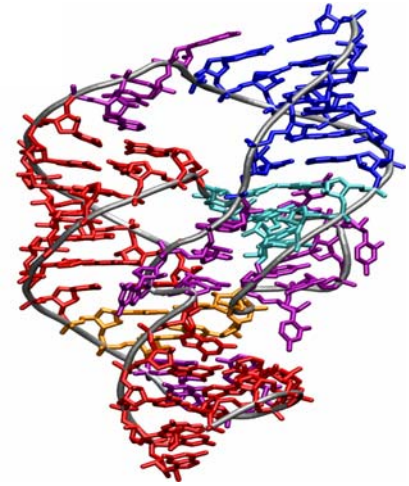
INSTITUTE OF BIOPHYSICS

ACADEMY OF SCIENCES OF THE CZECH REPUBLIC



RESEARCH REPORT 2005

IBP AS CR, BRNO 2005

Contents

INTRODUCTION

This was the first year of the new Institutional Research Plan (IRP) entitled „Biophysics of dynamic structures and functions of biological system“ which was approved by Academic Evaluation Committee towards the end of 2004. Owing to the positive outcome of the evaluation in which we were the best in the life sciences category, funding of the Institute was increased and now it exceeds 2 mil €/year. Approximately the same amount of finances was obtained from national and international grant agencies or other sources.

The Institute was very successful in grant applications, particularly in the National Program of Centres of Basic Research. In life sciences, we participate in five Centers with the total number of twenty Centres in the Czech Republic. Several world-class scientists work in the Institute, lead the best laboratories and create competitive atmosphere among the groups. The Institute has a very favorable reputation in the region.

In continuation of the evaluation of the Institute and in order to preserve a good level of science, the management of the Institute in collaboration with Scientific Board developed an internal evaluation algorithm based on scientometry. The quality of research was estimated using the number of citations of our articles and the impact factors of journals with our publications. The results of the second internal evaluation in 2006 will be used to modify the financial support of individual laboratories from institutional sources.

In order to achieve faster economical development in the Czech Republic, EU will support research and innovation in some regions including Brno. IBP suggested to use the Structural funds (SF) for the creation of Mendel Research Center (MRC). MRC will be a large center consisting of several institutions, Institute of Biophysics, Institute of Analytical Chemistry, Institute of Scientific Instruments AS CR, Masaryk University (Faculty of Sciences, Faculty of Medicine and Faculty of Informatics) and Mendel University. The Center will be devoted to modern biology with a number of core facilities used transregionally and will concentrate to the scientific excelence, innovations and postgradual education.

MRC is planed as an International institute which will be not only open for scientists from abroad but the conditions will be competitive for cutting edge scientists. The potential of about 40 research groups of the region will be used to create new research capacities with top-level instrumentation and to attract scientists from abroad.

Stanislav Kozubek

MOLECULAR BIOPHYSICS AND PHARMACOLOGY

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Chiral differentiation of DNA adducts formed by enantiomeric analogues of antitumor cisplatin is sequence-dependent

1,2-GG intrastrand cross-links formed in DNA by the enantiomeric complexes [$PtCl_2$(R,R-2,3-diaminobutane (DAB))] and [$PtCl_2$(S,S-DAB)] were studied by biophysical methods. Molecular modeling revealed that structure of the cross-links formed at the TGGT sequence was affected by repulsion between the 5'-directed methyl group of the DAB ligand and the methyl group of the 5'-thymine of the TGGT fragment. Molecular dynamics simulations of the solvated platinated duplexes and our recent structural data indicated that the adduct of [$PtCl_2$(R,R-DAB)] alleviated this repulsion

by unwinding the TpG step, whereas the adduct of [$PtCl_2$(S,S-DAB)] avoided the unfavorable methyl-methyl interaction by decreasing the kink angle (Fig. 1). Electrophoretic retardation measurements on DNA duplexes containing 1,2-GG intrastrand cross-links of Pt(R,R-DAB)$^{2+}$ or Pt(S,S-DAB)$^{2+}$ at a CGGA site showed that in this sequence both enantiomers distorted the double helix to the identical extent similar to that found previously for the same sequence containing the cross-links of the parent antitumor (cisplatin). In addition, the adducts showed similar affinities toward the high-mobility-group (HMG) box 1 proteins. Hence, whereas the structural perturbation induced in DNA by 1,2-GG intrastrand cross-links of cisplatin does not depend largely on the bases flanking the cross-links, the perturbation related to GG cross-linking by bulkier platinum diamine derivatives does.

