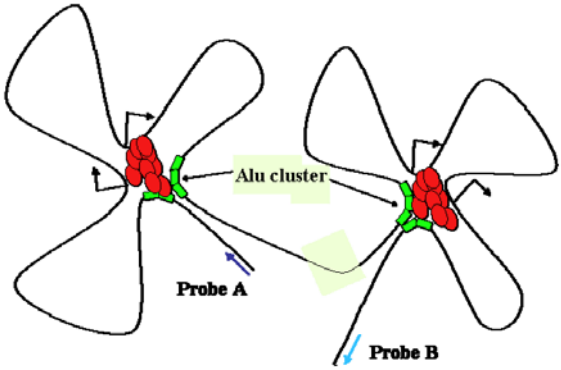
INSTITUTE OF BIOPHYSICS

ACADEMY OF SCIENCES OF THE CZECH REPUBLIC



RESEARCH REPORT 2006

IBP AS CR, BRNO 2006

CONTENTS

INTRODUCTION

In 2006, substantial changes have been made in the Institute of Biophysics, AS CR (IBP). The year 2006 was the first year, in which the internal evaluation of the scientific performance (introduced in 2005) was carried out and applied. The budgets of individual laboratories for 2007 were increased or decreased according to their results with maximum changes reaching 15% of the average group support. In addition, one laboratory was reduced to 50% owing to repeatedly low performance. The overall productivity of the Institute increased as compared to the previous year (both the total number of publications and the cumulative impact factor).

The organizational structure of the Institute underwent changes of larger and more productive laboratories to departments and smaller laboratories to independent groups. In departments, groups leaded by young, promising scientists have been established. This arrangement should give us more flexibility and enable easier and more effective application of the results of the internal evaluation.

Since 2007, the Institute changed its legal statute to public research organization. As a consequence, the Council of the Institute has been newly elected and the Director of the Institute has been newly selected and appointed by the President of the AS CR. The Rules of Organization, the Salary Rules, election and other regulations have been prepared by the management of the Institute and approved by the Council. In agreement with the recommendation of the Academy Council of the AS CR, we have introduced new rules for the determination of salaries that are now independent of age but depend on scientific performance. In addition, new scientific career structure has been adopted containing well defined rules for the achievement of higher positions.

We participated in the negotiations on the building of the Central European Institute of Technology (CEITEC), in which Mendel Research Center (MRC) represents an important component. CEITEC integrates both life sciences and material sciences together, including computing and information technologies. The application will be sent as a “Major Project” to the Operational Programme “Research and Development for Innovations”, part 1.2 “Centers of Ecellence”. The construction of the 3 GeV synchrotron in Brno represents another project that is under consideration.

Stanislav Kozubek

MOLECULAR BIOPHYSICS AND PHARMACOLOGY

HEAD

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Thermodynamic properties of damaged DNA and its recognition by xeroderma pigmentosum group A protein and replication protein A

The effects of the lesions induced by single, site-specific 1,2-GG or 1,3-GTG intrastrand adducts of *cis*-diamminedichloroplatinum(II) (cisplatin) formed in oligodeoxyribonucleotide duplexes on energetics of DNA were examined by means of differential scanning calorimetry. These effects were correlated with affinity of these duplexes for damaged-DNA binding-proteins XPA and RPA; this affinity was examined by gel electrophoresis. The results confirm that rigid DNA bending is the specific determinant responsible for high-affinity interactions of XPA with damaged DNA, but that an additional important factor, which affects affinity of XPA to damaged DNA, is a change of thermodynamic stability of DNA induced by the damage. In addition, the results also confirm that RPA preferentially

binds to DNA distorted so that hydrogen bonds between complementary bases are interrupted. RPA also binds to nondenaturational distortions in double-helical DNA, but affinity of RPA to these distortions is insensitive to alterations of thermodynamic stability of damaged DNA.

Deoxyribonuclease I footprinting reveals different DNA binding modes of bifunctional platinum complexes

Deoxyribonuclease I (DNase I) footprinting methodology was used to analyze oligodeoxyribonucleotide duplexes containing unique and single, site-specific adducts of trinuclear bifunctional platinum compound, [{*trans*-$PtCl(NH_3)_2$} μ-*trans*-$Pt(NH_3)_2\{H_2N(CH_2)_6NH_2\}_2]^{4+}$ (BBR3464) and the results were compared with DNase I footprints of some adducts of conventional mononuclear cisplatin. These examinations took into account the fact that the local conformation of the DNA at the sites of the contacts of DNase I with DNA phosphates, such as the minor groove width and depth, sequence-dependent flexibility and bendability of the double helix, are important determinants of sequence dependent binding to and cutting of DNA by DNase I. It was shown that various conformational perturbations induced by platinum binding in the major groove translated into the minor groove, allowing their detection by DNase I probing. The results also demonstrate the very high sensitivity of DNase I to DNA conformational alterations induced by platinum complexes so that the platinum adducts which induce specific local conformational alterations in DNA are differently recognized by DNase I.

Transplatin is cytotoxic when photoactivated: Enhanced formation of DNA cross-links

It is well-known that although cisplatin is an anticancer drug, its isomer transplatin [*trans*-diamminedichloroplatinum(II)] is not cytotoxic. We have shown that transplatin is almost as cytotoxic as cisplatin when treated cells (human keratinocytes HaCaT and ovarian cancer A2780 cells) are irradiated with UVA light (50 min, 1.77 mW cm^{-2}). Chemical studies show that light activates both chloride ligands of transplatin, and experiments on pSP73 plasmid DNA and a 23 base-pair DNA duplex show that irradiation can greatly enhance formation of interstrand cross-links and of DNA-protein cross-links (which are not formed in the dark). Comet assays showed that

UVA irradiation of transplatin-treated cells resulted in an increased inhibition of H_2O_2-induced DNA migration, supporting the conclusion that the cytotoxicity of photoactivated transplatin is mainly due to formation of DNA interstrand and DNA-protein cross-links.

Molecular aspects of antitumor effects of a new platinum(IV) drug

The new platinum(IV) complex *cis*,*trans*,*cis*-[$PtCl_2(CH_3COO)_2(NH_3)$(1-adamantylamine)] [adamplatin(IV)] seems promising for the perspective application in therapy of corresponding tumors. It is therefore of great interest to understand details of mechanisms underlying its biological efficacy. Cellular uptake of the drug, alterations in the target DNA induced by platinum drugs along with processing of platinum-induced damage to DNA and drug inactivation by sulfur-containing compounds belong to major pharmacological factors affecting antitumor effects of platinum compounds. The significance of these factors in the mechanism of antitumor effects of adamplatin(IV) was examined and the results were compared with those of the parallel studies performed with "conventional" cisplatin. The results show that deactivation of adamplatin(IV) by sulfur containing compounds (such as glutathione or metallothioneins) is likely to play a less significant role in the mechanism of resistance of tumor cells to adamplatin(IV) in contrast to the role of these reactions in the effects of cisplatin. Moreover, the treatment of tumor cells with adamplatin(IV) does not result in DNA modifications that would be markedly different from those produced by cisplatin. In contrast, the effects of other factors, such as enhanced accumulation of the drug in cells, strong inhibition of DNA polymerization by these adducts, lowered DNA repair, and DNA-protein cross-linking are different from the effects of these factors in the mechanism underlying activity of cisplatin. Hence, the differences between effects of adamplatin(IV) and cisplatin observed in the present work on molecular level may help understand the unique activity of adamplatin(IV).

Interactions of platinum complexes containing cationic, bicyclic, nonplanar piperidinopiperidine ligands with biological nucleophiles

The determination of the structures and DNA interactions and the reactions with glutathione and ubiquitin of complexes of the general formula *trans*-[$PtCl_2$(Am)(pip-pip)].HCl, where pip-pip is 4-piperidinopiperidine and Am

is NH_3, methylamine (MA), dimethylamine (DMA), *n*-propylamine (NPA), isopropylamine (IPA), *n*-butylamine (NBA), or cyclohexylamine (CHA), were performed. X-ray structures and NMR studies of the NH_3 and MA complexes showed that both pip rings were in the chair conformation and that the second pip ring is fluxional. The DNA binding studies showed that these complexes bind to calf thymus DNA nearly an order of magnitutde more quickly than cisplatin and form covalent adducts that stabilize the double helix. The binding of the pip-pip complexes to DNA results in high unwinding angles (~30°) and in the formation of ~25% interstrand cross-links. The pip-pip complexes reacted with glutathione more quickly than cisplatin and transplatin, and the rate of reaction decreased with increasing steric bulk of the ligand trans to the pip-pip. The reactions with ubiquitin resulted in monofunctional binding to Met1. Only the NH_3, MA, and DMA complexes reacted with ubiquitin in a slower and less efficient fashion than cisplatin.

Structural characterization, DNA interactions, and cytotoxicity of new transplatin analogues containing one aliphatic and one planar heterocyclic amine ligand

New analogues of clinically ineffective transplatin in which one ammine group was replaced by aliphatic and the other by a planar heterocyclic ligand, namely *trans*-[$PtCl_2$(isopropylamine)(3-(hydroxymethyl)-pyridine)], **1**, and *trans*-[$PtCl_2$(isopropylamine)(4-(hydroxymethyl)pyridine)], **2** have been described. The new compounds, in comparison with parent transplatin, exhibit radically enhanced activity in tumor cell lines both sensitive and in particular resistant to cisplatin. Concomitantly, the DNA binding mode of **1** and **2** compared to parent transplatin and other antitumor analogues of transplatin in which only one ammine group was replaced is also different. The results also suggest that the reactions of glutathione and metallothionein-2 with compounds **1** and **2** do not play a crucial role in their overall biological effects. In addition, the monofunctional adducts of **1** and **2** are quenched by glutathione considerably less than the adducts of transplatin, which may potentiate cytotoxic effects of these new platinum complexes.

Recognition of DNA modified by *trans*-[$PtCl_2NH_3$(4-hydroxymethylpyridine)] by tumor suppressor protein p53 and character of DNA adducts of this cytotoxic complex

trans-[$PtCl_2NH_3$(4-hydroxymethylpyridine)] (trans-PtHMP) is an analogue of clinically ineffective transplatin, which is cytotoxic in the human leukemia cancer cell line. As DNA is a major pharmacological target of antitumor platinum compounds, modifications of DNA by trans-PtHMP and recognition of these modifications by active tumor suppressor protein p53 were studied in cell-free media using the methods of molecular biology and biophysics. Our results demonstrate that the replacement of the NH_3 group in transplatin by the 4-hydroxymethylpyridine ligand affects the character of DNA adducts of parent transplatin. The binding of trans-PtHMP is slower, although equally sequence-specific. This platinum complex also forms on double-stranded DNA stable intrastrand and interstrand crosslinks, which distort DNA conformation in a unique way. The most pronounced conformational alterations are associated with a local DNA unwinding, which was considerably higher than that produced by other bifunctional platinum compounds. DNA adducts of trans-PtHMP also reduce the affinity of the p53 protein to its consensus DNA sequence. Thus, downstream effects modulated by recognition and binding of p53 protein to DNA distorted by trans-PtHMP and transplatin are not likely to be the same. It has been suggested that these different effects may contribute to different antitumor effects of these two transplatinum compounds.

DNA binding mode of ruthenium complexes and relationship to tumor cell toxicity

The review has been published in the journal Drug Resistance Updates, which summarizes results demonstrating that several ruthenium compounds exhibiting antitumor effects different from cisplatin or its analogues bind DNA and modify it differently than cisplatin or its analogues. Transition-metal-based compounds constitute a discrete class of chemotherapeutics, widely used in the clinic as antitumor and antiviral agents. Examples of established antitumor metallodrugs, routinely used in the clinic, are cisplatin and its analogues carboplatin and oxaliplatin. However, drug resistance and side effects have limited their clinical utility. These limitations have prompted a search for more effective and less toxic metal-based antitumor

agents. Some of the efforts have been directed in the design of non-platinum, transition-metal-based antitumor agents and ruthenium complexes have attracted much interest as alternative drugs to cisplatin in cancer chemotherapy. Ruthenium complexes demonstrate similar ligand exchange kinetics to those of platinum(II) antitumor drugs already used in the clinic while displaying only low toxicity. This is in part due to the ability of ruthenium complexes to mimic the binding of iron to molecules of biological significance, exploiting the mechanisms that the body has evolved for transport of iron. In addition, the redox potential between the different accessible oxidation states occupied by ruthenium complexes enables the body to catalyze oxidation and reduction reactions, depending on physiological environment. The biochemical changes that accompany cancer alter physiological environment, enabling ruthenium complexes to be selectively activated in cancer tissues. Due to different ligand geometry among their complexes, ruthenium compounds bind to DNA affecting its conformation differently than cisplatin and its analogues. In addition, nonnuclear targets, such as the mitochondrion and the cell surface, have also been implicated in the antineoplastic activity of some ruthenium complexes. Thus, ruthenium compounds offer the potential over antitumor platinum(II) complexes currently used in the clinic of reduced toxicity, a novel mechanism of action, the prospect of non-cross-resistance and a different spectrum of activity. In other words, some chemical properties make ruthenium compounds well suited for medicinal applications and as an alternative to platinum antitumor drugs in the treatment of cancer cells resistant to cisplatin. Although the pharmacological target for antitumor ruthenium compounds has not been unequivocally identified, there is a large body of evidence indicating that the cytotoxicity of many ruthenium complexes correlates with their ability to bind DNA although few exceptions have been reported.

Tröger's base scaffold in racemic and chiral fashion as a spacer for bisdistamycin formation. Synthesis and DNA binding study

'Head-to-head' oligo-N-methylpyrrole peptide dimers linked by a methano[1,5]diazocin scaffold are presented in racemic as well as chiral fashion. Their DNA binding activities were assayed on calf thymus DNA, poly(dA-dT)$_2$, and poly(dC-dG)$_2$ by NMR and ECD spectroscopies, and fluorescence probe displacement assay. The presented dimers prefer AT sequences, but show higher affinity to poly(dC-dG)$_2$ than distamycin A. The

(4R,9R) configuration of methanodiazocin bridge was found to be better suited for interaction with calf thymus DNA and poly(dA-dT)$_2$ than (4S,9S) configuration.

Sensitisation for cisplatin-induced apoptosis by isothiocyanate E-4IB leads to signalling pathways alterations

A new synthetic isothiocyanate (ITC) derivative, ethyl 4-isothiocyanatobutanoate (E-4IB), appeared to be an effective modulator of cellular proliferation and potent inducer of apoptosis. In cooperation with cisplatin, this compound exerted synergistic effects in human ovarian carcinoma A2780 cells. E4IB-sensitisation for cisplatin-induced apoptosis was investigated in more details. Sequential administration of both cytostatic agents led to increased intracellular platinum accumulation, glutathione level depletion and mitochondrial membrane potential dissipation. These events were accompanied with poly (ADP-ribosyl) polymerase cleavage, stimulation of caspase-3 activity, upregulation of p53, FasL and Gadd45a, cyclin B1 downregulation and an increase in mitogen-activated protein kinases JNK, ERK and p38 phosphorylation as well as PI3K level alterations. These results might have implications for developing new strategies aimed at therapeutic benefit of natural or synthetic ITCs in cooperation with various anticancer drugs.

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

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

GA CR 204/03/H016, Structural biophysics of macromolecules. Principal investigator: V. Brabec, 2003 - 2007

GA CR 203/05/2032, Raman spectroscopy of DNA modifited by antitumor metal-based compounds. Principal investigator: O. Vrána, 2005 -2007

GA CR 305/05/2030, New approaches to cancer chemotherapy by metal-based drugs. Principal investigator: V. Brabec, 2005 - 2007

IGA MH 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

NIH (USA), 1R01CA78754 Mechanistic studies on new platinum clinical agens. Principal investigator: V. Brabec, 2005 - 2007

The Wellcome Trust (UK), 073646/Z/03/Z, Platinum and ruthenium complexes. From DNA damage to cancer chemotherapy. Principal investigator: V. Brabec, 2004 – 2007

Kontakt, AIP, Czech-Greek project within Czech-Greek intergovernmental scientific and technical cooperation in 2006-2007. Synthesis, characterization and study of the nucleolytic activity of novel selective chemical nucleases, based on heteronuclear metallopeptides Ru(II)-Ni(II). Principal investigator: J. Malina, 2006 - 2007

5. FP EU, HPRN-CT-2002-00175, Structural effects arising from major groove DNA recognition by metallo-supramolecular cylinders. Principal investigator: V. Brabec, 2002 – 2007

ME, LC06030, Center of Basic Research, Biomolecular Center, Co-principal investigator: V. Brabec, 2006 - 2010

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

ME, 1P05OC070, Intracellular and extracellular targets of antitumor activity and toxicity of ruthenium complexes. Principal investigator: V. Brabec, 2006

ME, 1P05OC071, Characterization of metalloproteins, key molecules for biological processes. Principal investigator: V. Brabec, 2006

ME, 1P05OC072, Non-covalent DNA recognition strategies for design and synthesis of new metallo-drugs. Principal investigator: O. Nováková, 2006

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Valik, M., Malina, J., Palivec, L., Foltýnová, J., Tkadlecová, M., Urbanová, M., Brabec, V., Král, V.: *Tröger's base scaffold in racemic and chiral fashion as a spacer for bisdistamycin formation. Synthesis and DNA binding study*. Tetrahedron, 62, 2006, 8591–8600.

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Childs, L.J., Malina, J., Rolfsnes, B.E., Pascu, M., Prieto, M.L., Broome, M.L., Rodger, P.M., Sletten, E., Moreno, V., Rodger, A., Hannon, M.J.: *A DNA-binding copper(I) metallosupramolecular cylinder that acts as an artificial nuclease*. *Chem. Eur. J.* 12, 2006, 4919-4927.

Zhi, Z.-l., Dražan, V., Wolfbeis, O.S., Mirsky, V.M.: *Electrocatalytic activity of DNA on electrodes as an indication of hybridisation*. Bioelectrochem., 68, 2006, 1-6.

PhD thesis defended in 2006

Mgr. Kateřina Chválová, Protein recognition of DNA modified by platinum antitumor complexes

Mgr. Kristýna Stehlíková, Biophysical analysis of the mechanism of the antitumor action of new anticancer drugs

Mgr. Jana Zehnulová, Interactions of DNA with antitumor polynuclear platinum complexes

MOLECULAR ANALYSIS OF PLANT DEVELOPMENT

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Ectopic over-expression of the maize ß-glucosidase Zm-p60.1 perturbs cytokinin homeostasis in transgenic tobacco

The activity of the phytohormone cytokinin depends on a complex interplay of factors such as its metabolism, transport, stability and cellular/tissue localization. O-glucosides of zeatin-type cytokinins are postulated to be storage and/or transport forms, and are readily deglucosylated. Transgenic tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) plants were constructed over-expressing *Zm-p60.1*, a maize β-glucosidase capable of releasing active cytokinins from O- and N3-glucosides, to analyze its potential to perturb zeatin metabolism *in planta*. Zm-p60.1 in chloroplasts isolated from transgenic leaves has an apparent K_m more than ten-fold lower than purified enzyme *in vitro*. Adult transgenic plants grown in the absence of exogenous zeatin were morphologically indistinguishable from wild-type although differences in phytohormone levels were observed. When grown on medium containing zeatin, inhibition of root elongation was apparent in all seedlings 14 days after sowing (DAS). Between 14 and 21 DAS, the transgenic seedlings accumulated fresh weight leading later (28 to 32 DAS) to ectopic growths at the base of the hypocotyl. The development of ectopic structures correlated with the presence of the enzyme as demonstrated by histochemical staining.

