGG)_n$ motif expansion.

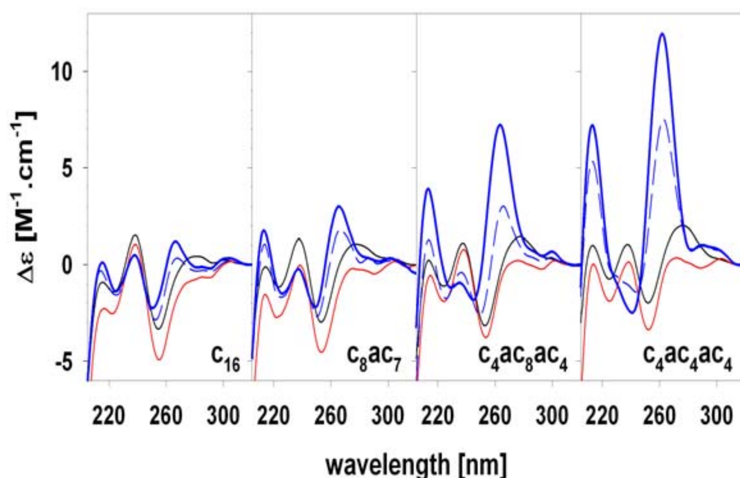

Figure 2: CD spectra of $(CGG)_{16}$ (c stands for CGG in the figure) and of its analogs interrupted by AGG triplets (a stands for AGG) in the presence of ethanol that stabilizes guanine quadruplexes. Ethanol was added to DNA fragments in 1mM phosphate buffer (black) up to 50% (red) and 60% (blue) concentrations. The quadruplex is formed with a slow kinetics. The spectra were measured after one (dashed) and five (full) days. The arising CD spectra (positive maxima at 265 and 210 nm) are characteristic of parallel quadruplexes. The figure demonstrates that the interrupting AGG triplets stabilize quadruplex formation.

Granted projects

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

GA CR 204/07/0057, Tetraplexes in the human genome. Principal investigator: M. Vorlíčková, 2007 - 2009

GA CR 202/07/0094, Biophysics and bioinformatics of human genome regions with an extreme nucleotide distribution. Principal investigator: J. Kypr, 2007 - 2009

IGA MZ CR NR 9147-3/2007, Pathological microsatelite expansion in the human genome. Principal investigator: J. Kypr, 2007 - 2009

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Plant Developmental Genetics

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The lab studies basic questions of developmental and evolutionary genetics on model angiosperm plants. Using numerous molecular techniques we analyze expression patterns of genes controlling sexual development. These pathways are genetically mapped and DNA sequences are compared and analyzed to study evo-devo (evolution and development) relationship.

The human Y—probably because of its nonrecombining nature—has lost 97% of its genes since X and Y chromosomes started to diverge. There are clear signs of degeneration in the *Drosophila miranda* neoY chromosome (an autosome fused to the Y chromosome), with neoY genes showing faster protein evolution, accumulation of unpreferred codons, more insertions of transposable elements, and lower levels of expression than neoX genes. In the many other taxa with sex chromosomes, Y degeneration has hardly been studied. In plants, many genes are expressed in pollen, and strong pollen selection may oppose the degeneration of plant Y chromosomes. *Silene latifolia* is a dioecious plant with young heteromorphic sex chromosomes. Here we test whether the *S. latifolia* Y chromosome is undergoing genetic

degeneration by analyzing seven sex-linked genes. *S. latifolia* Y-linked genes tend to evolve faster at the protein level than their X-linked homologs, and they have lower expression levels. Several Y gene introns have increased in length, with evidence for transposable-element accumulation. We detect signs of degeneration in most of the Y-linked gene sequences analyzed, similar to those of animal Y-linked and neo-Y chromosome genes. This research is realized in a close collaboration with Dr. Gabriel Marais (Lyon University I).