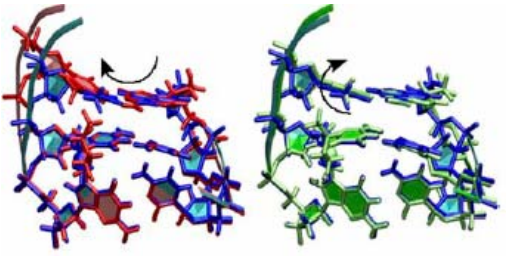

Fig 1. Unwinding of the TpG step stabilized by an $\alpha\gamma$ transition (A); the adduct of [$PtCl_2$(S,S-DAB)] avoided the unfavorable methyl-methyl interaction by decreasing the kink angle (B)

TGG fragments of models for the duplex containing TGGT sequence cross-linked with Pt(R,R-DAB)$^{2+}$ (red, left) and Pt(S,S-DAB)$^{2+}$ (green, right), both superimposed with the model for the adduct with *cis*-Pt$(NH_3)_2^{2+}$ (blue). Structures were averaged over the production period of the MD simulations and subsequently energy-minimized using 1000 cycles of the conjugate gradient minimizer of AMBER6.0. The structures were least-squares fitted using the Pt-G*pG* residue, which is nearly invariant in all three adducts. The arrows indicate how the two Pt(DAB)$^{2+}$ adducts avoid the methyl methyl clash.

DNA adducts of the enantiomers of the platinum(II) complexes of the ahaz ligand (ahaz = 3-aminohexahydroazepine) and recognition of these adducts by HMG domain proteins

The bending, unwinding, and structural changes in DNA caused by the binding of each of the enantiomers of the platinum(II) complexes of the ahaz ligand (R- and S-[$PtCl_2$(ahaz)], ahaz = 3-aminohexahydroazepine) have been studied using 20–23 bp oligonucleotides containing TGGT and CGGA-binding sites as has the recognition of the adducts by HMG domain proteins. The domain A of HMGB1 (HMGB1a protein) binds to the adduct formed by the R enantiomer at the CGGA sequence with a similar high affinity as it does to the adduct of antitumor cisplatin, and to the adduct formed by the S enantiomer with a slightly lower affinity. In contrast, HMGB1a binds much more weakly to the ahaz adducts than to the cisplatin adducts formed at the TGGT sequence, with the binding to the adduct formed by the R enantiomer being weakest. Each enantiomer and cisplatin cause unwinding of both sequences that is in the narrow range, 19–22°. There are modest but significant differences in the degree of bending induced, with the S enantiomer causing the least bending, cisplatin intermediate, and the R enantiomer the most. Molecular modeling of the {Pt(ahaz)}/GG adducts in 8-bp models reveals significant differences in the local distortion at the GG-binding sites depending on the flanking bases and shows that interactions between the thymine methyl groups and the ahaz ligand are likely to inhibit bending of the TGGT sequence.

Conformation, protein recognition and repair of DNA interstrand and intrastrand cross-links of antitumor trans-[$PtCl_2(NH_3)$(thiazole)]

Replacement of one ammine in clinically ineffective *trans*-[$PtCl_2(NH_3)_2$] (transplatin) by a planar N-heterocycle, thiazole, results in significantly enhanced cytotoxicity. Unlike classical' cisplatin {*cis*-[$PtCl_2(NH_3)_2$]} or transplatin, modification of DNA by this prototypical cytotoxic transplatinum complex *trans*-[$PtCl_2(NH_3)$(thiazole)] (trans-PtTz) leads to monofunctional and bifunctional intra or interstrand adducts in roughly equal proportions. DNA fragments containing site-specific bifunctional DNA adducts of trans-PtTz were prepared. The structural distortions induced in DNA by these adducts and their consequences for HMG protein

recognition, DNA polymerization and nucleotide excision repair were assessed in cell-free media by biochemical methods. Whereas monofunctional adducts of trans-PtTz behave similar to the major intrastrand adduct of cisplatin [J. Kasparkova, O. Novakova, N. Farrell and V. Brabec (2003) Biochemistry, 42, 792-800], bifunctional cross-links behave distinctly differently. The results suggest that the multiple DNA lesions available to trans-planaramine complexes may all contribute substantially to their cytotoxicity so that the overall drug cytotoxicity could be the sum of the contributions of each of these adducts. However, acquisition of drug resistance could be a relatively rare event, since it would have to entail resistance to or tolerance of multiple, structurally dissimilar DNA lesions.