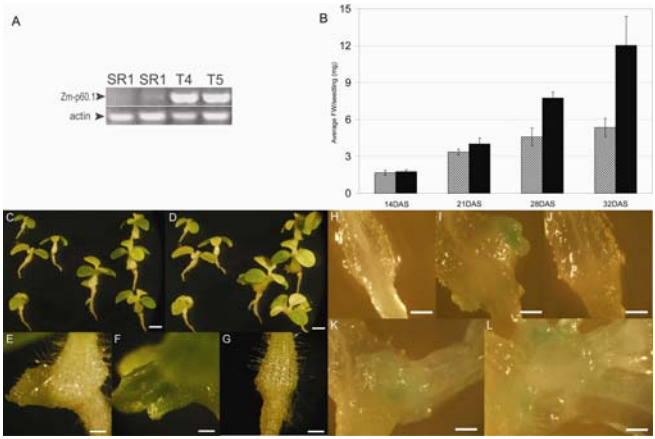

Fig. 1. RT-PCR, FW comparison, morphology and histochemical staining. (A) RT-PCR of transformed tobacco seedlings. Bands corresponding to the *Zm-p60.1* cDNA were detectable only in transformants (T4, T5), but were absent from the wild type (SR1). (B) Fresh weight comparison of seedlings growing on zeatin-containing medium. Average FW (mg seedling^{-1}) of transgenic (black) and wild-type (striped) seedlings when grown on medium containing zeatin. (C-G) Morphological changes in transgenic seedlings grown on zeatin-containing medium. (C, D) Representative photographs of wild-type (left) and transgenic (right) seedlings growing on the same plate at 28 DAS (C) and 32 DAS (D). Green blade-like structure is clearly seen in D. (E, F) Detail of the transgenic seedling in C and D respectively. G, Detail of corresponding portion of SR1 seedling at 32 DAS. (H-L) Histochemical staining for β-glucosidase. (H, J) Representative photographs of SR1 seedlings stained for β-glucosidase at 28 DAS (H) and 32 DAS (J). (I) Representative photograph of a transgenic seedling at 28 DAS. Note the restriction of staining to the ectopic structure. (K, L) Representative photographs of transgenic seedlings stained for β-glucosidase at 32 DAS. Note the patchy staining pattern in the blade-like structures. Bars in C, D = 0.5 cm; in E-L = 1 mm.

Cytokinin quantification showed that transgenic seedlings grown on medium containing zeatin accumulate active metabolites like zeatin riboside and zeatin riboside phosphate and this might lead to the observed changes. The presence of the enzyme around the base of the hypocotyl and later, in the ectopic structures themselves, suggests that the development of these structures is due to the perturbance in zeatin metabolism caused by the ectopic presence of Zm-p60.1.

A new, sensitive method for enzyme kinetic studies of scarce glucosides

The maize β-glucosidase Zm-p60.1 is important for the regulation of plant development through its role in the targeted release of free cytokinins from cytokinin-O-glucosides, their inactive storage forms. Enzyme kinetics studies using these scarce substrates close to physiological concentrations are difficult due to two reasons, namely (i) available methods are mainly suited for end-point kinetics and (ii) these methods are not sufficiently sensitive when using scarce glucoside substrates. We developed a glucose assay using a system comprising three enzymes β-glucosidase, glucose oxidase and horseradish peroxidase, with the new substrate N-acetyl-3,7-dihydroxyphenoxazine - Amplex UltraRed reagent. In comparison with the other methods this method is more sensitive, precise, and accurate. The assay is rapid and hence suited for continuous kinetics, it is readily adapted to suit automated procedures, and potential applications include its use in studying the physiological role(s) of enzymes that cleave scarce glucoside substrates.

Granted projects

GA AS CR IAA600040612, Functional analysis of *Shooting* gene in Arabidopsis and tobacco. Principal investigator: B. Brzobohatý, Co-principal investigator: I. Macháčková, 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, Co-principal investigator: B. Brzobohatý, Co-principal investigator: P. Ilík, 2006-2008

GA AS CR IAA600380507, Mechanisms maintaining hormonal homeostasis in plant cells. Principal investigator: J. Kamínek, Co-principal investigator: B. Brzobohatý 2005 -2007

Publications

Kiran, N.S., Polanská, L., Fohlerová, R., Mazura, P., Válková, M., Šmeral, M., Zouhar, J., Malbeck, J., Dobrev, P., Macháčková, I., Brzobohatý, B.: *Ectopic over-expression of the maize β-glucosidase Zm-p60.1 perturbs cytokinin homeostasis in transgenic tobacco*. J. Exp. Bot., 57, 2006, 985-996.

Mazura, P., Fohlerová, R., Brzobohatý, B., Kiran, N.S., Janda, L.: *A new, sensitive method for enzyme kinetic studies of scarce glucosides*. J. Biochem. Bioph. Meth., 68, 2006, 55-63.

PhD thesis defended in 2006

Nagavalli S. Kiran, Ectopic over-expression of the maize β-glucosidase Zm-p60.1 as a molecular tool to probe subcellular compartmentation of cytokinin metabolism

Mgr. Jana Hradilová, Analysis of the expression pattern in the *AHP* gene family in *Arabidopsis thaliana*

DNA-Molecular Complexes

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Molecular evolution of plant telomeres and telomerases. Mapping of position of the second evolutionary switch in telomere synthesis in plants of the Asparagales order – from human-type telomeres to alternative telomeres

Although telomere sequences are considered to be highly conserved, there are switch-points in plant telomere evolution that are congruent with species' phylogenies. When Asparagales diverged (see Fig. 1.), the

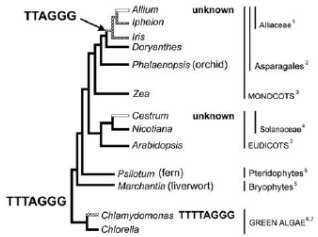

Fig. 1. Summary of knowledge about different telomere types in green plnts. The predominant telomeric sequence motif is TTTAGGG present in the green algae *Chlorella* and most plants studied . Variotion in this sequence is seen in Asparagales (TTTAGGG to TTAGGG) and the green alga *Chlamydomonas* (TTTAGGG to TTTTAGGG). The loss of the minisatelite telomeric sequence has been observed in the eudicot *Cestrum* (and sister genera *Sessea* and *Vestia* of Solanaceae) and in *Allium* (Asparageles). Superscript indicate references: (1) this work, (2) Sýkorová et al. (2003c), (3) Richards and Ausubel (1998), (4) Sýkorová et al. (2003a), (5) Suzuki (2004), (6) Petráček et al. (1990), (7) Higashiyama et al. (1995).

Arabidopsis-type telomeric minisatellite repeat (TTTAGGG)n was first replaced by a human-type (TTAGGG)n repeat, and both were lost in *Allium cepa* (Alliaceae). We aimed to discover (1) when this loss occurred during divergence of Alliaceae and, (2) if (TTAGGG)n repeats were replaced by other known telomeric minisatellites. Slot-blot hybridization, fluorescent in situ hybridization, BAL31 digestion, asymmetric PCR, and cloning were used to identify and localize candidate telomeric sequences in species of *Nothoscordum*, *Miersia*, *Ipheion*, *Tulbaghia*, *Gethyum*, *Gilliesia*, *Leucocoryne*, *Tristagma*, and representatives of the three major *Allium* clades. Alliaceae genera other than *Allium* have human (TTAGGG)-type telomeric repeats that form telomeres. In *Allium*, only Tetrahymena-type

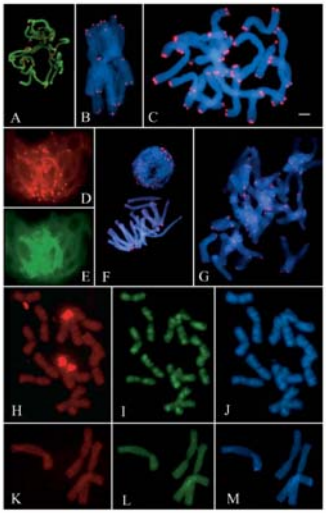

Fig. 2. Fig. 2: Fluorescence micrographs showing in situ labelling of chromosomes of Alliaceae species to localize telomeric sequences. (A) PRINS using dUTP conjugated with tetramethylrhodamine (red fluorescence) and (B–M) FISH with telomeric probes labelled with biotin/avidin-Cy3 (red fluorescence) or digoxigenin/antidigoxigenin-Cy3 (green fluorescence). Alliaceae species (A–G) outside *Allium* and within (H–M) *Allium*. (A) Metaphase of *Ipheion uniflorum* labelled by PRINS with Telo2 primer (red) and counterstained with DAPI (green). (B) Metaphase of *Tristagma bivalve* probed with HUSB concatemers (red) and counterstained for DNA with DAPI (blue). (C) Metaphase of *Nothoscordum striatum* probed with HUSB concatemers (red) and counterstained for DNA with DAPI (blue). (D, E) Late prophase of *Miersia chilensis* probed with HUSB concatemers and TTSB concatemers showing substantial telomeric signal with HUSB (D, red fluorescence) and small-scattered signals at the telomeres with TTSB (E, green fluorescence). (F) Metaphase and interphase of *Miersia chilensis* probed with HUSB concatemers (red fluorescence) and counterstained with DAPI (blue). Note signal at both ends of the chromosome arms. The chromosomes are strongly telocentric, and those telomeres near the centromere tend to be larger. At interphase, two clusters of telomeric signal are at each end of the nucleus, and chromosomes are organized in a Rabl (1885)-like orientation. (G) Metaphase of *Leucocoryne coquimbensis* probed with HUSB (red fluorescence) and counterstained with DAPI (blue). (H–J) Metaphase of *Allium cernuum* probed for (H) rDNA (red), (I) TTSB concatemers (green) and (J) DAPI stained for DNA (blue). Note there is no labelling by TTSB. (K–M) Partial metaphase of *A. schuberti* labelled with (K) HUSB concatemers (red), (L) TTSB concatemers (green) and (M) DAPI stained for DNA (blue). Note no labelling by TTSB or HUSB. Bar = 10 μm for all images except (F) where it is 10 μm.

(TTGGGG) repeats were ubiquitous in the genome, but they were not localized to telomeres. Likewise, the consensus telomeric repeats in *Arabidopsis*, human, *Bombyx* (TTAGG), *Chlamydomonas* (TTTTAGGG), and *Oxytricha* (TTTTGGGG) are absent in *Allium* telomeres. Alliaceae with human-type telomeres share telomere structures with related Asparagales species (see Fig. 2). We demonstrate that in the *Allium* ancestor human-type telomeric repeats were lost from telomeres and were not replaced by any investigated alternative minisatellite repeats. However, human and other types of minisatellite telomeric repeats are interspersed in some *Allium* genomes and their genomic signatures coincide with *Allium* clades (see Fig. 3).

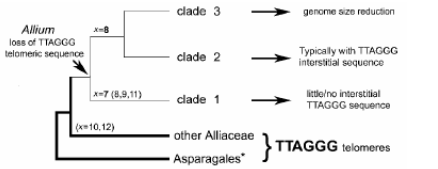

Fig. 3. Summary of the results in a phylogenetic context showing those Alliaceae with TTAGGG minisatelites at telomeres, and the genomic signatures that typically represent Allium clades . The genus Allium is separated from other Alliaceae that have telomeres formed by TTAGGG minisatelite sequence as in other Asparagales (*note Asparagales contain families with different telomeric types, see Fig. 1). Species in Allium are split into three clades; clade 1 differs by chromosome number (x) and species are unified by the absence of hybridization signals for HUSB, ATSB, BOSB, CHSB probes; clade 2 has strong signals for the HUSB probe resulting from interstitial TTAGGG sequences; clade 3 has various patterns of slot-blot hybridization and conteins species with significantly smaller genomes.

Molecular evolution of plant telomeres and telomerases. Molecular causes of the switch between synthesis of plant- and human-type telomeres: Characterisation of plant telomerases synthesising human-type telomeres

The order of monocotyledonous plants Asparagales is attractive for studies of telomere evolution as it includes three phylogenetically distinct groups with telomeres composed of TTTAGGG (Arabidopsis-type), TTAGGG (human-type) and unknown alternative sequences, respectively. To analyze the molecular causes of these switches in telomere sequence (synthesis), genes coding for the catalytic telomerase subunit (TERT) of representative species in the first two groups have been cloned. Multiple alignments of the sequences, together with other TERT sequences in databases, suggested candidate amino acid substitutions grouped in the Asparagales TERT synthesizing the human-type repeat that could have contributed to the changed telomere sequence. Among these, mutations in the C motif are of

special interest due to its functional importance in TERT (Fig. 4).

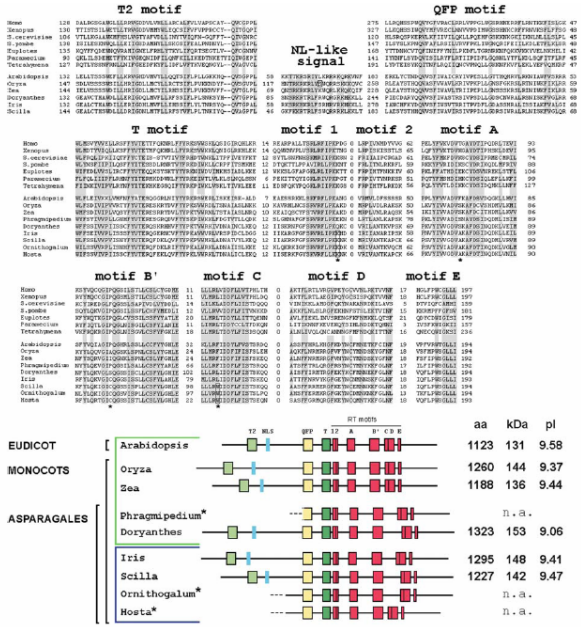

Fig. 4. Alignment of conserved telomerase motifs (above) and scheme of organization (below) of plant TERTs. The conserved motifs are as described previously (Nakamura et al., 1997; Malik et al., 2000; Xia et al., 2000), except for the nuclear localization-like signal (NL-like signal) predicted by Prosite and PSORT. The distance (number of amino acids) from the two termini and between the motifs are indicated, shaded amino acids are either identical or represent conservative substitutions for homologs included in this alignment. Conserved amino acids changes in motifs are indicated by asterisks , those discussed in detail are framed (including th eposition of Ser in the NL-like signal, see Results). The scheme below compares major features of telomerases such as motifs, linker regions, the length (aa) , size (kDa) or isolelectric point (pl) as predicted by Winstar (n.a. - not analysed). the partially sequenced TERTs are marked by arterisks, the taxonomy of species is shown on the left with the plant telomerases synthesizing *Arabidopsis*-type telomeres grouped in green bracket and telomerases synthesizing human-type telomeres in blue bracket.

Furthermore, two different modes of initial elongation of the substrate primer (denoted as *HLT* and *BLT*) were observed in Asparagales telomerases producing human-like repeats, which could be attributed to interactions between the telomerase RNA subunit (TR) and the substrate (Fig. 5) .

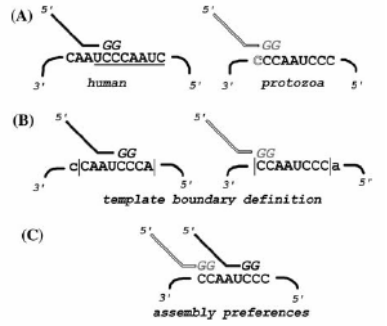

Fig. 5. Template regions. (A) human and protozoan template regions and possible template usage of the CAMVGG substrate primer by the HLT (black) or BLT (grey) group of Asparagales telomerases synthe sizing human-type telomeres. The region conserved throughout all vertebrates is underlined ; (B) the difference could be caused also by changed template boundary definition in otherwise similar template region, or (C) by different assembly preferences on the same template.

Granted projects

GA CR 521/05/0055, Molecular evolution and functional analysis of components of plant telomeres and telomerases. Principal investigator: J. Fajkus, 2005 - 2007

GA ASCR IAA600040505, Telomerase-independent mechanisms of telomere synthesis and loss. Principal investigator: J. Fajkus, 2005 - 2009

GA ASCR 204/04/P105, New evolutionary forms of telomeres and telomerases in plants. Principal investigator: E. Sýkorová, 2004 - 2006

ME LC06004, Integrated research of plant genome. Principal investigator: Boris Vyskot, 2006-2010

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Fajkus, J: *Detection of telomerase activity by the TRAP assay and its variants and alternatives*. Clin Chim Acta, 371, 2006, 25-31.

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Sykorova, E., Fajkus, J., Meznikova, M., Lim, K.Y., Neplechova, K., Blattner, F.R., Chase, M.W. and Leitch, A.R: *Minisatellite telomeres occur in the family Alliaceae but are lost in Allium*. American Journal of Botany, 93, 2006, 814-823.

Sykorova, E., Leitch, A.R. and Fajkus, J: *Asparagales Telomerases which Synthesize the Human Type of Telomeres.* Plant Mol Biol, **60**, 2006, 633-646.

PhD thesis defended in 2006

Mgr. Petra Schrumpfová, Characterisation of telomere-binding proteins.

BIOPHYSICAL CHEMISTRY AND MOLECULAR ONCOLOGY

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In 2006 the research pursued in Laboratory of Biophysical Chemistry and Molecular Oncology was concentrated to three main fields:

Field I: Electrochemistry of nucleic acids, 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 tumor suppressor protein p53 and its homologues

In the field I, a systematic study of the electrochemical behavior of synthetic oligonucleotides (ODNs) (that represent the basis of the DNA biosensors) at electrodes was continued. This research included development of sensitive analytical techniques applicable in bioanalysis and in the sensor development.

A sensitive voltammetric detection of different ODN samples at the surface-roughened glassy carbon electrode (GCE) was developed. An atomic force microscope (AFM) was used for characterization of the surface roughness of the mechanically polished GCE by different abrasion particles. The proposed electrochemical method for the detection of different ODNs was based on the following steps: (i) before voltammetric measurement the acidic hydrolysis of the ODN (ahODN) sample was performed; (ii) the second step includes a potential-controlled reduction of Cu(11) and accumulation of the purine base residue-Cu(I) complex (ahODN-Cu(I) complex) at the GCE; (iii) finally, followed the anodic stripping of the electrochemically accumulated ahODN-Cu(I) complex from the GCE. The anodic stripping of the accumulated ahODN-Cu(I) complex produced a well-developed voltammetric signal (peak I) around +0.28 V. A good correlation was obtained between the number of purine units within the ODN-chain and the height of the peak I of the electrochemically accumulated ahODN-Cu(I) complex.

Gold amalgam-alloy electrode (AuAE) was applied for a sensitive voltammetric detection of different purine-containing ODNs. The detection of ODNs was based on a similar procedure as described above. The proposed electrochemical method was used for (a) analysis of the length of ODNs containing only adenine units (from 10 to 80), and (b) determination of the number of purine units within the 30-mer ODNs containing a random sequence of both purine and pyrimidine units. The detection of acid-hydrolyzed 80-mer (A_{80}) was possible down to 200 pM at the AuAE. Similarly sensitive detection of different ODNs can be also performed at the platinum amalgam-alloy (PtAE) and copper amalgam-alloy (CuAE) contrary to a lower sensitivity attained with the silver amalgam-alloy (AgAE) electrode.

A simple technique was introduced for amplified ODN sensing in microliter volumes, based on solution streaming (bubbling) with an inert gas that

drives the motion of a 30-uL droplet of the analyte solution accommodating a three-electrode circuit (inverted drop microcell). In the presence of copper ions, this setup offers an about 50-times improvement in the sensitivity of ODN detection. At the carbon paste electrode, the analysis was based on the enhancement of the oxidation peaks of purine bases (adenine and guanine) by the anodic stripping of the electrochemically accumulated Cu(I)-ahODN complex (see above). We used the proposed method for (i) the determination of the percentage content of adenine and guanine units within analyzed ODN samples at subnanomolar concentrations and (ii) estimation of the $(TTC)_n$ triplet repeat length using DNA hybridization with reporter probes $(GAA)_n$.

Tensammetric responses of different synthetic ODNs at the platinum solid amalgam electrode (PtAE) were studied. The optimization of the AC voltammetric measurement of ODNs included the studies of the effects of the thickness of the amalgam layer, and the solid amalgam composition (platinum, copper, gold and silver), on the intensity and potential of the AC voltammetric peak 1 (due to reorientation of ODN segments adsorbed at the electrode via ODN sugar-phosphate backbone) or peak 3 (due to desorption and/or reorientation of the ODN segments adsorbed through their hydrophobic base residues). The height of the AC voltammetric peak 3 and potential difference between the AC voltammetric peaks 1 and 3 depended on the ODN lengths as well as proportion of purine (adenine and/or adenine + guanine) units to the total nucleotide number in an ODN molecule. Analytical applications of these phenomena were proposed.