The dioecious plant *Silene latifolia* possesses evolutionarily young sex chromosomes, and so serves as a model system to study the early stages of sex chromosome evolution. Sex chromosomes often differ distinctly from autosomes in both their structure and patterns of evolution. The *Silene latifolia* Y chromosome is particularly unique due to its large size, which contrasts with smaller, degenerate mammalian Y chromosomes. It is thought that the suppression of recombination on the *S. latifolia* Y chromosome could have resulted in the accumulation of repetitive sequences that account for its large size. Here we used fluorescence *in situ* hybridization (FISH) to study the chromosomal distribution of various microsatellites in *S. latifolia* including all possible mono-, di- and tri-nucleotides. Our results demonstrate that a majority of microsatellites are accumulated on the q-arm of the Y chromosome, which stopped recombining relatively recently and has had less time to accumulate repetitive DNA sequences compared to the p-arm (Fig. 1). Based on these facts we can speculate that microsatellites have accumulated in regions that pre-date the genome expansion, supporting the view that the accumulation of repetitive DNA occurred prior to, not because of the degeneration of genes.

We carried out a global survey of all major types of transposable elements in *Silene latifolia*, a model species with sex chromosomes that are in the early stages of their evolution. A shotgun genomic library was screened with genomic DNA to isolate and characterize the most abundant elements. We found that the most common types of elements were the subtelomeric tandem repeat X-43.1 and Gypsy retrotransposons, followed by Copia retrotransposons and LINE non-LTR elements. SINE elements and DNA transposons were less abundant. We also amplified transposable elements with degenerate primers and used them to screen the library. The localization of elements by FISH revealed that most of the Copia elements were accumulated on the Y chromosome. Surprisingly, one type of Gypsy element, which was similar to Ogre elements known from legumes, was

almost absent on the Y chromosome but otherwise uniformly distributed on all chromosomes. Other types of elements were ubiquitous on all chromosomes. Moreover, we isolated and characterized two new tandem repeats. One of them, STAR-C, was localized at the centromeres of all chromosomes except the Y chromosome, where it was present on the p-arm. Its variant, STAR-Y, carrying a small deletion, was specifically localized on the q-arm of the Y chromosome. The second tandem repeat, TR1, co-localized with the 45S rDNA cluster in the subtelomeres of five pairs of autosomes. FISH analysis of other Silene species revealed that some elements (e.g., Ogre-like elements) are confined to the section Elisanthe while others (e.g. Copia or Athila-like elements) are present also in more distant species. Similarly, the centromeric satellite STAR-C was conserved in the genus *Silene* whereas the subtelomeric satellite X-43.1 was specific for Elisanthe section. Altogether, our data provide an overview of the repetitive sequences in *Silene latifolia* and revealed that genomic distribution and evolutionary dynamics differ among various repetitive elements.

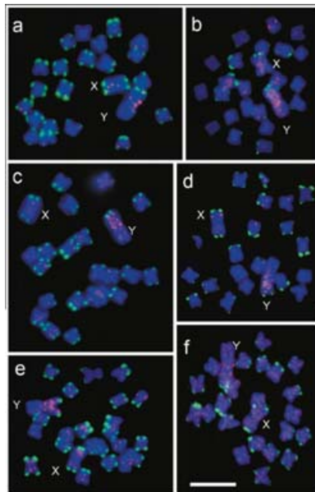

Figure 1: Mitotic metaphase chromosomes of male *S. latifolia* were hybridized with various labeled microsatellite-containing oligonucleotides: (a) - d$(A)_{30}$, (b) - $(C)_{30}$, (c) - $(CA)_{20}$, (d) - $(GA)_{20}$, (e) - $(GC)_{20}$, (f) - $(TA)_{20}$. Chromosomes were counterstained with DAPI (blue); microsatellite probes were directly labeled with Cy3 during synthesis (red signals). The subtelomeric repeat X43.1, which marks the q-arm of the Y chromosome was labeled with SpectrumGreen-conjugated nucleotides (green signals). The X and Y chromosomes are indicated, bar = 10µm.