Structural characterization and DNA interactions of new cytotoxic transplatin analogues containing one planar and one nonplanar heterocyclic amine ligand

trans-Diaminedicholoroplatinum(II) complexes with one planar and one non-planar heterocyclic amine ligand were designed as new potential antitumor drugs. The X-ray crystallographic structures of *trans*-[$PtCl_2$(4-picoline)(piperidine)] and *trans*-[$PtCl_2$(4-picoline)(piperazine)]·HCl revealed that the piperidine and piperazine ligands bind to the platinum through the equatorial position and that the ligands adopt the chair conformation. The nonplatinated amine of the piperazine can form hydrogen bonds with atoms that are approximately 0.75 nm away from the Pt binding site. DNA is considered a major pharmacological target of platinum compounds. Hence, to expand the database correlating structural features of platinum compounds and DNA distortions induced by these compounds, which may facilitate identification of more effective anticancer platinum drugs, we describe the DNA binding mode in a cell-free medium of *trans*-[$PtCl_2$(4-picoline)(piperidine)] and *trans*-[$PtCl_2$(4-picoline)(piperazine)]·HCl. Interestingly, the overall impact of the replacement of the second ammine group in transplatin by the heterocyclic ligands appears to change the character of the global conformational changes induced in DNA towards that induced by cisplatin. The clinical ineffectiveness of the parent transplatin has been proposed to be also associated with its reduced

capability to form bifunctional adducts in double-helical DNA. The results of the present work support the view that replacement of both ammine groups of transplatin by heterocyclic ligands enhances cytotoxicity probably due to the marked enhancement of the stability of intrastrand cross-links in double-helical DNA.

Structure and unique interactions with DNA of a cationic trans-platinum complex with the nonplanar bicyclic piperidinopiperidine ligand

We described a new type of platinum complex that has a trans configuration, is asymmetric, has a singly charged nonplanar semifluxional bicyclic ligand, that binds extremely rapidly to DNA by direct substitution (not requiring initial aquation as does cisplatin), distorts DNA in a unique manner and circumvents all of the known cisplatin resistance mechanism in human ovarian cancer cell lines (Fig. 2).

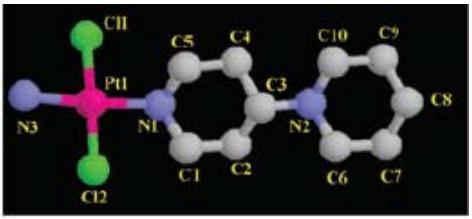

Fig. 2. The X-ray crystal structure of the complex *trans*-[$PtCl_2(NH_3)$(pip-pip)]·HCl

Effects of monofunctional adducts of platinum(II) complexes on thermodynamic stability and energetics of DNA duplexes

Effects of adducts of [$PtCl(NH_3)_3$]Cl or chlorodiethylenetriamine-platinum(II) chloride on DNA stability were studied with emphasis on thermodynamic origins of that stability. Oligodeoxyribonucleotide duplexes (15-bp) containing the single, site-specific monofunctional adduct at G-residues of the central sequences TGT/ACA or 5'-AGT/ 5'-ACT were prepared and analyzed by differential scanning calorimetry, temperature-

dependent ultraviolet absorption and circular dichroism. The unfolding of the platinated duplexes was accompanied by relatively small unfavorable free energy terms. This destabilization was enthalpic in origin. On the other hand, a relatively large reduction of melting temperature (T_m) was observed as a consequence of the monofunctional adduct in the TGT sequence, whereas T_m due to the adduct in the AGT sequence was reduced only slightly. We also examined the efficiency of the mammalian nucleotide excision repair system to remove from DNA the monofunctional adducts and found that these lesions were not recognized by this repair system. Thus, rather thermodynamic than thermal characterization of DNA adducts of monofunctional platinum compounds is a property implicated in the modulation of downstream effects such as protein recognition and repair.