Elimination voltammetry with linear scan (EVLS) was applied to the measurements of reduction signals of adenine (A) and cytosine (C) residues in short synthetic hetero-ODNs with different sequences of A and C. Compared to linear sweep voltammetry (LSV) and other usual voltammetric methods, the EVLS was capable of resolving the overlapped A and C signals, specifically by using the elimination function which eliminates the charging and kinetic currents (I-c, I-k) and conserves the diffusion current (I-d). This elimination function yields a well readable peak - counterpeak signal for adsorbed species, including the ODNs. The EVLS yielded two separated peaks in dependence on A-C sequences and pH and proved useful for qualitative and quantitative studies of short ODNs as well as for the development of electrochemical sensors.

The behavior of thiol-end-labeled ODNs at mercury electrodes was for the first time studied. It has been shown that such ODNs can form self-assembled monolayer (SAM) at the mercury surface. Changes in voltammetric signals due to cytosine or due to Hg-S bond reduction indicated changes in positioning of the HS-(TTC)7 molecules at the electrode with increasing HS-(TTC)7 concentration from lying flat with respect to SAMs, forming upright-standing molecules. This SAM behaved as an insulator not allowing electron transfer between [Ru(NH3)6] 3+ and the electrode. Different adsorption modes of thiolated and thiol-free ODNs were observed.

In addition to the basic ODN studies, the research oriented toward application of electrochemical biosensors in analysis of nucleotide sequences in synthetic as well as genomic (PCR-amplified) DNAs was continued. These investigations included testing novel experimental setups, application of electroactive indicators and introduction of new electrode types in analysis of electrochemically labeled DNAs:

Molecular diagnostics of inherited neurodegenerative disorders such as fragile X syndrome or Friedreich ataxia (FRDA) is usually based on analysis of the length of trinucleotide repetitive sequences in certain loci of genomic DNA. A single-surface sensor based on multiple hybridization of the expanded triplet repeat with short labeled reporter probe (spanning several trinucleotides) was proposed. Target DNA (tDNA) was adsorbed onto surface of a pyrolytic graphite or screen-printed carbon electrode. Biotin-labeled reporter probe (RP) was hybridized with the immobilized tDNA followed by binding of streptavidin-alkaline phosphatase (ALP) conjugate. The ALP catalyzes production of an electroactive indicator (1-naphthol) which is detected voltammetrically on the same electrode. Detection of $(GAA)_n$•$(TTC)_n$ triplet repeat expansion in nanogram quantities of PCR-amplified tDNAs, including amplicons of patients' genomic DNA, was demonstrated. This technique allows differentiation between normal and pathological alleles of *X25* gene related to the FRDA.

A bis-intercalator echinomycin (ECHI) and a simple intercalator [Co(phen)(3)](3+) were used as novel electrochemical redox indicators to detect DNA hybridization at gold electrodes (AuE). In order to minimize the nonspecific adsorption of oligonucleotides (ODN), the thiol-derivatized

oligonucleotides were immobilized onto AuE in the first step, and the exposition of AuE to 6-mercapto-1-hexanol (MCH) followed in the second step of this procedure. In this arrangement good reproducibility and discrimination between single-stranded (ss) probe and double-stranded (ds) hybrid DNA were obtained. While both redox indicators showed a good ability to discriminate between the ss and ds DNA, the signals of ECHI were by an order of magnitude higher than those of [Co(phen)(3)](3+) in agreement with stronger DNA binding by the bis-intercalator as compared to the simple intercalator. In addition, DNA single-base mismatch (DNA point mutation) was detected by means of ECHI.

The complex of osmium tetroxide with 2,2'-bipyridine has been utilized as an electroactive marker of DNA. The catalytic current due to evolution of hydrogen in voltammetry of DNA modified by complex of osmium tetroxide with 2,2'-bipyridine (DNA-Os,bipy) was studied using solid amalgam electrodes (modified with mercury menisci) of silver (m-AgSAE), copper (m-CuSAE), gold, and of combined bismuth and silver. Besides the hanging mercury drop electrode (HMDE), the catalytic current was observed only on m-AgSAE and m-CuSAE. The m-AgSAE was successfully applied as detection electrode in double-surface DNA hybridization experiments offering highly specific discrimination between complementary (target) and non-specific DNAs, as well as determination of the length of a repetitive DNA sequence. The m-AgSAE proved a convenient alternative to the HMDE or carbon electrodes used in previous work.

In the field II the work included basic research of the electrochemical behavior of peptides and proteins, as well as utilization of electrochemical techniques in studies of protein denaturation and in bioanalytical applications.

Voltammetric techniques have been used to investigate the electrochemical behavior of two phytochelatins ("plant metallothioneins", cysteine-rich peptides involved in detoxification of heavy metals in plants): heptapeptide (gamma-Glu–Cys)3–Gly and pentapeptide (gamma-Glu–Cys)2–Gly, tripeptide glutathione gamma-Glu–Cys–Gly and its fragments: dipeptides Cys–Gly and gamma-Glu–Cys at the hanging mercury drop electrode in the presence of cobalt(II) ions. Most interesting results were obtained with

direct current voltammetry in the potential region of -0.80 V to -1.80 V. Differential pulse voltammetry of the same solutions of Co(II) with peptides gave more complicated voltammograms with overlapping peaks, probably due to the influence of adsorption at slow scan rates necessarily used in this method. However, in using Brdicka catalytic currents for analytical purposes, differential pulse voltammograms seem to be more helpful. It has been shown that the electrochemical techniques allow distinguishing among phytochelatins, glutathione, and its fragments.

Further, we utilized electrochemical analysis to monitor accumulation of the PCs in the TBY-2 cells exposed to cadmium ions. Measurements of a characteristic PC signal at mercury electrode in the presence of cobalt ions made it possible to detect changes in the cellular PC levels during the time of cultivation. Upon TBY-2 cultivation in the presence of cytotoxic cadmium concentrations (50 uM), the PC levels remarkably increased during the pre-apoptotic phase and reached a limiting value at cultivation times coinciding with apoptosis trigger. The PC level observed for a sub-cytotoxic cadmium concentration (10 uM) was about 3-times lower than that observed for the 50 uM cadmium after 5 days of exposure. We show that using a simple electrochemical analysis, synthesis of PCs in plant cells can be easily followed in parallel with other tests of the cellular response to the toxic heavy metal stress.

Native and denatured states of bovine serum albumin (BSA) were studied by d.c. polarographic and voltammetric Brdicka catalytic responses (BCR) in cobalt-containing solution and by constant current chronopotentiometric stripping analysis (CPSA) in borate buffer, pH 9.3. Denatured BSA at a concentration of 90 nM produced catalytic peak H (around -1.8 V vs. Ag/AgCl/3 M KCl). This peak was about 50-fold higher than that of the native protein under the same conditions. Qualitatively similar results were obtained also with other proteins in native and denatured states, such as human serum albumin, c-globulin, myoglobin and alpha-crystallin. 2 nM denatured BSA produced a well-developed CPS peak (at accumulation time 5 min) while native BSA yielded almost no signal under the same conditions.

In the field III, the studies on structure and interactions of the tumor suppressor protein p53 and its homologues were continued. These

investigations were oriented particularly toward the sequence- and structure-selective DNA binding of the wild-type and mutated proteins and their ability to recognize damaged DNA, as well as toward functional consequences of the p53-DNA binding.

The p53 protein can recognize DNA modified with an antitumor agent cisplatin (cisPt-DNA). Interactions of the p53 with the cisPt-DNA were studied using p53 deletion mutants and via modulation of the p53-DNA binding by changes of the protein redox state. Isolated p53 C-terminal domain (CTD) bound to the cisPt-DNA with a significantly higher affinity than to the unmodified DNA. On the other hand, p53 constructs involving the core domain but lacking the C-terminal DNA binding site (CTDBS) exhibited only small binding preference for the cisPt-DNA. Oxidation of cysteine residues within the CD of posttranslationally unmodified full length p53 did not affect its ability to recognize cisPt-DNA. Blocking of the p53 CTDBS by a monoclonal antibody Bp53-10.1 resulted in abolishment of the isolated CTD binding to the cisPt-DNA. These results demonstrate a crucial role of the basic region of the p53 CTD (aa 363-382) in the cisPt-DNA recognition.

Proteins p53 and p73 are known as transcription factors involved in the cell cycle control, regulation of cell development and/or in apoptotic pathways. Both proteins bind to response elements (p53 DNA binding sites, p53DBS) typically consisting of two copies of a motif RRRCWWGYYY. Global DNA modification with cisplatin differentially inhibited sequence-specific DNA binding of the p53 or p73 proteins to various p53DBS, depending on the nucleotide sequence of the given target site. Relative sensitivities of the protein-DNA binding towards the cisplatin DNA treatment correlated with occurrence of sequence motifs forming stable bifunctional adducts with the drug (namely, GG and AG doublets) within the target sites. Binding of both proteins to mutated p53DBS's from which these motifs had been eliminated was only negligibly affected by the cisplatin treatment. Distinct effects of cisplatin DNA modification on recognition of different response elements by the p53 family proteins may have impacts on regulation pathways in cisplatin-treated cells.

Using a competition assay on agarose gels it was found that the p53DBS in longer DNA fragments are better targets for the protein binding than the

same sequences in shorter DNAs. Semi-quantitative evaluation of the competition experiments showed a correlation between the relative p53-DNA binding and the DNA length. These observations are consistent with a model of the p53-DNA interactions involving one-dimensional migration of the p53 protein along the DNA for distances of about 1000 bp while searching for its target sites. Positioning of the p53 target in the DNA fragment did not substantially affect the apparent p53-DNA binding, suggesting that p53 can slide along the DNA in a bi-directional manner. In contrast to full-length p53, the isolated core domain did not show any significant correlation between sequence-specific DNA binding and fragment length.

Most human tumors contain inactivated p53 protein, either by mutations and/or functional deactivation. Restoration of wild-type p53 function could be one of the key tools in new anticancer therapy. Using an electromobility shift assay, we investigated the effect of temperature on DNA binding of wild-type and mutant p53 proteins. We showed that analysis of the DNA-binding capacity of mutant p53 proteins is complicated by the temperature at which the assay is performed. Furthermore, neither the ability to bind DNA nor conformational analysis accurately defines the transcriptional activity of human tumor-derived p53 mutant proteins. Some p53 mutants can bind DNA and adopt a wild-type conformation in vitro, but are transcriptionally inactive in vivo. Therefore, the common use of purified proteins and in vitro determinations of DNA binding and conformation are not sufficient indicators of the functional properties of mutant p53.

Granted projects

GA AS CR IAA4004402, Electrochemical detectors of DNA hybridization and their applications in DNA diagnostics. Principal investigator: M. Fojta, 2004 - 2007

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

GA AS CR B500040502, Mutants of tumor suppressor protein p53 and regulation of their DNA binding activity. Principal investigator: V. Brázda, 2005 – 2007

GA AC 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 A4004404, Interaction of biopolymers with ligands and detection of their conformation changes at interfaces by electrochemical and optical methods. Principal investigator: V.Vetterl, 2004 - 2006

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: Luděk Havran, 2006-2008

GACR 203/04/1325, New approaches in development of electrochemical sensors for DNA damage. Principal investigator: M. Fojta, 2004-2006

GACR 301/04/P025, Influence of DNA superhelicity on sequence specific and structure selective binding of the p53 protein. Principal investigator: V. Brázda, 2004-2006

GACR 301/05/0416, Development of novel therapeutic strategies through sensitising tumour cells to ani-cancer drugs by targeting p53-kinases and p53 homologues. Principal investigator: B. Vojtěšek, co-investigator: M. Fojta, 2005-2007

GACR 203/05/0043, Conjugates of nucleobases with metal complexes as electroactive markers. Application of labelled oligonucleotides in electrochemical DNA sensors. Co-investigator: L. Havran, 2005-2007

AS CR KAN 400310651, Nanotechnologies for protein and gene diagnostics. Principal investigator: F. Foret, Co-investigator: E. Paleček, 2006 – 2010

AS CR KAN 200040651, Electrochemical and optical analysis of biomacromolecules at the microelectrodes modified by an electroactive material nanolayer. Principal Investigator: S. Hasoň 2006-2010

ME LC06035, Centre of biophysical chemistry, bioelectrochemistry and bioanalysis. New tools for genomics, proteomics and biomedicine. Coordinator: M. Fojta, 2006-2010

ME 1 M0528, Stomatological Research Center. Coordinator: Jiří Vaněk Co-investigator: V.Vetterl, 2005 - 2009

ME 1K04119, Interactions of mutant p53 proteins with genomic DNA *in vitro* and *in vivo.* Principal investigator: M. Fojta, key person: M. Brázdová, September 2004-August 2007

ME FRVŠ 1372/2006/F4/a, New practical exercises for the lecture "Introduction to molecular biophysics". Principal investigator: V.Vetterl, 2006

ME FRVŠ 1003/2006, Use of Bacteria in Electrochemistry. Principal investigator: L. Fojt, 2006

MIT 1H-PK/42, Research and development of a new-type electrochemical biosensor for the detection of nukleotide sequences and genotoxic agents in the environment. Project leader: M. Fojta, guarantor: E. Palecek, May 2004 – April 2007

6FP EU 502983, Mutant p53 as a target for improved cancer treatment. Principal investigator: E. Paleček, 2004-2008

MERG-6-CT-2005-014875, Novel interactions of mutant p53 with genomic DNA in vitro and in vivo. Fellow: M.Brázdová, Scientist in charge: E. Paleček, 2005-2006

Publications

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Mgr. Kateřina Cahová, Electrochemical Analysis of DNA Damage

RNDr. Veronika Ostatná, Study of Mechanism of DNA-Protein Interactions at Surfaces

Mgr. Hana Pivoňková, Interaction of Tumor Suppressor Protein p53 and its Homologues with Specific DNA Structures

EXPERIMENTAL HEMATOLOGY

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The efforts of the Laboratory of Experimental Hematology have been targeted in 2006 at continuation of our endeavors to obtain more detailed knowledge on the regulatory roles of various factors in mouse hematopoiesis and on the possibilities to favorably influence the course of regeneration of hematopoiesis suppressed by ionizing radiation.

A series of studies has been aimed at ascertaining whether meloxicam, a cyclooxygenase-2 inhibitor, given preirradiation can influence the course of the postirradiation hematopoietic recovery. Meloxicam was administered in a single intraperitoneal injection to mice one hour before their exposure to a sublethal dose of 6.5 Gy of gamma-rays. In comparison with saline-treated controls, significantly higher numbers of hematopoietic progenitor cells for granulocytes and macrophages (GM-CFC), progenitor cells for erythrocytes (BFU-E), as well as peripheral blood neutrophils and erythrocytes were found in the postirradiation period. These findings suggest that meloxicam effects should be further studied from the point of view of its possible use as a part of the treatment regimen for hematopoietic radiation injury in the man.

In another experiments we have investigated effects of N^6-(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA), a selective adenosine A_3 receptor agonist, on the granulocytic system in femoral marrow of mice depleted by the cytotoxic drug 5-fluorouracil. In the phase

of the highest cell depletion IB-MECA was injected i.p. at single doses of 200 nmol/kg given either once or twice daily in 2- and 4-day regimens starting on day 1 after 5-fluorouracil administration; the effects were evaluated on days 3 and 5, respectively. The general effect of IB-MECA in all these experiments was an enhancement of the counts of morphologically recognizable proliferative granulocytic cells, interpreted as evidence of the differentiation of committed progenitor cells. A more expressive effect was observed after IB-MECA injected twice daily. It was found that the induction of the strong differentiation pressures by IB-MECA given twice daily shortly after 5-fluorouracil treatment can be counterproductive due to the preponderance of differentiaton processes over the proliferation control. In additional experiments it has been shown that the use of the 2-day administration of IB-MECA given twice daily in the recovery phase, i.e. on days 5 and 6 after 5-fluorouracil administration, does not induce stimulatory effects. Thus, the dosing and timing of IB-MECA treatment determines its effectivity in stimulating granulopoiesis under conditions of myelosuppression.

We have also performed a series of in vitro experiments which have been aimed at finding out if adenosine is able to co-operate with selected hematopoietic growth factors and cytokines, namely with granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), interleukin-3 (IL-3), and interleukin-11 (IL-11), in inducing the growth of colonies from hematopoietic progenitor cells for granulocytes and macrophages (GM-CFC) from normal bone marrow cells *in vitro*. Adenosine was found not to produce any colonies when present in the cultures as the only potential stimulator. All the tested cytokines and growth factors were observed to induce the growth of distinct numbers of GM-CFC colonies, with the exception of IL-11. When suboptimum concentrations of the evaluated cytokines and growth factors were tested in the cultures when present concomitantly with various concentrations of adenosine, mutually potentiating effects were found in the case of IL-3 and SCF. These results confirm the role of adenosine in regulation of granulopoiesis and predict IL-3 and SCF as candidates for further *in vivo* studies of their combined administration with adenosine.

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

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MOLECULAR EPIGENETICS

HEAD

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Trans-generation inheritance of methylation patterns in a tobacco transgene triggered by posttranscriptional silencing

One of the most prominent features of DNA methylation is the faithful propagation of its genomic pattern from one cellular generation to the next. Symmetrical CG methylation provides a model of how epigenetic information is passed along to the next generations in both plants and animals. We have studied the inheritance of the epigenetic state of tobacco transgenes whose expression and DNA methylation status were modified by exposure to a silencer locus. The *npt*II reporter genes residing in the target loci were posttranscriptionally silenced (PTGS) in hybrids by a homologous silencing locus organized as an inverted repeat. We show that in hybrids the coding region of the target *npt*II genes was almost exclusively methylated at CG configurations with exception of a few non-symmetrical sites located close to the 3´ end. On the other hand homologous sequences in the trigger were heavily methylated at both CG and non-CG motifs. After segregation of the silencing locus, the CG methylation but not the non-CG methylation was transmitted to the progeny.

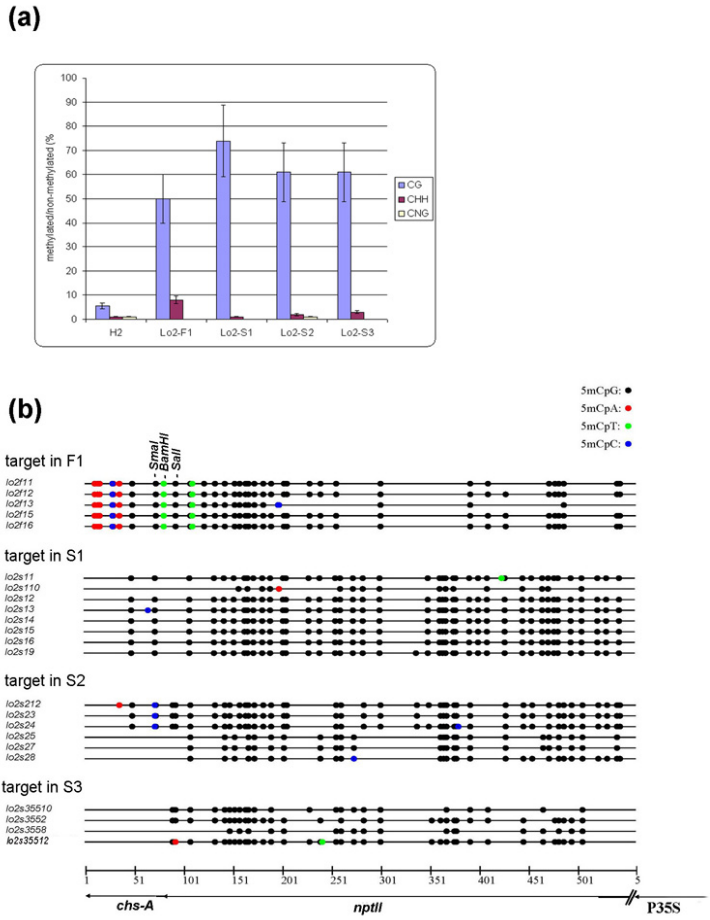

Fig. 1. Inheritance of target locus methylation after segregation of the silencing trigger analyzed by bisulfite sequencing.