The unique pattern of repeat distribution is found on the Y chromosome, where some elements are accumulated while other elements are conspicuously absent, which probably reflects different forces shaping the Y chromosome. This research is realized in a close collaboration with Prof. Alex Widmer (ETH Zurich).

In addition to *S. latifolia, S. diclinis*, and *S. dioica*, there is also another group of dioecious *Silene* species consisting of *S. otites* and its closely related species. So far, the studied species of this group are characterized by the absence of heteromorphic sex chromosomes. This fact, together with the supposed nondioecious origin of the genus *Silene*, suggests that the sex chromosomes in *S. otites* and its closely related species are evolutionarily younger than those in *S. latifolia*. It was also suggested (on the basis of the sequencing of rDNA internal transcribed spacer loci) that sex determination evolved independently in the group of species around *S. latifolia* and in the group of species around *S. otites*. Even though the bootstraps supporting this hypothesis are rather low, the hypothesis of the recent and independent origin of sex determination in the species related to *S. otites* certainly deserves great attention. If it is true, these two groups of species could be suitable and complementary models for the study of early sex chromosome evolution. This type of system could also address important questions relating to mechanisms of sex chromosome evolution such as the suppression of recombination and evolutionary changes of sex-linked genes. Our efforts were concentrated on three basic questions: (1) Is the origin of sex determination in *S. colpophylla* independent from that of *S. latifolia*?, (2) Have the sex chromosomes in *S. colpophylla* evolved from the same pair of autosomes as the sex chromosomes in *S. latifolia*?, and (3) What is the type of sex determination in *S. colpophylla*? Our results show either that there is a homomorphic sex chromosome pair in this species or that at least the difference between heterochromosomes is much less prominent than in S. latifolia. Because of the supposed homomorphic character of the sex chromosomes in S. colpophylla, we decided to use molecular markers to further investigate the sex-determining system. To obtain a sufficient number of markers, we used the AFLP technique. To determine the heterogametic sex, we used an approach that was based on the study of two subsequent generations—the parental and F1 generation. This approach would enable us to identify the heterogametic sex even if none of the markers were completely sex linked. We found 45 segregating AFLP markers, but only 3 showed sex linkage. Two markers (B4, C11) from the pollen donor cosegregated predominantly with the female sex in the

progeny. This pattern of inheritance suggests that these markers are linked to the X chromosome. One marker (B5) showed cosegregation mostly with the male sex, suggesting that the B5 marker is linked to the Y chromosome. In total, these data reveal that males in S. colpophylla, rather than females, are the heterogametic sex. Synthesis of the data obtained by genetic mapping of the three chosen genes and the AFLP markers enabled us to define two separate linkage groups for S. colpophylla (Fig. 2). Our results further support the idea that the sex chromosomes of S. colpophylla and S. latifolia have evolved from different pairs of autosomes. In conclusion, the finding that sex chromosomes in S. colpophylla have evolved from a different pair of autosomes further supports the view that the genus Silene is an excellent model for studying the evolution of sex chromosomes and sex determination because of the diversity of evolutionary patterns represented within it. This research was done in collaboration with Prof. Ioan Negrutiu (ENS de Lyon).

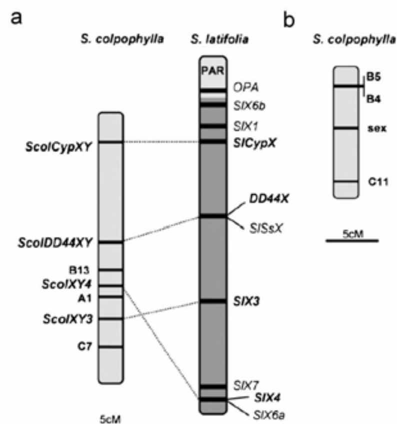

Figure 2: Results of the genetic mapping in *S. colpophylla*. (a) Linkage group containing three AFLP loci and homologs of the genes that are sex linked in *S. latifolia* compared with the *S. latifolia* X chromosome map based on the data of D. Bergero (pseudoautosomal region is marked as PAR; homologs are connected by dotted lines). (b) Linkage group containing the sex-determining region (sex) and three AFLP loci.