(a) Summary of methylation patterns in the locus 2 target. Target in parental HoLo2 line (H2), F1 hybrid (F1-Lo2) and three generations of individuals that did not inherit the trigger (F2-Lo2, F3-Lo2 and F4-Lo2).

(b) Distribution of cytosine methylation in all Cs. CG – black dots; non-CG – red, green and blue dots.

In these segregants we observed overall increase of CG methylation in the target genes associated with a re-distribution from the 3' towards the middle of the coding region. This pattern was inherited with some fluctuation for at

least two additional generations suggesting that CG methylation is not cleared during meiosis. These epiallelic variants re-expressed the reporter gene immediately after segregation of the trigger showing that relatively dense CG methylation (~60-80%) imprinted on the most of the coding region (>500 bp) did not reduce expression as compared to the non-methylated locus before the contact with the silencer. We propose that the genic CG methylation seen in euchromatic regions of the genome may originate from ancient PTGS events as a result of adventitiously produced methylation-directing RNA molecules.

Transcription activity of rRNA genes correlates with a tendency towards intergenomic homogenization in *Nicotiana* allotetraploids

There are two aspects of rDNA biology that have been studied extensively for many years, these aspects are: (1) epigenetic silencing or nucleolar dominance, whereby ribosomal RNA (rRNA) genes or rDNA loci from one particular parent in a hybrid or allopolyploid are transcriptionally silent and; (2) rDNA unit homogenization whereby one particular unit overwrites pre-existing units, probably mediated by recombination or copy number expansion/contraction mechanism that lead to the fast evolution of novel families, a process called concerted evolution. In this study we use a range of natural allopolyploids and synthetic F1 hybrids and polyploids to determine if these two fundamental processes are linked. We examine rDNA inheritance and expression patterns in three natural *Nicotiana* allopolyploids (closest living descendents of diploid parents are given), *N. rustica* (*N. paniculata* x *N. undulata*), *N. tabacum* (*N. sylvestris* x *N. tomentosiformis*) and *N. arentsii* (*N. undulata* x *N. wigandioides*), and synthetic F1 hybrids and allopolyploids.

The extent of interlocus rDNA homogenization decreases *viz*: *N. arentsii* > *N. tabacum* > *N. rustica*. The persistence of parental rDNA units in one of the subgenomes associates with their transcription inactivity and likely heterochromatization. Of synthetic hybrids and polyploids only *N. paniculata* x *N. undulata* show strong uniparental transcriptional silencing of rDNA triggered already in F1. Epigenetic patterns of expression established early in allopolyploid nucleus formation may render units susceptible or resistant to homogenization over longer time-frames. We propose that nucleolus-associated transcription leaves rDNA units vulnerable to homogenization, while epigenetically inactivated units, well-separated from the nucleolus, remain unconverted.

Granted projects

GA CR 521-04-0075, Epigenetic regulation of gene expression in transgenic and endogenous loci of higher plants. Principal investigator: Aleš Kovařík, 2004-2006

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

GACR 204/06/1432, Nicotiana sylvestris DNA-(cytosine-5)-methyl-transferases. Principal investigator: Jaroslav Fulneček, 2006-2008

GACR 204/05/0687, The impacts of interspecific hybridization and allotetraploidization on evolution of plant genomes. Principal investigator: Roman Matyášek, 2005-2007

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MOLECULAR CYTOMETRY AND CYTOLOGY

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Higher-order chromatin changes induced by an oncogenic transcription factor PML/RARa

Gross modifications in chromatin texture and global changes of gene expression are constant features of cancer cells. The underlying mechanisms, however, as well as their mechanistic links remain largely unknown. In collaboration with the European Institute of Oncology in Milan we finished studies of the effects of the PML-RAR oncogenic transcription factor on higher-order chromatin structure and their consequences on gene expression. We found that 30% of the >1000 transcriptional targets of PML-RAR are distributed in the genome as gene clusters, do not possess PML-RAR - specific DNA-recognition elements (RAREs) within their promoters, and are flanked by one or more clusters of 30-40 Alu repeats each containing one RARE (Alu-RAREs). Chromatin analysis revealed that PML-RAR is first recruited at clusters of Alu-RAREs, where it accumulates at high density, and then at the transcription start sites (TSSs) of adjacent genes, through the formation of multiple DNA

loops. High-resolution *in situ* imaging showed co-localization of PML-RAR with clusters of co-regulated genes and long-range chromatin changes of the corresponding chromosomal regions. Treatment with drugs that target PML-RAR and induce tumor regression (RA and TSA) reverted higher-order chromatin changes and transcriptional repression of clustered genes. These findings demonstrate that higher-order chromatin changes are early events following PML-RAR oncogene expression and that they cause global changes of gene expression. Since also wild-type RARs bind Alu-RAREs and Alu repeats contain DNA-recognition elements for different transcription factors, specific recruitment to transposable elements and induction of higher-order chromatin changes might function as a general mechanism of transcriptional regulation (see Fig. 1).

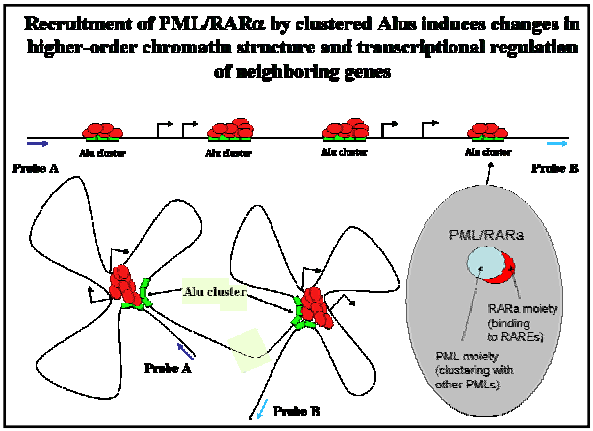

Fig. 1. New model of gene down-regulation by PML/RARa fusion oncoprotein in APL. PML/RARa (red) binds to the degenerated RARE elements in Alu-RARE repeats (green), a subfamily of Alus found to contain these specific sequences. Alu-RAREs are clustered in the human genome, which results in formation of massive PML/RARa aggregates, interacting with other Alu-RAREs and promoters of down-regulated genes (selected most probably on the basis of epigenetic characteristics). These interactions – mediated by the „sticky" PML moiety of PML/RARa take – place on long distances (proofed for 2Mb at present) and result in gene down-regulation (by changes of higher-order chromatin structure, preventing the binding of transcription factors and/or local heterochromatinization mediated by HDACs).

Plasmid DNA trafficking in the cytoplasm and the cell nucleus

Once plasmid DNA has entered the cell, it traverses the cytoplasm to reach the cell nucleus. Microtubule and actin networks play a crucial role in plasmid movement toward the cell nucleus, similarly, as it was found in

viruses. We observed strong binding activity of plasmid DNA-lipid complexes to both networks, their immobilization at actin filaments and directional motion of plasmids along the microtubules. In addition, actin filaments play an important role in decomposition of huge aggregates of plasmid DNA/lipids complexes at the cell periphery to smaller ones. Disruption of one of these networks led to the abortion of plasmid transport to the cell nucleus, decreasing mobility of plasmids and accumulation of plasmid DNA in huge aggregates at the cell periphery. These findings confirm an idea on the indispensable role of both types of cytoskeleton in gene delivery. The networks constitute two functionally different but connected systems where complexes of the plasmid DNA/lipids bound to the actin filaments and moved in short distances to microtubules. Than the microtubule network constitutes the high-ways for long-distance transport of plasmid DNA as a cargo toward the cell nucleus (Fig.2).

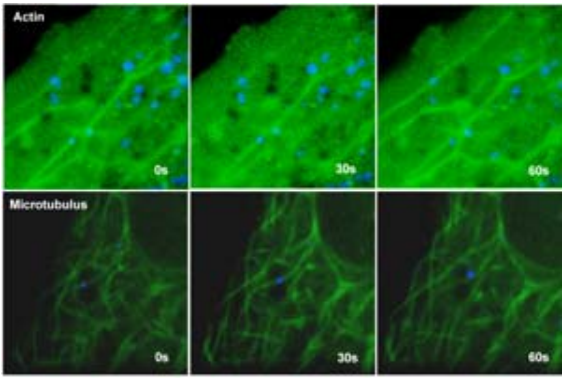

Fig. 2. Movement of plasmid DNA-lipid complexes in live cells visualized simultaneously with cytoskeletal networks. The upper row of images taken at successive time points shows the highly constrained motion of plasmid DNA-lipid complexes (blue) bound to actin filaments (green), visualized by means of the actin-Lumio fusion protein. The bottom row of images demonstrates the directional movement of two plasmid DNA-lipid complexes (blue) along a microtubule towards the cell nucleus (n) during 1 min. Scale bar 1 μm.

Functional role of the nuclear actin in plasmid DNA movement and targeting was investigated. As already mentioned in previous report, the most frequent sites of plasmid targeting in the cell nucleus were those in which the genome integrity was impaired. Plasmid DNA frequently localized in double stranded DNA breaks (DSB) induced spontaneously or

by ionizing radiation and detected as sites with phosphorylated H2AX. Plasmids move to these sites, probably, to integrate into the host genome. Inhibition of actin polymerization by latrunculin B perturbed plasmid transport not only throughout the cytoplasm but also inside the nucleus and resulted in stopping of plasmid co-localization with newly induced DSBs. It indicates the crucial role of polymeric actin in plasmid intranuclear transport.

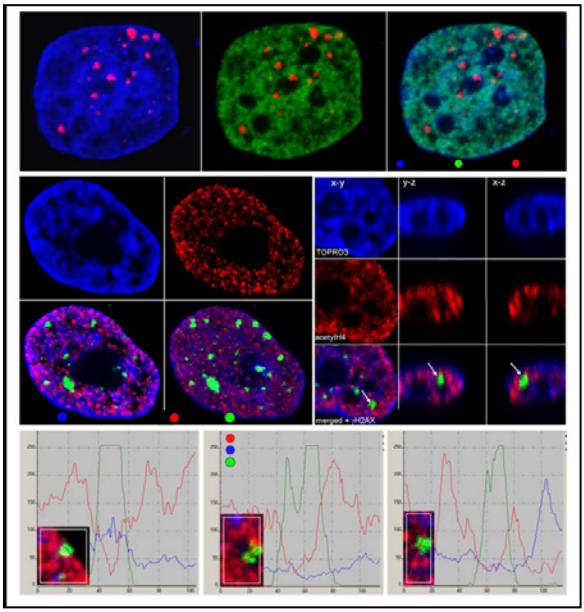

Fig. 3. Localization of DSBs detected as γH2AX in the cell nucleus of human fibroblasts irradiated with 1.5 Gy of γ-rays. **A.** Cell nucleus transfected with GFP-H2B 3D fixed 30 min PI and immunostained with anti-γH2AX (red). The nucleus was labelled also with TOPRO-3 to compare the distribution of chromatin density detected by this labeling and that of H2B-GFP. **B**. Left side: x-y sections through the nucleus showing chromatin density (blue), location of acetylated H4K12 (red) and γH2AX (green) foci. Maximal image is also shown. Right side: 3D projections of a nucleus showing chromatin density (blue), distribution of acetylated histone H4K12 and location of γH2AX. The arrow indicates the location of a γH2AX focus in the space of the nucleus. Location of γH2AX in low-dense chromatin can be seen as well as its contact with the signals of acetylated histone (white arrow). **C.** RGB-profiles of chromatin density (blue) and intensity of acetylated histone H4K12 (red) in the site of location of randomly selected γH2AX foci (green) along the pathways indicated at inserted images. (x-axis: relative fluorescent intensity, arbitrary >100 RFI for dense chromatin, y-axis: millimetres [mm])

Chromatin dynamics during DSB repair

The most serious damage induced to DNA is interruption of its integrity resulting in double-strand breaks (DSBs). Improper repair of DNA DSBs can lead to the development of cancer. Once a cancer has developed, radiation and chemotherapies are used to damage DNA in order to kill the tumour cells. Thus, recognition how cells respond to DNA damage is critical for understanding both the cancer development and therapy. Immediately after DSBs induction, histone H2AX, the variant of H2A is phosphorylated by ATM. Phosphorylated H2AX, called γH2AX, can be detected within minutes after the introduction of DSBs and is involved in the recruitment of other known proteins of DNA.

We showed that DSBs induced in euchromatin as well as in heterochromatin by exposure of human cells to γ-rays are repaired in low-dense chromatin (Fig. 3). Extensive chromatin decondenzation in the vicinity of DSBs was observed 30 minutes after irradiation. Slight movement of sporadic DSB loci on short distances was noticed in living cells within this time. Chromatin decondenzation around DSBs results frequently in their looping out into the chromatin domains of low density. In these regions, the clustering (contact or fusion) of DSB foci was seen in vivo and in situ after cell fixation. The majority of the clustered foci were repaired within 240 min but some of them persisted in the nucleus for several days after irradiation, indicating hardly repaired damages. We suppose that the repair of DSB in clustered foci might lead to misjoining of ends and to exchange aberrations.

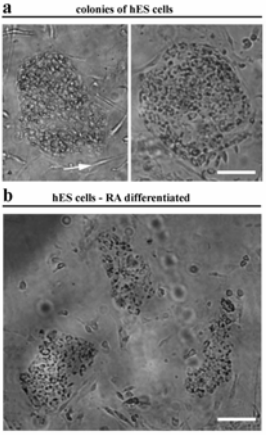

Fig. 4. Light microscope observation of hES cells induced to differentiation. (a) Pluripotent hES growing on a feeder layer of MEF cells (white arrow) and forming specific colonies. (b) Differentiation of hES cells induced by *all-trans* retinoic acid (ATRA) for 48, in the_absence of bFGF factor. Bars indicate 150 μm.

Structure of chromatin in human embryonic stem cells

Human embryonic stem cells (hES) are unique in their pluripotency and capacity for self-renewal. We studied the differences in the level of chromatin condensation, nuclear possitioning of some genes and distributions of proteins in pluripotent and *all-trans* retinoic acid differentiated hES cells (Fig. 4). The *Oct4* (6p21.33) gene, responsible for hES cell pluripotency, the *C-myc* (8q24.21) gene that controls the cell cycle progression and the HP1 protein (heterochromatin protein 1) were investigated. Unlike differentiated hES cells, pluripotent hES cell populations were characterized by a high level of decondensation of the territories of chromosomes 6 (HSA6) and 8 (HSA8). In pluripotent hES cells, the *Oct4* genes were located in many cases on largely extended chromatin loops, outside their respective chromosome territories. This phenomenon was not observed for the *Oct4* genes in differentiated hES cells and for the *C-myc* genes in both cell types studied (Fig. 5). The high level of chromatin decondensation in hES cells also influenced the nuclear distribution of all variants of HP1 proteins, particularly, HP1□ did not form distinct foci, as usually observed in most other cell types. Our experiments showed that unlike *C-myc*, the *Oct4* gene arrangement and the HP1 proteins distributions reflect a high degree of chromatin decondensation in hES cells.

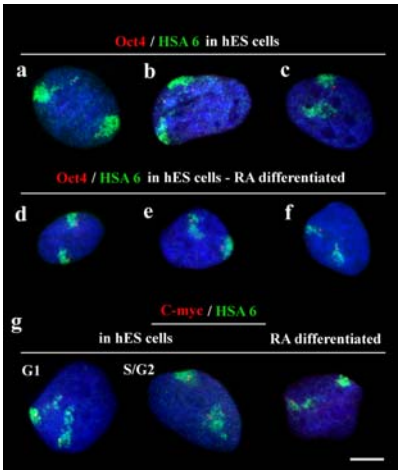

Fig. 5. Nuclear positioning of *Oct4* and *C-myc* genes within interphase nuclei and chromosome territories. (a) *Oct4* gene (red) location on a chromatin loop greatly extended from a chromosome 6 territory (green). The second *Oct4* gene was positioned the periphery of the other chromosome 6 territory. (b) Positioning of both *Oct4* genes on chromatin loops, distant from the compact part of the chromosome 6 territories. (c) Both *Oct4* genes were positioned at the peripheries of their respective chromosome territories. (d-f) Representative examples of *Oct4* gene location at the periphery of the chromosome 6 territories in ATRA-differentiated hES cells. (g) The left panel is an example of *C-myc* localized within the chromosome 8 territories of a pluripotent hES cell in the G1 phase of the cell cycle. The middle panel shows the location of the *C-myc* gene at the periphery of HSA 8 in the S/G2 phase of a pluripotent hES cell. The right panel represents the *C-myc* gene location at the chromosome periphery in an ATRA-differentiated hES cell. Bar indicates 6 μm.

The highly decondensed chromatin structures seem to be responsible for hES cell pluripotency due to their accessibility to regulatory molecules. Differentiated hES cells were characterized by a significantly different nuclear arrangement of the chromatin structures studied.

Transcription sites of the *c-myc* gene

The nuclear and territorial location of the *c-myc* gene, its epigenetic characteristics, and nuclear positioning of the *c-myc* transcription sites were investigated in relation to nuclear domains involved in RNA synthesis and processing. The p1 promoter and selected coding sequences of the *c-myc* gene were H3(K9) acetylated and H3(K4) di-methylated, showing no heterochromatin markers, regardless of the cell type and independently of the karyotype. The *c-myc* genes were located non-randomly within the interphase nucleus, with an average position at 70% of the nuclear radius. However, the *c-myc* transcription sites were found internally, at 40% of the nuclear radius, and were associated with the periphery of the centrally located nucleoli. Up-regulation of the *APC* gene reduced both the level of the c-MYC protein and the number of *c-myc* transcription sites. This correlated well with the decreased number of cells in the late G1- and early S-phases of the cell cycle, during which the *c-myc* gene is mainly transcribed (Fig. 6). In most cases, the *c-myc* gene was characterized by a single transcription site, which co-localized with the RNAP II region and was associated with the periphery of its chromosome territory. In 20% of cells, the *c-myc* transcriptional complex was released from the site of synthesis. The *c-myc* pre-mRNA was associated with nuclear speckles (SC-35 domains) in 65% of nuclei, but with promyelocytic leukaemia (PML) bodies in only 22% of nuclei. We suggest that *c-myc* gene transcription and *c-myc* pre-mRNA processing take place close to nucleoli, with the participation of factors contained in those SC-35 domains located in the most internal parts of the cell nucleus.

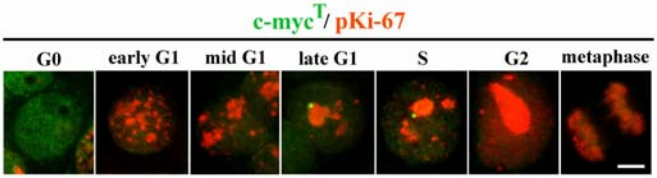

Fig. 6. Results of immuno-RNA-FISH experiments showed that the *c-myc* gene is transcribed in late G1 phase of the cell cycle, and in most cases its transcription continues in S-phase. Red regions are pKi-67 positive cells and green signals are *c-myc* transcription sites. Bar indicates 2.5 μm.

Epigenetic characteristics of multiple myeloma cells

Multiple myeloma is a plasma cell malignancy localized in the bone marrow. Abnormalities of genome concern wide spectrum of chromosomal aberrations, which type and frequency correlate with the stage of disease and drug response. However, no typical prognostic factor is still known. We focused on two multiple myeloma cell lines: ARH77 ($CD138^-$) and MOLP-8 ($CD138^+$). This model is very interesting for its analogy with the bone marrow microenvironment where $CD138^-$ cells are considered as a physiological component and $CD138^+$ as a malignant cell population. Our study was aimed at genes that are critical for the mentioned type of malignancy for which epigenetic characteristics and the levels of corresponding proteins were investigated. We tested effects of clinically used approaches such as gamma-irradiation and cytostatic treatments (bortezomib, dexametahoson and melphalan). In comparison with MOLP-8 cells, we detected increased level of p53 protein in ARH77 cells which was stable, regardless of cytostatic treatment. Bortezomib, melphalan and gamma-irradiation increased the level of p53 in MOLP-8 cells. Histone H3(K9) acetylation and di-methylation were investigated using ChIP-PCR technology for the c-myc gene. Changes in these epigenetic markers were determined not only in multiple myeloma cell lines, but also in several patients suffering from multiple myeloma.

Granted projects

GA CR 202/04/0907, High-resolution cytometry of living cells. Principal investigator: S. Kozubek, 2004 – 2006

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

GA CR 204/06/0978, Postranslation modifications of histones after cell treatment by inhibitors of histone deacetylases and during induction of cell differentation. Principal investigator: E. Bártová, 2006-2008

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

GA AS CR A1065203, The use of multiple optical tweezers to controlled manipulation and rotation of micro objects. Principal investigator:

P. Zemánek, ISI AS CR Brno, Co-principal investigator: E. Lukášová, 2002 – 2006

GA AS CR A5004306, Structure of human genome. Principal investigator: S. Kozubek, 2004-2008

ME, COST 1P050C084, Dynamic structure and function of the cell nucleus after irradiation, principal investigator: S. Kozubek, 2005-2007

IGA MH CR 1A8241-3, New possibility of diagnostics of leukaemia using the technology of DNA microarrays. Principal investigator: S. Kozubek, 2004-2006

ME LC06027, Monoclonal Gammopathy and Multiple Myeloma, Basic Research Centre, Co-principal investigator: E. Bártová, 2006-2010

6. FP EU, LSHG-CT-2003-503441, 3D Genome structure and function. Principal investigator: R. van Driel, Co-principal investigator: S. Kozubek, 2004-2007

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CYTOKINETICS

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Laboratory of Cytokinetics focused on the research of potential role of lipid membrane elements and their derivatives in cell signalling. Particularly, the effects of environmental substances, such as lipid nutrition components (essential polyunsaturated fatty acids and butyrate) and xenobiotics (cytostatics and environmental organic pollutants) on regulattion of cytokinetics, i. e. cell proliferation, differentiation and apoptosis are studied. Using both tumour and non-tumourigenic cells, new types of interactions of lipid/phospholipid components, anticancer drugs (non-steroidal anti-inflammatory drugs-NSAIDs, cytostatics) or selected environmental pollutants (polycyclic aromatic hydrocarbons, PCBs, dioxins) with

physiological regulators of cytokinetics (cytokines, growth factors) are being investigated. A special attention is being paid to the effects of the investigated substances on biophysical properties of cell membranes, oxido-reduction processes, cell communication and signalling and to consequences of these modulations to deregulation of the cytokinetics and intercellular communication. The results are exploited in the field of ecotoxicology and in cancer prevention/therapy.

Cellular and molecular physiology of lipids

Dietary lipids are important modulators of behaviour of colonic epithelial cells playing important role in colon carcinogenesis. Among them, butyrate, short-chain fatty acid produced by microbial fermentation of fibre, and essential polyunsaturated fatty acids (PUFAs) are known to influence colonic cell kinetics. Based on differences among the normal and cancer cells, we investigated the response of human colonic cell lines derived from normal fetal tissue (FHC) and colon adenocarcinoma (HT-29, HCT116) to sodium butyrate (NaBt), arachidonic acid, docosahexaenoic acid and/or their combination. While FHC and HT-29 cells differentiated after NaBt treatment, the response of HCT116 cells was primarily apoptotic. Interestingly, the response of normal FHC cells, particularly in cell cycle changes and apoptosis, was more intensive. Combined treatment with NaBt and PUFAs decreased colonic cell proliferation, increased apoptosis (FHC, HCT116) and/or suppressed differentiation (FHC, HT-29) as compared to single agents, in a cell line-dependent manner. The apoptotic response to PUFAs, as well as to their combinations with NaBt was stronger in normal FHC cells in comparison with cancer cells.

Mcl-1, an anti-apoptotic member of Bcl-2 family, functions as an early critical regulator in the apoptotic signalling leading to the activation of the mitochondrial pathway. Enhanced levels of Mcl-1 have been demonstrated in several cancers to protect the cells from cell death induced by a variety of apoptotic stimuli, which might contribute to their malignant phenotype. We demonstrated that TRAIL is responsible for up-regulation of Mcl-1 protein level in HT-29 human colon adenocarcinoma cell line. This effect was completely abolished by inhibition of the MEK/ERK pathway, which concomitantly potentiated TRAIL-induced apoptosis (Vaculova et al., 2006).

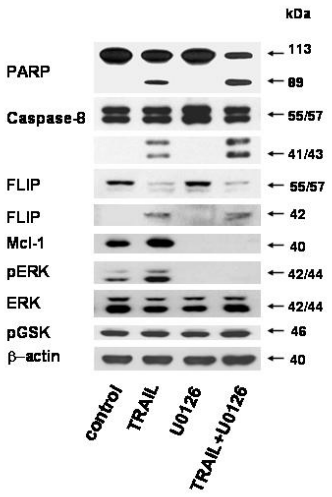

Fig. 1. PARP, pro-caspase-8 and $cFLIP_L$ cleavage, Mcl-1 protein level, phosphorylated and total ERK1/2 levels, and phosphorylated GSK3β in HT-29 cells pretreated with U0126 (10 μM, 45 min.) and then treated with TRAIL (100 ng/ml, 4 hrs), detected by Western blotting.

Moreover, we examined, whether the Mcl-1 up-regulation could be a more general phenomenon also for other colon cell lines and if there is any correlation between the cell sensitivity/resistance to TRAIL-induced apoptosis and Mcl-1 protein level. We also showed a significant TRAIL-induced up-regulation of Mcl-1 protein level in HCT116 colon cancer cell line, while significantly weaker response was observed in normal human fetal colon cell line FHC. The mechanisms responsible for the Mcl-1 up-regulation in colon cancer cells were studied at the different levels of the intracellular signalling pathways. Using RT-PCR, we demonstrated that TRAIL-induced up-regulation of Mcl-1 protein level is preceded by an increase in the amount of Mcl-1 mRNA. This suggests that TRAIL may be responsible for activation of signalling pathways leading to stimulation of mcl-1 gene transcription in colon cancer cells. As inhibition of MEK/ERK pathway completely abrogated the described effects at the level of Mcl-1 mRNA, we suggest that MEK/ERK pathway is involved in the TRAIL-induced Mcl-1 transcription in colon cancer cells.

We also elaborated the topic of cell adhesion and detachment-induced apoptosis – anoikis. Using previously established model of non-adherent cellular cultivation, we studied changes in cytokinetics and adhesion properties between adherently and non-adherently cultivated normal FHC and cancer HT-29 colon cell lines. We found that during the non-adherent cultivation the induction of apoptosis is associated with increased expression of specific integrins and E-cadherin. The cancer HT-29 cells were more sensitive to the conditions of non-adherent cultivation than the normal FHC cells.

The response of normal and cancer colon cells was also compared with regard to hypericin- mediated photodynamic therapy (PDT). Normal FHC cells were more resistant to PDT than the cancer HT-29 cells despite of higher intracellular hypericin incorporation. However, the reactive oxygen species (ROS) production and decrease of MMP after PDT were markedly lower in FHC cells. In HT-29 cells, the possibility of improvement of therapeutic effectiveness of hypericin-mediated PDT by pre-treatment of cells with 5-lipoxygenase (LOX) inhibitor MK-886 was investigated. This type of therapy clearly shifted the type of cell death from necrosis to apoptosis, when compared with MK-886 alone. Moreover, some combined modalities (especially 48 h pre-treatment and combination of low doses of both agents) significantly potentiated the apoptotic effects of hypericin alone. Combined treatment of increasing concentrations of MK-886 and hypericin caused massive changes in the cell cycle progression (accumulation of cells in the S-phase or G0/G1 phase depending on MK-886 concentration). These results indicate that combined treatment involving PDT and LOX inhibitor MK-886 may improve therapeutic effectiveness of PDT (Klebán et al. 2006).

Growth factors in cancer cell signalling

Tissue microenvironment plays an important role in tumour initiation and progression. Growth factors - cytokines - play crucial role in cancer development and some of them belong to the significant autocrine/paracrine factors produced by various cell types in tumour microenvironment. Because of their important role during tumour progression, modulation of their signal transduction represent potential target for anti-cancer therapy. Chronic inflammation may cause cancer and its progression in several organs such as in prostate or colon. Finding of ways for modulation of signals and pathways leading to the chronic inflammation seem to be reasonable for establishment of strategy for chemoprevention and treatment of these diseases.

In 2006 we started with new studies focusing especially on (1) functional role of GDF-15 (member of transforming growth beta family) in the effects of cyclooxygenase inhibitors (non-steroidal anti-inflammatory drugs (NSAIDs) and in interaction of NSAIDs and 1-alpha, 25-dihydroxyvitamin D3, (2) role of PUFAs in modulation of signalling pathway of pro-

inflammatory cytokine IL-6, (3) modulation of neuroendocrine differentiation (NED) of prostate cancer.

We have demonstrated that treatment of androgen-sensitive prostate cancer line LNCaP by NSAIDs leads to quick arrest of cells in G0/G1 phase of cell cycle. This effect is associated with increased level of GDF-15 and $p21^{Waf1/Cip1}$protein. Using RNA interference we have shown that $p21^{Waf1/Cip1}$ protein plays essential role in the inhibition of proliferation after the treatment with NSAIDs. We have further conducted studies aimed at possible role of PI3K/Akt in effects of NSAIDs. We have found that NSAIDs treatment of LNCaP cells leads to increase in activity (phosphorylation) of pro-survival kinase Akt which is in positive correlation with $p21^{Waf1/Cip1}$ phosphorylation at Thr 145. These observations suggest that a novel posttranslational mechanism of the effects of NSAIDs may exist. Similarly to NSAIDs, we have found that $p21^{Waf1/Cip1}$ might be a key protein in the increased inhibition of proliferation after the combined treatment by NSAIDs and 1-alpha, 25-dihydroxyvitamin D3.

Using treatment of LNCaP cellswith IL-6, we have further characterized its signalling pathways that might be affected by docosahexaeonic acid (DHA). We have demonstrated that pretreatment of LNCaP cells by DHA led to reduction of level of phosphorylated STAT3 after IL-6 treatment. Results of STAT3 EMSA show that DHA pretreatment of LNCaP cells completely abolished formation of DNA/STAT3 complex. However, DHA did not affect IL-6-induced Ras activation. These results demonstrate possible mechanism of anti-inflammatory and chemopreventive effects of n-3 PUFAs in prostate cancer.

Cellular and molecular toxicology

Diverse environmental organic pollutants are known or suspected carcinogens that have been reported to possess tumour-initiating and/or tumour-promoting properties. It is our aim to characterize their effects at molecular and cellular level that might be linked to carcinogenesis, reproductive or developmental impairment.

In 2006, we have continued our studies on environmental toxicants, aimed especially at the effects associated with activation of aryl hydrocarbon receptor (AhR), as well as AhR interactions with endogenous regulatory

molecules, such as proinflammatory cytokines. Our work concentrated on the AhR-dependent gene transcription, deregulation of cell proliferation and cell-to-cell communication. Principal model compounds included heterocyclic aromatic compounds (7-*H*-dibenzo[*c,g*]carbazole and its methylated derivatives), polycyclic aromatic hydrocarbons (PAHs) and their methylated derivatives, and flavonoids. We have concentrated mostly on compounds that are largely ignored by the current risk analysis or environmental protection bodies, such as e.g. monomethylated benz[a]anthracenes, chrysenes, phenanthrenes and PAHs with a large molecular weight.

Our results showed that compounds with high AhR-mediated activity are able to disrupt cell proliferation control in epithelial cells in a manner similar to unsubstituted PAHs or persistent organochlorine pollutants (Vondráček et al., 2006). Some of them are also able to disrupt gap junctional intercellular communication; however, this effect appears to be largely AhR-independent. Using siRNA targeted against AhR and dominant negative AhR construct, we have confirmed that induction of cell proliferation in contact-inhibited cells is AhR-dependent, while it does not depend on activation of mitogen-activated protein kinases (MAPKs) (Andrysík et al., 2006; 2007). Our data also suggest that a balance between genotoxic and nongenotoxic effects of PAHs, such as a widespread environmental carcinogen benzo[*a*]pyrene (BaP), plays a crucial role in determination of fate of cell populations affected by these compounds. Activation of AhR may lead not only to induction of enzymes (such as cytochrome P450 1A1, 1B1 or aldo-keto reductases) involved in metabolic activation of PAHs to the ultimate carcinogens, dihydrodiol epoxides, but it might also determine cell proliferation, thus allowing for survival and proliferation of cells potentially carrying mutated DNA (Andrysík et al., 2007). These results have implications for our understanding to chemical carcinogenesis and evaluation of risks associated with exposure to chemical carcinogens, such as PAHs.

Inhibitors of arachidonic acid metabolism in the effects of cytokines

The studies investigating the effects of combined treatment in leukemic cell lines HL-60 and U937 with death-ligand TNF-alpha and a) inhibitor of 5-LOX MK-886 (Štika J. et al., 2006) or differentiation agent DMSO were

completed (Vondráček J. et al., 2006). These results have shown that these combinations might potentiate differentiation and/or apoptosis of leukemic cells. This topic was continued by investigation of signalling pathways included in the induction of differentiation of leukemic cells. Our research focused on the role of different mitogen-activated protein kinases (MAPKs), which mediate reaction of cells to stress conditions (including the activation of signalling pathways of cytokines TNF-alpha and TGF-beta1 or Vitamin D3) by modulating expression of so called early response genes.

In the first model, the differentiation of human myeloid HL-60 cells was induced by treatment with cytokines TNF-alpha, TGF-beta1 and MK-886. Here, the activation of c-Jun N-terminal kinase (JNK), MAPK p38 and p44/42 kinase (ERK1/2) signalling pathways, which regulate cell survival versus apoptosis was investigated. Our results indicate that these kinases are dynamically up- and down-regulated in differentiation- and viability-dependent manner. In the second model, the role of MAPKs was studied during monocytic differentiation induced by dihydroxyvitamin D3 (VD3), which is significantly potentiated by 5-LOX inhibitors MK-886 and AA-861 in HL-60 cells. Using MAPKs inhibitors: p38 - SB202180, JNK - SP600125 a p44/p42 - U0126, the involvement of particular kinases in myeloid cell differentiation was studied. Moreover, the association with changes of antiapoptotic Mcl-1 protein level was investigated. These results suggest involvement of MAPK in regulation of monocytic differentiation induced by VD3 in combination with 5-LOX inhibitors, and imply a significant role of dynamic changes of Mcl-1 protein.

The effects of novel cytostatics

Generally, these studies were aimed to investigate the ability of novel Pt(IV) complex with adamantylamine, coded LA-12, and/or its reduced counterpart with lower oxidation state Pt(II) - LA-9, to overcome intrinsic cisplatin resistance. LA-12 was able to overcome intrinsic resistance to cisplatin in ovarian cancer cell line SK-OV-3. Stable S-phase arrest as well as decreased level of proliferation were results of incubation of SK-OV-3 cells with LA-12. In the contrary, LA-9, the reduced Pt(II) counterpart of LA-12, and cisplatin, commonly used platinating drug, caused only transient S-phase arrest and less pronounced cytotoxicity (Horvath, V., 2006).

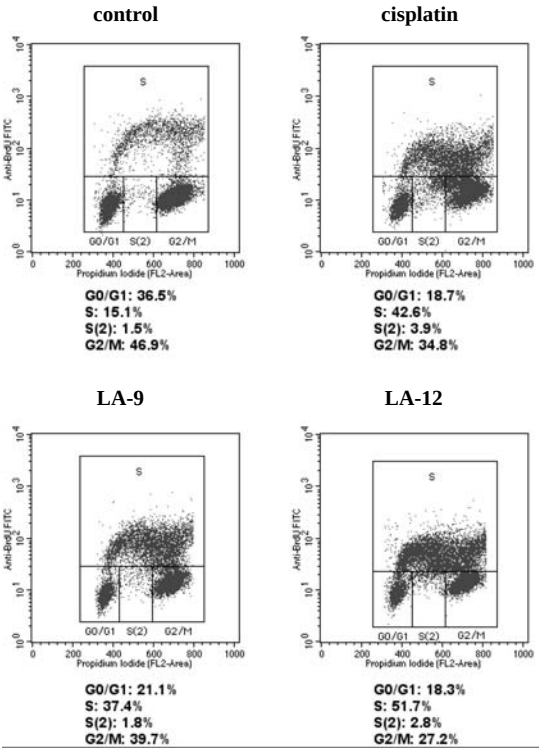

Fig. 2. Effects of cisplatin, LA-9 or LA-12 on percentage of SK-OV-3 cells in S-phase of the cell cycle and DNA synthesis. Cells were metabolically labeled with 5-bromo-2´-deoxyuridine (BrdU) nd then assessed for cell cycle distribution and DNA synthesis rate by flow cytometry (BrdU and propidium iodide double staining). Compartment S(2) represents cells in the S-phase of the cycle which do not synthesize DNA.

Further investigations of different cell cycle modulations by Pt(II) and Pt(IV) drugs might reveal signalling pathways acting as DNA damage sensors and elucidate their roles in decreased proliferation/increased cell death after treatment with these anticancer agents .

Granted projects

GA CR 524/04/0895, Mechanisms of cell death induced by dietary lipid components and endogenous apoptotic regulators in colon epithelial cells. Principal investigator: J. Hofmanová, 2004 - 2006

GA AS CR KJB6004407, Interactions of genotoxic and nongenotoxic effects of polycyclic aromatic hydrocarbons in regulation of cell proliferation. Principal investigator: J. Vondráček, 2004 - 2006

GA CR 524/05/0595, Interactions of physiological growth regulators, arachidonic acid and xenobiotics. Principal investigator: A. Kozubík, 2005 - 2007

GA AS CR KJB500040508, Cell adhesion and anoikis of intestinal cells - role of TNF family members, AA metabolism, and differentiation. Principal investigator: M. Hýžďalová, 2005 - 2007

GA AS CR IQS5000405070, 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

ESF-ME, Improvement of qualification and flexibility of Ph.D. students of the Faculty of medicine, PU. Principal co-investigator: A. Kozubík, 2006-2007

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PhD thesis defended in 2006

Ing. Viktor Horváth, High effectiveness of platinum complexes with adamantylamine in overcoming resistance to cisplatin and suppressing proliferation of ovarian cancer cells in vitro

DNA BIOPHYSICS AND GENOME BIOINFORMATICS

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KAREL NEJEDLÝ

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TECHNICAL ASSISTENT

SOŇA KAŠKOVÁ

The laboratory studies regularities of primary, secondary and tertiary structures of genomic molecules of DNA, their restriction and PCR fragments, and synthetic oligonucleotides using spectroscopic, electrophoretic, photochemical, PCR and computer methods. It is the aim of the studies to find how the genomic molecules of DNA arose, how they function and how they undergo changes. A particular attention is focused on the role of DNA unusual structures in the human genome.

Studies continued of the longest (A+T) and (G+C) runs in the human and other genomes. First we generated randomized sequence of the human genome where the longest (A+T) and (G+C) runs were found to be 46 and 26 nucleotides in length, respectively. Then we focused on (A+T) and (G+C) runs in the human genome that were longer. The first interesting finding was that such long (A+T) as well as (G+C) runs do exist in the human genome and that they are very long and abundant. The longest (A+T) block has the length of 1040 nucleotides in the human genome. It occurs in the 16^{th} human chromosome. Other longest (A+T) runs have 661, 654 and 643 nucleotides in length. There are 1449 (A+T) blocks longer than 100 nucleotides in the human genome. The blocks where the (A+T) content

is higher than 90% are naturally still much longer. Two of them have more than 3 kilobases and still further 22 are longer than 2 kilobases.
The (G+C) blocks are significantly shorter in the human genome than the (A+T) blocks, but they are still much longer than in the randomized sequence. The longest (G+C) block has 261 nucleotides in the human genome. It occurs on the 8^{th} chromosome. Further 8 (G+C) blocks are longer than 100 nucleotides. The longest block with a higher than 90% (G+C) content occurs on chromosome 2 and has the length of 983 nucleotides. Nine further such blocks are longer than 300 nucleotides.