The key mechanisms controlling mitotic progression are also effective in meiosis, although extensive modifications have evolved, particularly during the first reductional division, to allow the formation of four haploid nuclei after two successive rounds of chromosome separation. Whereas meiosis I

is characterized by several unique processes, such as chiasmata formation, monoorientation of sister kinetochores and protection of centromeric cohesion, the second meiotic division resembles mitosis. Furthermore, whereas CDK activity is only partially destroyed at the end of meiosis I, preventing entry into S phase and chromosome reduplication, complete CDK inactivation must occur at the end of the second meiotic division to allow the transition to a subsequent G1 phase. Studies in frogs and fission yeast indicate that a high level of CDK activity is retained in meiotic interkinesis by reduced proteolysis as well as by increased synthesis of meiotic cyclins. However, mechanisms leading to a differential regulation of CDK activity after the first and second meiotic divisions are still only poorly understood. In our study, we characterized a novel *Arabidopsis thaliana* gene (*SMG7*) that is crucial for completion of the meiotic cell cycle. The gene encodes a protein that possesses an evolutionarily conserved EST1 domain and exhibits strong homology to human SMG6 (EST1A) and SMG7 (EST1C) proteins, which are implicated in meiosis consists of two nuclear divisions that are separated by a short interkinesis. Here we show that the SMG7 protein, which plays an evolutionarily conserved role in nonsense-mediated RNA decay in animals and yeast, is essential for the progression from anaphase to telophase in the second meiotic division in *Arabidopsis*. *Arabidopsis SMG7* is an essential gene, the disruption of which causes embryonic lethality. Plants carrying a hypomorphic *smg7* mutation exhibit an elevated level of transcripts containing premature stop codons. This suggests that the role of SMG7 in NMD is conserved in plants. Furthermore, hypomorphic *smg7* alleles render mutant plants sterile by causing an unusual cell-cycle arrest in anaphase II that is characterized by delayed chromosome decondensation and aberrant rearrangement of the meiotic spindle. The *smg7* phenotype was mimicked by exposing meiocytes to the proteasome inhibitor MG115. Together, these data indicate that SMG7 counteracts cyclin-dependent kinase (CDK) activity at the end of meiosis, and reveal a novel link between SMG7 and regulation of the meiotic cell cycle. This work was realized in collaboration with Dr. Karel Riha (Gregor Mendel Institute, Vienna).

Granted projects

GA CR 204/05/H505, Plant developmental genetics. Principal investigator: B. Vyskot, 2005 - 2008

GA CR 521/06/0056, Cytogenetic mapping of plant sex chromosomes. Principal investigator: B. Vyskot, 2006 - 2008

MSMT LC06004, Dynamics of plant genome. Principal investigator: B. Vyskot, 2006 - 2010

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 CR 522/06/0380, The study of genome instabilities in Arabidopsis mutants deficient for DNA repair and checkpoint proteins. Principal investigator: J. Široký, 2006 - 2008

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

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Siroky, J.: *Cytogenetics for the study of telomere function in plants* – Cytogenetic and Genome Research, 122, 2008, 374-379.

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Cermak, T., Kubat Z., Hobza R., Koblizkova A., Widmer A., Macas J., Vyskot B., Kejnovsky, E. : Catalogue of repetitive sequences in *Silene latifolia* and their distribution in connection with sex chromosomes evolution. - Chromosome Research, 16, 2008, 961-976.

Hobza, R., Widmer, A.: Efficient molecular sexing in dioecious *Silene latifolia* and
S. dioica and paternity analysis in F1 hybrids. - Molecular Ecology Resources, 8, 2008, 1274-1276.