The chimp genome is highly homologous to the human genome so that one would expect a similar occurrence of extremely long (A+T) and (G+C) blocks. Surprisingly, this is not true. The longest chimp (A+T) block occurs on chromosome 14 and has only 719 nucleotides in length. The (A+T) blocks are generally much shorter in the chimp genome compared to the human genome. The same is true with the (G+C) blocks. The ten longest 100% and 90% (A+T) blocks have the average length of only 79% and 61% in chimp compared to human, respectively. The analogous percentages are 64% and 54% for the G+C) blocks.

The next organism we analyzed was rat. Its genome contains fairly long 100% (A+T) blocks (86% of the human value) but short 90% (A+T) blocks (36%). The (G+C) blocks are relatively long in rat. Mouse has shorter (A+T) as well as (G+C) blocks than rat. Chicken is interesting by relatively long (G+C) blocks (134% and 105% of the human value) but (A+T) blocks are short. The last higher eukaryote was dog whose values were not extreme in any case except for short 90% (A+T) blocks. Finally we analysed 100% (A+T) and 100% (G+C) blocks in a number of further organisms. Arabidopsis contains one 131 nucleotide (A+T) block whereas the longest 100% (G+C) block has 27 nucleotides. Caenorhabditis is rich in long (A+T) blocks while (G+C) blocks are short. Drosophila is also very rich in long (A+T) blocks but it contains no (G+C) block longer than 20 nucleotides. On the other hand, Deinococcus contains no long (A+T) block but contains relatively long (G+C) blocks. Yeast contains one very long (A+T) block and a 21 nucleotide (G+C) block. The longest (A+T) and (G+C) blocks have 30 and 21 nucleotides, respectively, in E.coli.

Next we studied neighbourhood of the long (A+T) and (G+C) blocks in the human genome. The 100% (A+T) blocks were immediately followed by sequences having about 60% (A+T), i.e. the average value of the whole genome. In other words, the (A+T) blocks are discrete and are not embeeded in the genome through any connectors having the (A+T) value between 60% and 100%. In contrast, the (G+C) blocks are different. They are embeeded in the genome through connectors where the (G+C) content gradually drops to a value of 50%. The connectors are longer than a kilobase. Even 2 kilobases before and after the 100% (G+C) blocks the (G+C) content does not decrease to the level of 40%, i.e. the average value in the whole genome.

The long (A+T) and (G+C) blocks have unique biophysical properties and we believe that the properties play a role in the genome functioning. Further studies are in progress in this direction.

We develop methods to study secondary structure transitions in pieces of genomic DNA. The methods are based on the observation that UV light damages DNA and that the damage depends on the DNA secondary structure. In the first part of this project we detected the damage using restrictases. Now we have extended the detection by a primer extension approach. It belongs to its advantages to detect the damage with a single nucleotide resolution. We have applied the primer extension method to the pUC19 DNA and mainly to its polylinker region. We induced the B-to-A conformational transition in a linearized pUC19 DNA, irradiated the DNA with UV light in various stages of the transition and detected the damage by primer extension. The primer extension approach is based on the fact that damaged bases in the template cause pausing of a polymerase, which gives rise to shorter fragments ending at the sites of damage. The shorter fragments are separated on sequencing gels.

We observed that the most damaged bases were located in B-form trinucleotides containing both pyrimidine bases, i.e. TTC, TCT, CTC and CTT. Another highly sensitive target was the CCCC tetramer. The amount of the damaged bases decreased in the course of the B-A transition. Some of the damage even disappeared in the A-form, which concerns mainly CCCC and CCC. On the contrary, the decrease of the damage during the B-A transition was less significant with thymine dimers. We found differences

in the B-form and A-form patterns of UV light-damaged bases within the pUC19 polylinker region embedded in the long (the whole plasmid) and short (a 127 bp fragment) molecule of DNA. The B-A transition of the fragment was found less cooperative than with the linearized plasmid, which was confirmed by parallel CD spectroscopy analysis.

Granted projects

GA AS CR A1004201, Biophysical properties of (guanine+cytosine) and (adenine+thymine) regions in the DNA molecules of human chromosomes.Principal investigator: Jaroslav Kypr, 2002-2006

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FREE RADICAL PATHOPHYSIOLOGY

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Carvedilol as a constituent of preservation solution – the effect on liver function and injury

High amount of patients waiting for liver transplantation along with the lack of donor organs lead physicians to use of marginal grafts, as are steatotic livers. Steatotic grafts are more prone to preservation injury and consequently, to primary dysfunction or nonfunction after transplantation. Therefore, there is an effort to improve the properties of preservation solutions to diminish the preservation injury. The aim of the study was to investigate the potential use of carvedilol (multiple action drug - beta blocker, antioxidant, vasodilating and antihypertensive properties) as the constituent of preservation solution.

Homozygous (obese, with steatotic liver) and heterozygous (lean, with nonsteatotic liver) Zucker rats were used. The livers of anaesthetized animals were flushed *in situ* with cold University of Wisconsin solution adjusted with carvedilol (10 μM). Then the livers were withdrawn and stored in UW solution at 4^{o}C for 24 hours. After the storage the livers were exposed at lab temperature for 30 min and flushed with 60 ml of Ringer-

lactate solution. Than the livers were connected to recirculating perfusion system and perfused for 120 min with warm (37°C) oxygenated Krebs-Hensleit solution. The samples of Ringer-lactate solution as well as the samples of perfusate were taken at time points t0, t30, t60, t90, t120. The livers were frozen on dry ice immediately at the end of perfusion and stored at –80°C. Perfusate flow rate, bile output, BSP clearance and amount of BSP in the bile were estimated. In the perfusate, nitrites, MDA levels, the levels of transaminases (AST, ALT) and cytokines (TNF-alpha, IL-10) were measured. In the liver tissue, COX-2 and iNOS expression was measured.

In steatotic livers, carvedilol significantly decreased MDA and transaminase levels in the perfusate. There was observed a significant increase in bile output, increased amount of BSP in the bile, BSP clearance and perfusate flow rate. Carvedilol significantly increased both TNF-alpha and IL-10 production with the very positive correlation between both cytokines (R^2 = 0.97). None of the described parameters was significantly changed in nonsteatotic livers. Very low expression of COX-2 was detected in all experimental groups, without significant differences among controls and treated groups. The expression of iNOS was not observed in any group, which is consistent with the fact, that there were not measurable concentrations of nitrites in the perfusate. In conclusion, carvedilol decreased liver injury and oxidative stress and had significant favourable effect on liver function in the model used. However, the increasing level of TNF alpha is alarming even though it is accompanied by the induction of strong anti-inflammatory response.

The use of terrestrial bacteria with natural bioluminescence in ecotoxicology

Marine luminescent bacteria are used in environmental toxicity testing due to their ease of use and the potential to rapidly report on toxic events. However it is well known that the use of marine bioluminescent bacteria also brings some imperfections (the necessity to keep the optimal temperature, salinity, pH). We assumed that these imperfections can be overcome by the use of *Photorhabdus* species - the only known terrestrial bacteria with natural bioluminescence. Eight presently known *Photorhabdus* sp. and subsp. were cultivated in different media in wide range of temperatures, pH and salinities and their bioluminescence was

compared with the bioluminescence of *Vibrio fischeri* and *Photobacterium leiognathi* (marine bacteria). Bioluminescence of *Photorhabdus temperata* CCM 7078^T and *Photorhabdus luminescens* subsp. *thracensis* CCM 7095^T was the highest and the most stable among terrestrial bacteria tested. That is the reason why these two bacterial strains were selected as the most advantageous for the testing of toxicity. The influence of inorganic polutants (mainly heavy metals) on the bacterial bioluminescence was analysed in following experiments. Results show that the sensitivity of terrestrial bacteria is comparable with marine bacteria.

Antiinflammatory and antioxidative properties of serotonin

The effect of serotonin in a concentration range of 10^{-7} M - 10^{-3} M on the oxidative burst of phagocytes was studied using luminol-enhanced chemiluminescence method. Serotonin inhibited the CL response of opsonized zymosan particle – OZP, phorbol myristate acetate – PMA, formyl-Methionyl-Leucyl-Alanine – fMLP and calcium ionophore – Ca-I activated phagocytes in human whole blood in a dose dependent manner.

Since serotonin could act as a true scavenger of reactive oxygen species (ROS) generated during the respiratory burst of stimulated phagocytes, the aim of one of the studies was to compare direct scavenging antioxidative effects of serotonin and its precursors (L-tryptophan and 5-hydroxytryptophan) and metabolites (N-acetyl-5-hydroxytryptamine and melatonin). The effects of studied compounds in a concentration range of 10^{-7} M - 10^{-3} M were studied using fluorescence ORAC and HORAC methods. The ORAC method measures the antioxidant scavenging activity against peroxyl radical induced by 2,2'-azobis(2-amidinopropane) dihydrochloride, while the HORAC method measures the metal-chelating activity of antioxidants in the conditions of Fenton-like reactions and hence the protecting ability against formation of hydroxyl radical. 5-hydroxytryptophan and N-acetyl-5-hydroxytryptamine exerted comparable antioxidative properties to peroxyl radical as serotonin. L-tryptophan and melatonin exerted only negligible antioxidative potential to peroxyl radical. The protecting ability against formation of hydroxyl radical (HORAC method) decreased in the rank of L-tryptophan > 5-hydroxytryptophan > serotonin > N-acetyl-5-hydroxytryptamine > melatonin. It can be concluded that serotonin scavenges ROS due to its unique chemical structure,

especially due to the type and position of substituents on the indole molecule.

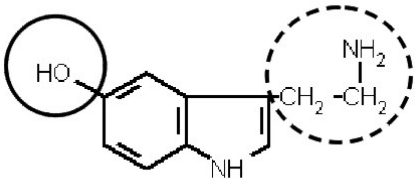

Fig. 1: Chemical structure of serotonin (5-hydroxytryptamine) with marked substituents on the indole molecule. Especially hydroxyl group at carbon 5 plays a crucial role in the activity of serotonin.

The inhibition effects of the tested compounds on the generation of NO were examined in lipopolysaccharide (LPS, 1 mg/ml)-stimulated murine RAW 264.7 macrophages. The total activity showing the level of nitrite production was measured by Griess method. Primary screening tests were done at a sample concentration of 5 mM, and no cytotoxicity was observed at this concentration (cell viability 95%). Only 5-hydroxy-L-tryptophan exhibited marked cytotoxicity (cell viability 30%), and therefore the concentration was decreased to 1 mM. Serotonin, 5-hydroxytryptophan and N-acetyl-5-hydroxytryptamine strongly inhibited NO production. Melatonin and L-tryptophan did not inhibit production of nitrates at any tested concentration. Lower concentrations of tested compounds did not inhibit production of nitrites by LPS stimulated macrophages. On the other hand, inducible NO synthase protein levels were determined using Western blot analysis. LPS-activated macrophages exposed to serotonin, 5-hydroxytryptophan and N-acetyl-5-hydroxytryptamin contained significantly dose-dependently lower levels of inducible NO synthase protein compared to macrophages treated with LPS only.

Effects of collagen on platelet aggregation

Platelet aggregation in the presence/absence of both non-modified and oxidatively modified collagens was measured using an aggregometer (turbidimetry is the principle of the method). Thrombin in a concentration

of 0.025 U/ml was used as a reference sample. All collagens were used in a concentration of 1.1 mg/ml. The total volume of samples was 0.470 ml.

All collagen samples were measured in 7 human volunteers, 3 times in each case. It is obvious from the results that the native (or non-modified collagen) was able to induce platelet aggregation little bit more than thrombin. On the other hand, all the modified collagens lost the ability to induce platelet aggregation, approximately to the same extent.

Role of reactive oxygen and nitrogen species (RONS) in the activation of phagocytes by endotoxin

RONS are important inflammatory mediators produced under endotoxin challenge by lung macrophages and lung epithelial cells. RONS are included in redox sensitive signaling pathways induced by endotoxin stimulation. RONS are suggested to potentiate these signaling pathways which lead to increase their own production by induction of RONS producing enzymes. Our work was focused on modulation of endotoxin induced production of nitric oxide (NO) and an expression of inducible NO synthase (iNOS) by mouse macrophages. Therefore we tested wide range of cell permeable antioxidants and inhibitors of RONS producing enzymes and analyzed modulation of iNOS expression together with NO production by endotoxin stimulated macrophages. Our data showed that inhibitors of enzymes producing superoxide and NO (Apocynin and Diphenyleneiodonium chloride) significantly down regulated both iNOS expression and NO production. Similarly antioxidants increasing intracellular concentration of reduced thiols (Glutathione and N-acetyl cystein) and analog of vitamin (Trolox) decreased iNOS expression and NO production. Interestingly, water soluble antioxidant ascorbic acid did not reveal inhibitory effects on iNOS expression and NO production. However, our data in general confirmed redox sensitivity of cellular signaling pathways activated by endotoxin treatment. This could open new supporting therapeutic approaches for treatment of lung inflammatory diseases.

Further, regulation of NADPH oxidase, the enzyme responsible for superoxide production by stimulated phagocytes, was evaluated in human blood phagocytes activated by endotoxin. In contrast to production of NO by mouse macrophages, endotoxin did not significantly induce production

of superoxide. However, endotoxin revealed “priming” effect on blood phagocytes. A consequent stimulation of endotoxin pre-treated phagocytes by other stimuli leaded to significantly higher production of superoxide compared to endotoxin non-treated phagocytes. To clarify molecular mechanisms of observed “priming” effect of endotoxin the activation of selected signaling pathways (ERK1/2 and p38) and phosphorylation of regulatory subunit of NADPH oxidase 47phox was evaluated. Our data showed that the phosphorylation of the NADPH subunit 47phox is involved in observed phenomenon of “priming” of phagocytes by endotoxin.

Enhancement of antinociception by coadministration of nonsteroidal anti-inflammatory drugs and soluble epoxide hydrolase inhibitors

Combination therapies have long been used to treat inflammation while reducing side effects. The present study was designed to evaluate the therapeutic potential of combination treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) and previously undescribed soluble epoxide hydrolase inhibitors (sEHIs) in lipopolysaccharide (LPS)-challenged mice. NSAIDs inhibit cyclooxygenase (COX) enzymes and thereby decrease production of metabolites that lead to pain and inflammation. The sEHIs, such as 12-(3-adamantan-1-yl-ureido)-dodecanoic acid butyl ester, stabilize anti-inflammatory epoxy-eicosatrienoic acids, which indirectly reduce the expression of COX-2 protein. Here we demonstrate that the combination therapy of NSAIDs and sEHIs produces significantly beneficial effects that are additive for alleviating pain and enhanced effects in reducing COX-2 protein expression and shifting oxylipin metabolomic profiles. When administered alone, AUDA-BE decreased protein expression of COX-2 of control mice treated with LPS only without altering COX-1 expression and decreased PGE2 levels compared with LPS-treated mice not receiving any therapeutic intervention. When AUDA-BE was used in combination with low doses of indomethacin, celecoxib, or rofecoxib, PGE2 concentrations dropped significantly, without disrupting prostacyclin and thromboxane levels. These data suggest that these drug combinations (NSAIDs and sEHIs) produce a valuable beneficial analgesic and anti-inflammatory effect while prospectively decreasing side effects such as cardiovascular toxicity.

Heparins increase endothelial nitric oxide bioavailability by liberating vessel-immobilized myeloperoxidase

Clinical study focused on the role of myeloperoxidase (MPO), an abundant enzyme released from neutrophils and monocytes, in chronic coronary artery diseases (CAD) was performed. Neutrophils and monocytes are centrally linked to vascular inflammatory disease, and MPO has emerged as an important mechanistic participant in impaired vasomotor function. MPO binds to and transcytoses endothelial cells in a glycosaminoglycan-dependent manner, and MPO binding to the vessel wall is a prerequisite for MPO-dependent oxidation of endothelium-derived nitric oxide (NO) and impairment of endothelial function in animal models. In the present study, we investigated whether heparin mobilizes MPO from vascular compartments in humans and defined whether this translates into increased vascular NO bioavailability and function. Plasma MPO levels before and after heparin administration were assessed by ELISA in 109 patients undergoing coronary angiography. Whereas baseline plasma MPO levels did not differ between patients with or without angiographically detectable CAD, the increase in MPO plasma content on bolus heparin administration was higher in patients with CAD. Heparin treatment also improved endothelial NO bioavailability, as evidenced by flow-mediated dilation and by acetylcholine-induced changes in forearm blood flow. The extent of heparin-induced MPO release was correlated with improvement in endothelial function. Moreover, and consistent with this tenet, ex vivo heparin treatment of extracellular matrix proteins, cultured endothelial cells, and saphenous vein graft specimens from CAD patients decreased MPO burden. Mobilization of vessel-associated MPO may represent an important mechanism by which heparins exert antiinflammatory effects and increase vascular NO bioavailability. These data add to the growing body of evidence for a causal role of MPO in compromised vascular NO signaling in humans.

Granted projects

GA CR 524/04/0897, The role of serotonin in mutual interactions of platelets and professional phagocytes. Principal investigator: M. Číž, 2004 - 2006

GA CR 305/04/0896, Effects of carvedilol on metabolic activity of neutrophils and monocytes. Principal investigator: A. Lojek, 2004 - 2006

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

NATO CBP.EAP.CLG.982048, Collagen, platelet and neutrophil interactions with respect to wound healing. Principal investigator: M. Číž, 2006 – 2007

MEYS - Kontakt 66 Antioxidant and antiphagocytic properties of chemical and natural substances and drugs Principal investigator: Antonín Lojek, 2006-2007

MEYS - Kontakt 4-2006-11 Effect of ischemic preconditioning on reperfusion injury. Principal investigator: Antonín Lojek, 2006-2007

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PhD students defended in 2006

Mgr. Jana Franková, The influence of polysaccharides on immune cells and wound healing process

Mgr. Lucie Gallová, The modulation of the functional activity of phagocytes by pro-inflammatory, anti-inflammatory and anti-oxidative substancies

Mgr. Daniela Komrsková, Antioxidative mechanism in prevention of oxidative injury

STRUCTURE AND DYNAMICS OF NUCLEIC ACIDS

HEAD

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We have carried out a wide range of investigations of structural dynamics and molecular interactions of nucleic acids, using a variety of methods such as atomistic simulations, electronic structure calculations and bioinformatics.

RNA motifs called Kink turns are asymmetrical internal loops, characterized by sharp bend of phosphodiester backbone. Kink turns are often localized adjacent to the key functional sites in the ribosome. Extensive computational structure-dynamical study of selected Kink turn motifs (in the presence of explicit solvent) was performed. Using current state-of the art computational techniques, we complemented the knowledge about the structural and dynamical behavior these RNAs at atomic level, and significantly enriched the available information about molecular interactions in the ribosome. We found that RNA Kink turn motifs are constructed as flexible molecular RNA nano-hinges which are able to allow biologically significant motions of ribosomal segments during individual stages of proteosynthesis. Moreover, we found that RNA Kink turns are associated with a unique network of hydration sites that are intimately involved in their conformational dynamics. We also demonstrated that crucial role in observed hinge-like motions is played by a common A-minor motif tertiary interaction. Distinct iso-energetic conformational substates of A-minor motif are directly coupled with presence or absence of water molecule between the interacting bases. Independent implicit solvent

conformational search confirms the flexibility of K-turns around their x-ray geometries and identifies a second low-energy region with open structures that could correspond to K-turn geometries seen in solution experiments. An extended simulation of Kt-42 with adjacent rRNA Helices 43/44 (i.e. Factor binding site or GTPase associated center of the large ribosomal subunit; see figure) shows that the local Kt-42 elbow-like motion fully propagates beyond the K-turn. K-turns thus could mediate large-scale adjustments of distant RNA segments.

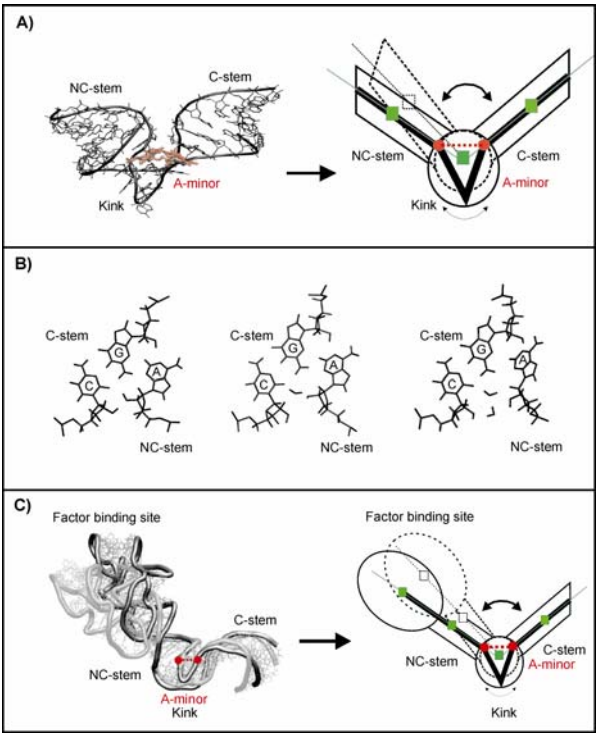

Fig. 1. A) V-shaped Kink turns are the most recurrent RNA motifs. MD simulations predict that Kink turns are uniquely flexible elbow-like RNA building blocks, where subtle local conformational changes in the kink area propagate as large-scale motions towards the attached helical stems. B) The local dynamics associated with Kink turn flexibility coupled with dynamical insertion of long-residency waters between the C/A nucleotides of the A-minor motif type I interaction, localized between the two helical arms. C) Large-scale elbow–like dynamics, observed in simulation of Helix 42-44 rRNA portion of the large ribosomal subunit, coupled with dynamical water insertion into the A-minor interaction in the universally conserved Kt-42.

Explicit solvent molecular dynamics simulations were carried out for sarcin-ricin domain (SRD) motifs from 23S (*E. coli*) and 28S (rat) rRNAs. The SRD motif consists of GAGA tetraloop, G-bulged cross-strand A-stack, flexible region and duplex part. Detailed analysis of the overall dynamics, base pairing, hydration, cation binding and other SRD features is presented. The SRD is surprisingly static in multiple 25 ns long simulations and lacks any non-local motions, with RMSd values between averaged MD and high-resolution X-ray structures 1-1.4 Å. Modest dynamics is observed in the tetraloop, namely, rotation of adenine in its apex and subtle reversible shift of the tetraloop with respect to the adjacent base pair. The deformed flexible region in low-resolution rat X-ray structure is repaired by simulations. The simulations reveal few backbone flips, which do not affect positions of bases and do not indicate a force field imbalance. Non-Watson-Crick base pairs are rigid and mediated by long-residency water molecules while there are several modest cation-binding sites around SRD. In summary, SRD is an unusually stiff rRNA building block. Its intrinsic structural and dynamical signatures seen in simulations are strikingly distinct from other rRNA motifs such as Loop E and Kink-turns.

Molecular dynamics simulations were employed to investigate the structure, dynamics, and local base-pair step deformability of the free helix 44 from small ribosomal subunit *(Thermus thermophilus)* and of a canonical A-RNA double helix. It was shown that helix 44 is bent in the crystal structure of the small subunit while the simulated helix 44 is intrinsically straight with substantial instantaneous bends that are isotropic.

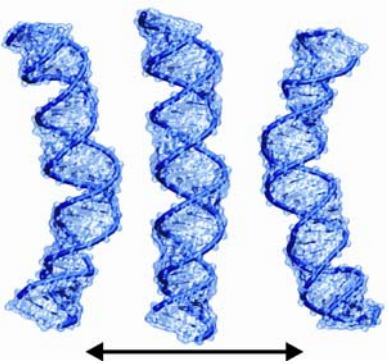

Fig. 2. Bending motion of helix 44 in the course of simulation. The duplex oscillates (left and right) around the averaged geometry (in the middle). The observed flexibility well correlates with the biological function.

The spontaneous motions seen in simulations achieve large degrees of bending seen in the crystal structure and would be entirely sufficient to allow the dynamics of the upper part of helix 44 evidenced by cryo-electron microscopic studies. Analysis of local base-pair step deformability reveals a patch of flexible steps in the upper part of helix 44 and in the area proximal to the bulge bases, suggesting that the upper part of helix 44 has enhanced flexibility. The simulations identify two conformational substates of the second bulge area (bottom part of the helix) with distinct base pairing. In agreement with nuclear magnetic resonance and x-ray studies, a flipped out conformational substate of conserved 1492A is seen in the first bulge area. Molecular dynamics simulations reveal a number of reversible α-γ backbone flips that correspond to transitions between two known A-RNA backbone families. The flipped substates do not cumulate along the trajectory and lead to a modest transient reduction of helical twist with no significant influence on the overall geometry of the duplexes. Despite their considerable flexibility, the simulated structures are very stable with no indication of substantial force field inaccuracies.

A comprehensive structural, evolutionary and molecular dynamics (MD) study of the G/U wobble basepairs in the ribosome was performed. These basepairs are classified according to their tertiary interactions, and sequence conservation at their positions is determined. G/U basepairs participating in tertiary interactions are more conserved than those lacking any interactions. Specific interactions occurring in the G/U shallow groove pocket-like packing interactions (P-interactions) and some phosphate backbone interactions (phosphate-in-pocket interactions)-lead to higher G/U conservation than others. Two salient cases of unique phylogenetic compensation are discovered. First, a P-interaction is conserved through a series of compensatory mutations involving all four participating nucleotides to preserve or restore the G/U in the optimal orientation. Second, a G/U basepair forming a P-interaction and another one forming a phosphate-in-pocket interaction are replaced by GNRA loops that maintain similar tertiary contacts. MD simulations were carried out on eight P-interactions.

Helices 90-92 of the 23S rRNA domain V were studied. Helices 90-92 are components of ribosomal A-site. Top of helix 92, called A-loop (5´-2587-2581-3´, numbering of *H.m.*: PDB code 1JJ2), is a segment of peptidyltranferase center and it may be crucial for the tRNA selection by ribosome. It is expected that the A-loop residuum (G2588) forms a regular Watson-Crick base pair with C75 of the incoming tRNA. In native rRNA structures a posttranscriptional 2´-O-ribose methylation of a residuum of the

A-loop (U2587) has been observed. It is supposed that the methylation can change a conformation of the A-loop. Helices 90-92 are classified as three-way-junction type C, containing two characteristic structural elements in the junction area: ribose zipper (two different A-minor motifs) and U-turn. Preliminary molecular dynamics (MD) study of this three-way-junction characterizes the movement of the system as the breathing of helices 90 and 92. Helix 91 is nested in the ribosome and surrounded by rRNA. It seems that breathing-like motion might be characteristic of this class of three-way-junctions.

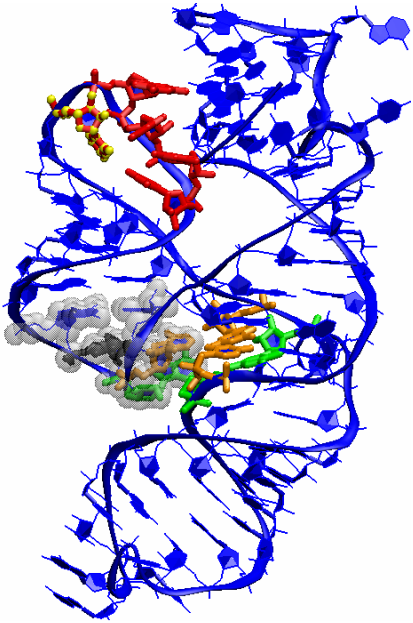

Fig. 3. X-ray structure of helices 90-92 (conserved bases of A-loop are in red). Residues of A-minor type I are in orange and residues of A-minor type II are in green. U-turn is in silver van der Waals representation, the methylated residue is shown as yellow.

Ribozymes are catalytically competent examples of highly structured noncoding RNAs, which are ubiquitous in the processing and regulation of genetic information. Using explicit-solvent molecular dynamics simulation, it was found that trapped long-residency water molecules are critical for catalytic core hydrogen bonding network in Hairpin ribozyme and only

occasionally exchange with bulk solvent as they pass through a breathing interdomain base stack. These highly structured water molecules line up in a string that may potentially also be involved in specific base catalysis. Our observations suggest important, still underappreciated roles for specifically bound water molecules in the structural dynamics and function of noncoding RNAs. Extensive simulations were also executed for Hepatitis Delta Virus ribozyme.

Titanocene derivatives exhibit high potential in the treatment of cisplatin resistant tumor types. We performed DFT calculations on the hydrated form of five drug candidates differing in the pendant arms attached to the aromatic rings. We found a qualitative correlation between the experimentally measured anticancer activity of alkylammonium-functionalized titanocene derivatives and the computed free energy change of the proton induced dissociation reaction of these compounds. The results indicate that differences in the cytotoxic activities could be related to the solvation properties of the protolysis products whereas no correlation was found with gas-phase properties of these molecules. Contrary to the free energy change of the protolysis reaction, other molecular properties, such as the geometrical parameters or the binding energies of the cyclopentadienyl rings in solution, do not correlate with the in vitro cytotoxic activity of these drug candidates. Our computational results suggest that more potent titanocene-based drug candidates may be obtained by improving the solubility of the protonated and substituted cyclopentadienyl rings.

Base-stacking energies in ten unique B-DNA base-pair steps and some other arrangements were evaluated by the quantum chemical (QM) second-order Moller-Plesset (MP2) method, complete basis set (CBS) extrapolation, and correction for triple (T) electron-correlation contributions. The CBS(T) calculations were compared with decade-old QM reference data and molecular mechanical AMBER force field. The new calculations show modest increases in stacking stabilization compared to the earlier benchmarks and surprisingly large sequence-dependent variation of stacking energies. The absolute force-field values are in better agreement with the new reference data, while relative discrepancies between quantum-chemical (QM) and force-field values increase modestly. Nevertheless, the force field provides good qualitative description of stacking, and there is no need to introduce additional pair-additive electrostatic terms, such as distributed multipoles or out-of-plane charges. The reference calculations are complemented by continuum-solvent assessment of solvent-screening effects.

Granted projects

GA CR 203/05/0388, Conformational dynamics of nucleic acids. Co-investigator: Jiří Šponer, 2005-2007

GA CR 203/05/0009, Structure and dynamics of DNA nitrogeneous bases, base pairs, oligonucleotides and their complexes with water, ions and drugs. Co-investigator: Jiří Šponer, 2005-2007

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

GR067507, Wellcome Trust International Senior Research Fellowship in Biomedical Science in Central Europe. Principal investigator: Jiří Šponer, 2003-2007

LC06030, **Biomolecular Center**; Ministry of Education of the Czech Republic; Principal investigator: V. Sklenář, Principal co-investigator: J. Šponer, 2006-2010

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ANALYSIS OF CHROMOSOMAL PROTEINS

HEAD

MICHAL ŠTROS

RESEARCH FELLOWS

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EVA POLANSKÁ

Discovery of a functional link between HMGB1 and telomeres

Mechanisms involved in regulation of maintenance of chromosome ends – telomeres – are subject to intensive investigation, namely due to the known links of telomere biology to the problems of genome stability, cancer and ageing. Numerous factors participating in regulation of telomere synthesis by the nucleoprotein complex of telomerase have been identified, including telomere- and telomerase-associated proteins, and structural transitions among multitude of possible telomere conformations (e.g., intra-strand and inter-strand tetraplex structures or telomeric loops).

Telomere function is apparently tightly linked to chromatin architecture, and analysis of contribution of other factors involved in chromatin dynamics is of special interest to current telomere biology. Many of the chromatin structural changes are mediated by a large and diverse superfamily of HMG (High Mobility Group) proteins, including the HMGB-type proteins (reviewed by Štros et al.: The HMG-box: a versatile protein domain occurring in a wide variety of DNA-binding proteins. *Cell. Mol. Life Sci.*, 2007, *submitted*). Recent reports indicated that lack of HMGB1 in mouse embryonic fibroblasts (MEFs) results in striking chromosomal instability (Giavara et al.: *Curr Biol.*, **15**, 68-72, 2005). In order to understand the mechanism of the chromosomal instability in HMGB1-deficient cells, we have studied the importance of HMGB1 for the telomeres structure (a collaborative project with the LDMC laboratory of Dr. J. Fajkus). We have demonstrated that knockout of *HMGB1* gene could bring about ~5-fold decrease in telomerase activity, general shortening of telomere length, and increased frequency of chromosomal aberrations in MEFs (Fig. 1). Rare cases of extremely expanded telomeres could also be

observed in *HMGB1*$^{-/-}$ MEFs, with no apparent changes in telomere chromatin structure and quantity of G-strand overhang-specific signals.

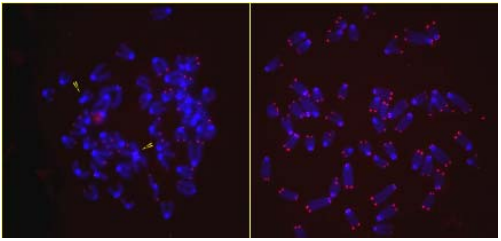

Fig. 1. Increased chromosomal instability in HMGB1-deficient mouse embryonic cells (MEFs). Fluorescence *in situ* hybridization of metaphase spreads from mouse wild-type (*HMGB1*$^{+/+}$) and *HMGB1*$^{-/-}$ MEFs. Telomeres (red) were detected with Cy3 labeled PNA probe, and DNA stained with DAPI (blue). Arrows indicate chromosomal abnormalities in *HMGB1*$^{-/-}$ MEFs. Parental and *HMGB1*$^{-/-}$ MEFs were kindly provided by M.E. Bianchi (Milano, Italy).

Higher activity of telomerase in *HMGB1*$^{+/+}$ MEFs may be a consequence of a direct physical interaction of HMGB1 with protein component of telomerase mTERT (as revealed by *in vitro* "pull-down" assay, Fig. 2), rather than due to *de novo* synthesis of the enzyme, as HMGB1 does *not* up-regulate the expression of *mTERT* in MEFs.

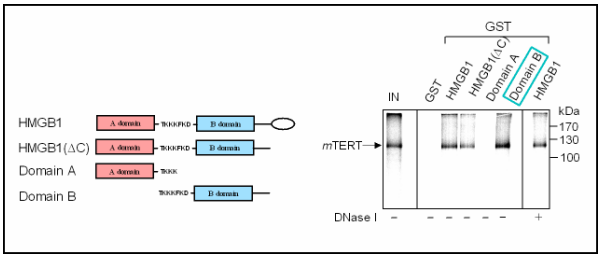

Fig. 2. HMGB1 interacts with mouse telomerase protein catalytic subunit (mTERT) *via* the HMG-box B-domain. HMGB1 and truncated forms were pre-bound to glutathione Sepharose beads. These beads were then incubated with [^{35}S]-labeled mTERT ("*in vitro* translation" in reticulocyte lysate). The Sepharose-bound proteins were resolved by PAAG electrophoresis, transferred on to the membrane and subjected to autoradiography. Input, 10% of the [^{35}S]-labeled mTERT used for the pull-down assay. Some PD experiments were also performed in the presence of 10 units of DNAse I in the incubation buffer as indicated.

Discovery of chromosomal abnormalities (Giavara et al.: *Curr Biol.*, **15**, 68-72, 2005) and telomere dysfunctions in HMGB1-deficient cells (Kunicka et al: HMGB1 protein associates with mammalian telomerase catalytic subunit

and stimulates its cellular activity. *Nucleic Acids Res.*, 2007, *submitted*) strongly suggests that HMGB1 protein is necessary for maintaining genomic stability. Our novel finding on telomere dysfunctions in HMGB1-deficient cells may be of interest for cancer research community in order to clarify whether some aspects of chromosomal instabilities in malignant cells might have originated from aberrant expression of HMGB1 frequently found in cancer cells.

Construction of phylogenetic tree of human proteins containing HMG-box domains

In the frame of the project to identify all known human proteins containing HMG-box domains and to complete an invited review paper on this subject (Štros et al.: The HMG-box: a versatile protein domain occurring in a wide variety of DNA-binding proteins. C*ell. Mol. Life Sci.*, 2007, *submitted*), we have constructed an amino acid sequence similarity tree to reveal a sequence relationship between the human HMG-box proteins. We have used amino acid sequence of the human HMGB1 HMG-box di-domain (A and B domain, aa 1-185) as query to identify HMG-box proteins in the human protein database (http://www.ebi.ac.uk/swissprot/) using BLASTP. The search revealed a total of 47 amino acid sequences of 15-193 kDa, exhibiting sequence similarity to the HMG-box domains of the human HMGB1 protein. From the tree in Fig. 2 follows that the human HMG-box proteins can be subdivided into seven groups. The first two groups contain variable numbers of HMG-boxes, whereas all other groups contain proteins with a single HMG-box. Group 1 consists of HMG-box proteins containing two HMG-boxes with the exception of a single HMG-box protein HMG4L of unknown function. This group contains HMGB-type proteins with two HMG-boxes including HMGB1-3, transcriptional activator/or repressor SP100, and HMG1L10 of unknown function. Group 2 consists of five proteins related to the group 1. The transcription factors TCF/LEF-1 (a group of proteins that regulate gene expression during cell differentiation) form group 3. Group 4 includes chromatin remodeling factors HMG20B, BRAF35 (also termed HMG20A), mismatch repair protein PMS1 and SOX-related protein BBX (bobby SOX homolog of a *Drosophila* protein). Group 5 represents the TOX family (T-lymphocyte specific factors) proteins implicated in the regulation of thymocyte selection. The sex-determining factor SRY and the SOX (SRY-related) proteins are arranged into group 6. These proteins can be further divided into several distinct subgroups: A (SRY), B1 (SOX1,2,3) and B2 (SOX14,21), C (SOX4,11,12), D (SOX5,6,13), E (SOX8,9,10), F (SOX7,17,18), G (SOX15) and H

(SOX30). Group 7 includes diverse HMG-box proteins: a SOX-related CIC protein, chromatin remodeling factors BAF57 and PB1, a suppressor of Wnt signaling HBP1 and protein WHSC1 (possibly involved in Wolf-Hirschhorn malformation syndrome).

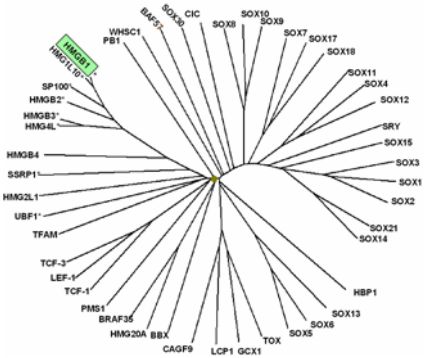

Fig. 3. Unrooted phylogenetic tree of human HMG-box proteins. The amino acid sequence of human HMGB1 AB di-domain (aa 1-185) was used as query to identify similar human HMG-box proteins in the Swiss-Prot database (http://www.ebi.ac.uk/swissprot/) using the protein-protein BLAST program (http://130.14.29.110/BLAST). The retrieved sequences were aligned by multiple sequence alignment that was used to construct the amino acid sequence similarity tree using the ClustalX software (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html). Asterisks mark sequences containing, in addition to the variable number of HMG-boxes, also the acidic C-terminal domains. The canonical HMGB1 protein is in green.

Granted projects

GA CR 204/05/2031, Understanding of HMGB1 involvement in functioning of tumor suppressor proteins and telomers. Principal investigator: M. Štros, 2005-2007

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

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CD Spectroscopy of Nucleic Acids

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Ethanol is a better inducer of DNA guanine tetraplexes than potassium cations

Guanine tetraplexes are biologically relevant alternatives of the Watson and Crick duplex of DNA. They play many important regulatory roles in eukaryotic cells. It is thought that potassium or other cations present in the cavity between consecutive guanine tetrads are an integral part of the tetraplexes. We have, however, shown using CD spectroscopy that ethanol induces the guanine tetraplexes like or even better than potassium cations (Fig. 1). There are many examples when ethanol stabilizes guanine tetraplexes with fragments when potassium cations fail to do so. Hence besides the A-form or Z-form, ethanol stabilizes another conformation of DNA, i. e. the guanine tetraplexes. The three conformers share the property of having a much better contact of their bases with the environment than the B-form, which is stabilized by water. Hence perhaps the lowered water activity and the enhanced base-solvent interactions stand behind the stabilization of the three DNA arrangements. The use of ethanol permits studies of guanine tetraplexes that cannot be induced by potassium cations or other tetraplex-promoting agents. The work demonstrates that still a broader spectrum of nucleotide sequences can fold into guanine tetraplexes than it has so far been thought. Moreower, aqueous ethanol solutions better simulate overcrowded environment existing in cells than aqueous solutions.