Prestigious International Projects

6. FP EU, LSHG-CT-2003-502983, Mutant p53 as a target for improved cancer treatment. Co-principal investigator: E. Paleček, 2004 - 2008

HHMI, INTNL 55005613, Platinum and ruthenium compounds. From DNA damage to cancer chemotherapy. Principal investigator: J. Kašpárková, 2005 - 2010

Prestigious National Projects

ME, LC535, Center of Basic Research, Dynamics and organization of chromosomes dureing the cell cycle. Principal investigator: I. Raška, Principal co- investigator: S. Kozubek, 2005 - 2009

ME, 1M0021622409, Center of Applied Research, Stomatological research center. Principal investigator: J. Vaněk, Principal co-investigator: V. Vetterl, 2005 - 2009

ME, LC06035, Center of Basic Research, Center of biophysical chemistry, bioelectrochemistry and bioanalysis. New instruments for genomics, proteomics and biomedicine. Principal investigator: M. Fojta, 2006 - 2010

ME, LC06004, Center of Basic Research, Integration of research activities to study the plant genome. Principal investigator: B. Vyskot, 2006 - 2010

ME, LC06030, Center of Basic Research, Biomolecular Center. Principal co-investigators: V. Brabec, J. Šponer, 2006 - 2010

ME, LC06027, Center of Basic Research for Monoclonac Gamapathy and Multiple Myeloma. Principal co-investigator: E. Bártová, 2006 - 2010

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

Masaryk University, Brno

Viktor Brabec: Seminar of the Department of molecular biophysics and pharmacology

Břetislav Brzobohatý: Structure and function of proteins

Milan Číž: Free radicals in animal physiology

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

Jiří Fajkus: Seminar of the Department functional genomics and proteomics

Jiří Fajkus, Eva Sýkorová: Analysis of chromatin structure (practical training)

Jiří Fajkus: Applied genomics and proteomics

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

Jiřina Hofmanová, Alois Kozubík: Health risks

Jiřina Hofmanová, Alois Kozubík: Genotoxicity and carcinogenesis

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

Jiřina Hofmanová, Alois Kozubík, Jiřina Procházková: Special methods of animal physiology

Eduard Kejnovský, Roman Hobza: Evolutionary genomics

Aleš Kovařík: Special methods of microorganism analysis

Stanislav Kozubek, Eva Bártová: Molecular physiology of the genome

Stanislav Kozubek, Martin Falk: Radiation biophysics

Alois Kozubík, Jiřina Hofmanová: Physiology of cell systems

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

Lukáš Kubala: Special physiology of blood

Antonín Lojek: Immunology

Olga Nováková: Biophysics - seminar

Jiřina Procházková: Scientific work methodology

Karel Souček, Eva Lincová: Analytical cytometry

Karel Souček: Journal club - cancer biology

Naďa Špačková: Structure and dynamics of nucleic acids

Jiří Šponer: Introduction into molecular biophysics

Jiří Šponer: Molecular interactions and their role in biology and chemistry

Vladimír Vetterl: Introduction to molecular biophysics

Oldřich Vrána: Experimental methods of biophysics

Vladimír Vetterl, František Jelen: Bioelectrochemistry 1

Vladimír Vetterl, František Jelen: Bioelectrochemistry 2

Vladimír Vetterl: Physical properties of biopolymers

Jan Vondráček: Applied chemistry and biochemistry

Jan Vondráček: Physiology of pharmaceuticals and toxic compounds

Boris Vyskot: Developmental genetics

Boris Vyskot: Special seminar in English

Jitka Žlůvová: Evolutionary developmental genetics of plants

PalackýUniversity Olomouc

Viktor Brabec: Biophysical seminar

Jana Kašpárková, Viktor Brabec: Molecular biophysics of mutagens, cancerogens and cytostatics