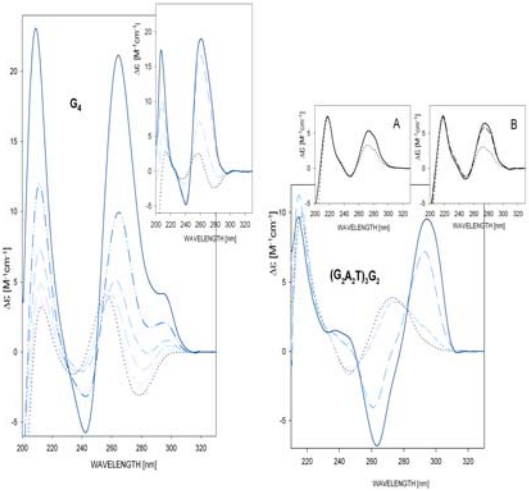

Fig. 1: CD spectra reflecting ethanol-induced tetraplex formation. Left: (GGGG): In 1mM Na phosphate (dotted line) no ordered structure is formed. Adding ethanol gives rise to a parallel stranded tetraplex, which is characterized by the huge positive band at 260 nm. The same tetraplex is formed in KCl-containing aqueous solution (insert). Right: $(GGAAT)_3G_2$: Adding ethanol to the oligonucleotide, which is a random singlestrand in 1 mM Na phosphate (dotted line) gives rise to an antiparallel tetraplex characterized by a CD spectrum with a positive and negative bands at 295 and 263 nm, respectively. Both, NaCl (insert A) or KCl (insert B) stabilize homoduplexes instead.

Modified bases contribute to a better understanding of the unusual conformations of the alternating guanine-adenine repeat strands of DNA

Purines are supposed to be the first bases existing in prebiotic nucleic acids. We have recently shown that guanines are a key constituent of single-, double- or four-stranded ordered structures, which may be relevant to an emergence of the first replicators on the Earth. Alternating guanine-adenine strands of DNA are known to self-associate into a parallel-stranded homoduplex at neutral pH, fold into an ordered single-stranded structure at acid pH, and adopt yet another ordered single-stranded conformer in aqueous ethanol. The unusual conformers melt cooperatively and exhibit distinct CD spectra suggestive of a substantial conformational order, but their molecular structures are not known yet. We have probed the molecular structures using guanine and adenine analogs lacking the N_7 atom, and thus unable of Hoogsteen pairing, or those restrained in the less frequent syn glycosidic orientation. The studies showed that the syn glycosidic

orientation of dA residues promoted the neutral homoduplex whereas the syn orientation of dG was incompatible with the homoduplex. In addition, Hoogsteen pairing of dA seemed to be a crucial property of the homoduplex whereas dG did not pair in this way. The situation was the same in both single-stranded conformers with the dG residues. On the other hand, the presence of N_7 was important with dA but its syn geometry was not favorable. The present data can be used as restraints to model the unusual molecular structures of the alternating guanine-adenine strands of DNA. These studies are in progress.

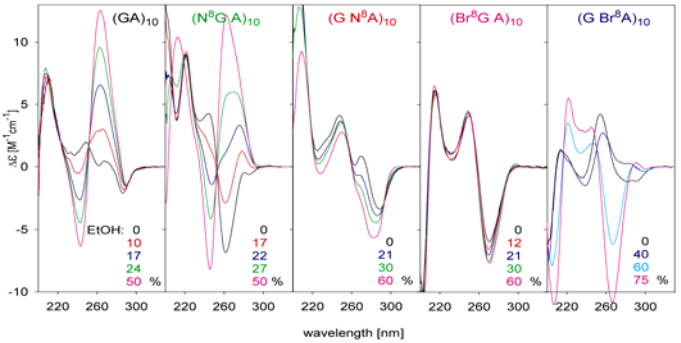

Fig. 2: CD spectra reflecting distinct structural behaviour of the studied $(GA)_{10}$ analogs induced by ethanol.

The thrombin binding aptamer forms a bimolecular tetraplex

Aptamers are nucleic acid molecules that specifically bind to various biologically important molecules. As such they represent a potential tool for therapeutic purposes. One of the most intensely studied aptamers is the DNA 15-mer GGTTGGTGTGGTTGG that specifically binds to thrombin and inhibits thrombin activity in the cascade of reactions resulting in blood clotting. Aptamer structural properties are important for optimizing its binding to the target molecule.

In the literature the thrombin binding aptamer is generally taken as a prototype of an intramolecular guanine tetraplex of DNA. Our results, however, show that this notion is not correct in aqueous solutions. This conclusion is based on a dependence of the CD spectra on the aptamer concentration, migration of the aptamer in polyacrylamide gels (Fig. 3, left), and the Ferguson analysis of the gel migration data. The obtained results document that the aptamer forms a bimolecular tetraplex in aqueous

solution (Fig. 3, right). We furthermore show that only an extension of the aptamer by a sequence containing further guanines, or an elongation of loop regions, cause that its tetraplex folding is intramolecular (Fig. 3, right).

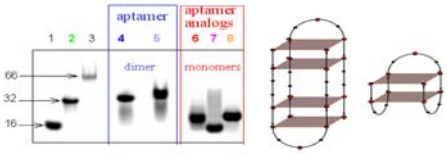

Fig. 3: (left) Native polyacrylamide gel electrophoresis run in the presence of K^+ ions. Lanes 1-3, markers, lanes 4 and 5, thrombin binding aptamer $G_2T_2G_2TGTG_2T_2G_2$ loaded at 1 mM and 0.06 mM nucleoside concentration, lane 6, 21-mer **GTAGGT**$G_2T_2G_2TGTG_2T_2G_2$, lane 7, the 19-mer G_2T_2**T**G_2T**T**GT**T**G_2T_2**T**G_2, lane 8, the 17-mer G_2T_2**A**$G_2TTAG_2T_2$**A**G_2. (right) the bimolecular model of the thrombin aptamer and the intramolecular model of their analogs. The red balls are guanines, black ones thymines.

Granted projects

GA AS CR A4004201, Tetraplexes of DNA and Their Occurrence in the Human Genome. Principal Investigator: M. Vorlíčková, 2002 – 2006

Publications

Vorlíčková, M., Bednářová, K., Kypr, J.: *Ethanol is a better inducer of DNA guanine tetraplexes than potassium cations.* Biopolymers, 82 , 2006, 253-260.

Fialová, M., Kypr, J., Vorlíčková, M.: *The thrombin binding aptamer GGTTGGTGTGGTTGG forms a bimolecular guanine tetraplex.* Biochem. Biophys. Res. Commun., 344, 2006, 50-54.

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PLANT DEVELOPMENTAL GENETICS

HEAD

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MARTINA KAŠÍKOVÁ

A majority of plant species are cosexuals forming male (stamens) and female (pistils) sexual organs in each flower. About 5% of species are strictly dioecious and form unisexual flowers, either male or female, on different individuals. A high number of plant species represent intermediate stages, i.e., different forms of flowers are present on one individual (e.g. monoecy) or sexually different individuals occur in populations of plants (e.g. gynoecy). It is well documented that these sexual forms also represent intermediate steps in the evolution of sexuality. Similarly as in animals, there are two basic mechanisms of sex determination in plants: genetic and environmental (hormonal). Among the dioecious species, only a few of them have evolved heteromorphic sex chromosomes, especially white campion (*Silene latifolia*) and common sorrel (*Rumex acetosa*). In these two classical species, different sex chromosome-based mechanisms have been described: white campion has the male dominant chromosome Y (the

mammalian type of sex determination), while in sorrel the sexuality is controlled by a ratio between the number of X chromosomes and the number of autosomal sets (the drosophila system). Recent molecular analyses show that the plant sex chromosomes are evolutionarily much younger compared with the sex chromosomes in animal species. This fact makes them the optimum models to study early stages of sex chromosome evolution. In our lab research efforts are focused on structure, evolution, and function of plant sex chromosomes.

The aim of this work was to isolate new DNA markers linked to the *Silene latifolia* Y chromosome.To do this we created a chromosome-specific plasmid library after DOP-PCR amplification of laser-microdissected Y-chromosomes. The library screening led to the isolation of several clones yielding mostly to exclusive male specific hybridization signals. Subsequent PCR confirmed the Y-unique linkage for one of the sequences. This DNA sequence called MK17 has no homology to any known DNA sequence and it is absent in related gynodioecious and hermaphroditic species. The mapping analysis using a panel of deletion mutants showed that MK17 is closely linked to the region controlling suppression of gynoecium development. Hence MK17 represents a valuable marker to isolate genes controlling the gynoecium development suppression on the Y chromosome of *S. latifolia*.

We have also found a pair of novel Ty3/gypsy retrotransposons in *S. latifolia*, consisting of a non-autonomous element *Retand-1* (3.7 kb) and its autonomous partner *Retand-2* (11.1 kb). These two elements have highly similar long terminal repeat (LTR) sequences but differ in the presence of the typical retroelement coding regions (*gag-pol* genes), most of which are missing in *Retand-1*. Moreover, *Retand-2* contains two additional open reading frames in antisense orientation localized between the *pol* gene and right LTR. *Retand* transcripts were detected in all organs tested (leaves, flower buds and roots) which, together with the high sequence similarity of LTRs in individual elements, indicates their recent transpositional activity. The autonomous elements are similarly abundant (2,700 copies) as non-autonomous ones (2,100 copies) in *S. latifolia* genome. *Retand* elements are also present in other *Silene* species, mostly in subtelomeric heterochromatin regions of all chromosomes. The only exception is the subtelomere of the short arm of the Y chromosome in *S. latifolia*, which is known to lack the

terminal heterochromatin. An interesting feature of the *Retand* elements is the presence of a tandem repeat sequence, which is more amplified in the non-autonomous *Retand-1*.

We have also characterized a novel tandem repeat called TRAYC, which has accumulated on the Y chromosome in *S. latifolia*. Its presence demonstrates that processes of satellite accumulation are at work even in this early stage of sex chromosome evolution. The presence of TRAYC in other species of the *Elisanthe* section suggests that this repeat had spread after the sex chromosomes evolved but before speciation within this section. TRAYC possesses a palindromic character and a strong potential to form secondary structures, which could play a role in satellite evolution. TRAYC accumulation is most prominent near the centromere of the Y chromosome. We propose a role for the centromere as a starting point for the cessation of recombination between the X and Y chromosomes. In collaboration with ETH University of Zurich we have also isolated a BAC7H5 clone preferentially hybridizing to the Y chromosome of *S. latifolia*. Sequence analysis revealed that this BAC7H5 contains part of the chloroplast genome, indicating that these chloroplast sequences have accumulated on the Y chromosome and also may contribute to its large size. We constructed Y chromosome- and X chromosomespecific libraries and screened them to find Y- and/or X-linked copies of chloroplast sequences. Sequence analysis revealed higher divergence of a non-genic region of the chloroplast sequences located on the Y chromosome while genic regions tested showed only very low (max 0.9%) divergence from their chloroplast homologues.

Most dioecious plant species are believed to derive from hermaphrodite ancestors. The regulatory pathways that have been modified during evolution of the hermaphrodite ancestors and led to the emergence of dioecious species still remain unknown. To identify the molecular mechanisms involved in female organ suppression in male flowers of *S. latifolia*, we looked for genes potentially involved in the establishment of floral organ and whorl boundaries. We identified homologs of *Arabidopsis thaliana SHOOTMERISTEMLESS* (*STM*) and *CUP SHAPED COTYLEDON* (*CUC*)*1* and *CUC2* genes in *S. latifolia*. Our phylogenetic analyses suggest that we identified true orthologs for both types of genes. Detailed expression analyses showed a conserved expression pattern for these genes between *S. latifolia* and *A. thaliana*, suggesting a conserved

function of the corresponding proteins. Comparative *in situ* hybridization experiments between male, female, and hermaphrodite individuals reveal that these genes show a malespecific pattern of expression before any morphological difference become apparent. Our results make *SlSTM* and *SlCUC* strong candidates for being involved in sex determination in *S. latifolia*. This research has been realized in collaboration with ENS de Lyon.

The genus *Silene* is a good model for studying evolution of the sex chromosomes, since it includes species that are hermaphroditic and dioecious, while maintain a basic chromosome number of $2n = 24$. For some combinations of *Silene* species it is possible to construct interspecific hybrids. Here, we present a detailed karyological analysis of a hybrid between the dioecious *Silene latifolia* as the maternal plant and a related species, hermaphroditic *Silene viscosa*, used as a pollen partner. Using genomic probes (the genomic *in situ* hybridization (GISH) technique), we were able to clearly discriminate parental genomes and to show that they are largely separated in distinct nuclear domains. Molecular GISH and fluorescence *in situ* hybridization (FISH) markers document that the hybrid genome of somatic cells was strictly additive and stable, and that it had 12 chromosomes originating from each parent, including the only X chromosome of *S. latifolia*. Meiotic analysis revealed that, although related, respective parental chromosomes did not pair or paired only partially, which resulted in frequent chromosome abnormalities such as bridges and irregular nondisjunctions. GISH and FISH markers clearly document that the larger genome of *S. latifolia* and its largest chromosome component, the X chromosome, were mostly employed in chromosome lagging and misdivision.

Stability and integrity of nuclear genome are studied in collaboration with the Gregor Mendel Institute in Vienna and Texas A&M University in Texas. The maintenance of the cell nucleus integrity is a vital condition for both errorless function of somatic cell and transmission of genetic information into generative cells. Cell-external and/or -internal factors can induce double-strand breaks in DNA, which may lead to nuclear destabilisation *via* rearrangements of chromosomes or eventual loss of genetic information. Eukaryotic cells have evolved a number of mechanisms to repair the double-strand breaks. They are repaired by

homologous recombination or non-homologous end joining (NHEJ). When broken chromosome ends are fused together by NHEJ, the resulting dicentric chromosomes can be detected as anaphase bridges during the subsequent mitosis. Telomeres in the absence of functional telomerase shorten, became unprotected, and are eventually recognized by the cell repair system as double stranded breaks. As result, chromosomes of *Arabidopsis thaliana* plants that are deficient in the gene for telomerase reverse transcriptase (TERT) are prone to chromosome fusions. We use *Arabidopsis tert* mutants as a model system for analyzing terminal chromosome fusions. We have developed a novel and sensitive cytogenetic assay for the identification and characterization of chromosome-terminal fusion events by employing fluorescence *in situ* hybridization (FISH) with multiple probes and a repeated hybridization approach. A mixture of chromosome-spoecific subtelomeric probes is applied successively in three FISH reactions to the slides containing mitotic anaphase figures with anaphase bridges. Each figure is registred by a CCD camera after each *in situ* hybridization procedure. By comparing the signals presented on the bridge in successive images the assessment of the particular chromosome arms involved in fusion is possible. This experimental setup enables unambiguous identification of individual chromosome ends employed in fusion events.

Granted projects

GA AS CR A6004304, Epigenetic consequences of telomere dysfunction in Arabidopsis thaliana. Principal investigator: B. Vyskot, 2003 - 2006

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

GA CR 521/05/2076, Studies on X- and Y-chromosome differentiation. Principal investigator: B. Janoušek, 2005 - 2007

GA CR 204/05/2097, Roles of repetitive DNA sequences in evolution of the sex chromosomes of *Silene latifolia*. Principal investigator: E. Kejnovský, 2005 - 2007

GA CR 522/03/0354, Cytogenetic study of nuclear chromatin in plants.Principal investigator: J. Široký, 2003 - 2005

GA CR 204/05/P505, Mechanisms of sex chromosome evolution. Principal investigator: J. Žlůvová, 2005 – 2007

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

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

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

Publications

Hobza, R., Hrušáková, P., Šafář, J., Bartoš, J., Janoušek, B., Žlůvová, J., Michu, E., Doležel, J., Vyskot, B.: *MK17, a specific marker closely linked to the gynoecium suppression region on the Y chromosome in Silene latifolia.* Theoretical and Applied Genetics, 113, 2006, 280-287.

Hobza, R., Lengerová, M., Svoboda, J., Kubeková, H., Kejnovský, E., Vyskot, B.: *An accumulation of tandem DNA repeats on the Y chromosome in Silene latifolia during early stages of sex chromosome evolution.* Chromosoma, 115, 2006, 376-382.

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Vyskot, B.: *Přenos epigenetické informace v liniích buněk somatické a zárodečné dráhy.* In: Molekulární biologie a genetika XII, Jonák, J., jun., Jonák, J. (eds.), Ústav molekulární genetiky AV ČR, Praha, 2006, p. 7-21 (ISBN: 80-902588-5-9)

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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

6. FP EU, LSHG-CT-2003-503441, 3D Genome structure and function. Principal investigator: R. van Driel, Co-principal investigator: S. Kozubek, 2004-2006

NIH, 1R01CA78754, Mechanistic studies on new platinum clinical agents. Principal investigator: V. Brabec, 2005 - 2007

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

The Wellcome Trust, 073646/Z/03/Z, Platinum and ruthenium complexes. From DNA damage to cancer chemotherapy Principal investigator: V. Brabec, 2004 – 2007

The Wellcome Trust, GR067507, Wellcome Trust International Senior Research Fellowship in Biomedical Science in Central Europe. Principal investigator: Jiří Šponer, 2003-2007

Prestigious National Projects

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

ME, 1M0021622409, Center of Applied Research, Stomatological research center. Principal investigator: J. Vaněk, 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

ME, LC06004, Center of Basic Research, Integrated research of the plan genome. Principal investigator: B. Vyskot

ME, LC06030, Center of Basic Research, Biomolecular Center. Co-principal investigators: V. Brabec, J. Šponer

ME, LC06027, Center of Basic Research for Monoclonac Gamapathy and Multiple Myeloma. Co-principal investigator: E. Bártová

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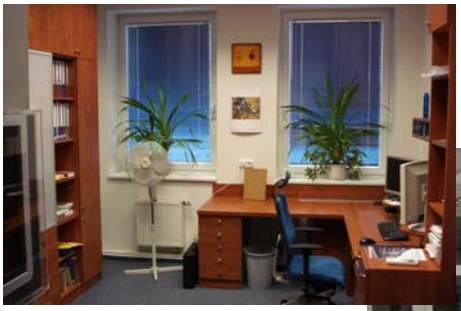
Standard services of the Center of Information Technologies (CIT) include maintenance of the local area network (LAN), the connection of the IBP LAN to Brno Academic Computer Network and to the Internet, maintenance of exchange and IP telephony, maintenance of the IBP e-mail server, including antivirus and antispam systems, maintenance of the IBP web server including design and data update, development and maintenance of computer hardware and software commonly used by all laboratories (servers, graphic workstations, PCs with Internet access) running under UNIX, MS Windows NT/2000/XP or MS Windows 95/98/ME. 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 2006 web pages of the IBP was completely redesigned. There were added new services: file depository – a web based tool for easy transfer of large amounts of data (up to 100 MB) over the network (http://www.ibp.cz/CIT/); reservation system – a web based tool for booking rooms like auditorium for lectures and instruments of common use (http://www.ibp.cz/local/rezervace.php).

RECONSTRUCTION OF THE INSTITUTE

In 2006, most laboratories and offices of the Institute have been reconstructed. In addition, the office of the director and management of the Institute were also renewed.



The 2nd European Workshop on the Analysis of Phagocyte Functions

was organised by the Laboratory of Free Radical Pathophysiology. The workshop was held at the Congress Centre of the Mendel University of Agriculture and Forestry at Křtiny from 15 to 17 June 2006. 25 delegates came from 6 countries including Finland, Spain, Italy, Hungary, Slovakia and Czech Republic. The workshop consisted of short oral presentations of all participants and fruitful discussion.

The main meeting topics were:

- Biology of phagocytes (enzymes of phagocytes, cooperation of phagocytes with other cell types, etc.)
- Phagocytes in ischemia/reperfusion, trauma and transplantation
- Pharmacological modulation of phagocyte functions
- Methodology for evaluation of phagocyte functions

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