Jana Kašpárková: Molecular biophysics

Jiří Šponer: Structure and dynamics of nucleic acids

Boris Vyskot: Developmental biology and genetics

Boris Vyskot: Epigenetics

Mendel University of Agriculture and Forestry in Brno

Břetislav Brzobohatý: Molecular plant physiology

Roman Hobza: Gene engineering I

Boris Vyskot: Gene engineering II

University of Veterinary and Pharmaceutical Sciences Brno

Eduard Kejnovský, Roman Hobza: Structure and evolution of genomes

Comenius University in Bratislava

Veronika Ostatná: Medicinal physics 1

Veronika Ostatná: Medicinal physics 2

Charles University in Prague

Jiří Šponer: Structure and dynamic of nucleic acids

University of South Bohemia in České Budějovice

Eduard Kejnovský, Roman Hobza: Evolutionary genomics

University of Ostrava

Boris Vyskot: Developmental biology

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CENTER OF INFORMATION TECHNOLOGIES (CIT)

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Standard services of the Center of Information Technologies (CIT) include maintenance of the local area network (LAN), the connection of the IBP LAN to Brno Academic Computer Network and to the Internet, maintenance of exchange and IP telephony, maintenance of the IBP e-mail server, including antivirus and antispam systems, maintenance of the IBP web server including design and data update, development and maintenance of computer hardware and software jointly used by all laboratories (servers, graphic workstations, PCs with Internet access) running under UNIX, MS Windows 2000/XP/Vista. CIT also provides consulting services for individual scientists.

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 two independent virus scanners 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 2008 the effort was focused to virtualization. There were installed two VMWare ESX 3.5 servers. After extensive testing the ESX servers were put into production environment. Several specialized servers (FreeBSD, Linux) were converted to virtual machines running above VMWare ESX server.

Redundant optical connection of the IBP computer network to the Brno metropolitan network in an independent path was build. It enables to maintain IBP network connectivity even in the case of a single break of the optical connection.

Electrochemistry of Nucleic Acids and Proteins. New Tools for Biotechnologies

This event was organized by Department of Biophysical Chemistry and Molecular Oncology and by Centre of Biophysical Chemistry, Bioelectrochemistry at Bioanalysis as a satellite symposium to the ESEAC 2008 meeting (held in Prague on June 16-19). The symposium took place on June 19-22 at the Orea Santon Hotel near the Brno Dam and brought together 70 participants from Belgium, UK, Germany, Sweden, Spain, Ireland, Slovakia, USA, Poland, Italy, South Africa, Japan, Russia, The Netherlands, Turkey and Czech involved in nucleic acids and protein electrochemistry, bioelectrochemistry and biosensor research and development. The organizers had the pleasure to welcome to the symposium world-recognized electrochemists, including Prof. Joseph Wang, Prof. Claudine Buess-Herman, Prof. Frieder Scheller, Prof. José Pingarron, Prof. Petr Zuman and others.

3rd European Workshop on the Analysis of Phagocyte Functions

The workshop was organized by the Department of Free Radical Pathophysiology at the Hotel Continental from 22nd to 24th June 2008. 43 scientists came from 15 countries including Croatia, France, Bulgaria, Germany, Finland, Denmark, Poland, Hungary, Slovakia, Turkey, Serbia, Ukraine, Cyprus, Canada and Czech Republic. Meeting topics included biology and physiology of phagocytes, methodology for evaluation of phagocyte function, production of phagocyte–derived reactive oxygen and nitrogen species, damage of biologically important molecules by these reactive species, lipid peroxidation as a consequence of functional changes of phagocytes, pathophysiology of phagocytes and pharmacological modulation of phagocyte functions.

The contributions of 13 young researchers aged below 35 years were judged by the committee of 4 members and the 3 best contributions were awarded: P. Zelnickova (Veterinary Research Institute, Brno), P. Hyrsl (Masaryk University, Brno), and M. Pekarova (Institute of Biophysics AS CR, Brno).

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