Thermal stability and energetics of 15-mer DNA duplex interstrand cross-linked by trans-diamminedichloroplatinum(II)

The effect of the location of the interstrand cross-link formed by transplatin on the thermal stability and energetics of 15-mer DNA duplex has been investigated. The duplex containing single, site-specific cross-link, thermodynamically equivalent model structures (hairpins) and nonmodified duplexes were characterized by differential scanning calorimetry, temperature-dependent UV absorption, and circular dichroism. The results demonstrate that the formation of the interstrand cross-link of transplatin does not affect pronouncedly thermodynamic stability of DNA: the cross-link induces no marked changes not only in enthalpy, but also in “reduced” (concentration independent) monomolecular transition entropy. These results are consistent with the previous observations that interstrand cross-links of transplatin structurally perturb DNA only to a relatively small extent. On the other hand, constraining the duplex with the interstrand cross-link of transplatin results in a significant increase in thermal stability that is primarily due to entropic effects: the cross-link reduces the molecularity of the oligomer system from bimolecular to monomolecular. Importantly, the position of the interstrand cross-link within the duplex modulates cooperativity of the melting transition of the duplex and consequently its thermal stability.

Conformation of DNA modified by monofunctional Ru(II) arene complexes: recognition by DNA-binding proteins and repair. Relationship to cytotoxicity

Organometallic ruthenium(II) arene complexes of the type [(η^6-arene)Ru(II)(en)Cl][PF_6] (en = ethylenediamine) constitute a relatively new group of anticancer compounds. To achieve a rational design of novel antitumor Ru(II) arene compounds, it is important to understand in detail the differences in DNA binding properties of these complexes and their possible relationship to cytotoxicities in different tumor cell lines. In this work, we studied the activity of two monofunctional Ru(II) arene complexes [(η^6-arene)Ru(II)(en)(Cl)]$^+$ (arene = tetrahydroanthracene and *p*-cymene, Ru-THA and Ru-CYM, respectively), in two tumor cell lines and conformational distortions induced by monofunctional adducts of these complexes, their recognition by DNA-binding proteins and repair, i.e. the most important factors that modulate the antitumor effects of related platinum drugs. These two ruthenium complexes were chosen as representatives of two different classes of Ru(II) arene compounds which modify DNA differently: one that may interact with DNA by intercalation (tricyclic-ring Ru-THA) and the other (mono-ring Ru-CYM) that cannot.

The presence of the arene ligand in this class of ruthenium complexes capable of noncovalent, hydrophobic interaction with DNA considerably enhances cytotoxicity in several tumor cell lines. An analysis of DNA duplexes modified by Ru-THA and Ru-CYM revealed substantial differences in the impact of their monofunctional adducts on the conformation and thermodynamic stability of DNA and DNA polymerization *in vitro*. In addition, the adducts of Ru-CYM are removed from DNA more efficiently than those of Ru-THA. Interestingly, the adducts of Ru(II) arene compounds are preferentially removed from DNA by mechanisms other than by nucleotide excision repair, which provides additional support for a mechanism underlying antitumor activity of Ru(II) arene compounds different from that of cisplatin. Hence, the character and extent of DNA distortion induced in DNA by the adducts of Ru(II) arene complexes and resulting thermodynamic destabilization of DNA control the biological effects of this class of ruthenium complexes.

Granted projects

GA AS CR A5004101/01, Structure, recognition and biochemistry of DNA modified by antitumor platinum drugs. Principal investigator: V. Brabec, 2001 - 2005

GA AS CR B5004301, Molecular mechanisms underlying anticancer effects of a new drug BBR3464 Principal investigator: J. Kašpárková, 2003 - 2005

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

ME, COST OC D.20 003, Intracellular and extracellular targets for antitumour activity and toxicity of ruthenium complexes Principal investigator: V. Brabec, 2002 - 2005

ME, COST OC D. 21 001, Characterization of metalloproteins, key molecules for biological processes Principal investigator: V. Brabec, 2002 - 2005

ME, COST OC D. 20 004 Non-covalent DNA recognition strategies for design and synthesis of new metallo-drugs Principal investigator: Olga Nováková, 2003 - 2005

ME, COST OC D 20 001, Biochemistry, structural and cellular biology of non-classical antitumor platinum compounds Principal investigator: V. Brabec, 2001 – 2005

ME, Kontakt, AIP Czech-Slovak project within Czech-Slovak intergovernmental scientific and technical cooperation in 2004-2005 Platinum complexes, From DNA damage to cancer chemotherapy Principal investigator: V. Brabec, 2004 - 2005

HHMI (USA), INTNL 55000313, Basis for new structure-pharmacological relationship of platinum antitumor drugs Principal investigator: J. Kašpárková, 2001 - 2005

NIH (USA), 1R01CA78754 Mechanistic studies on new platinum clinical agents 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

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

Publications

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Brabec, V., Kašpárkova, J.: *Modifications of DNA by platinum complexes: Relation to resistance of tumors to platinum antitumor drugs*. Drug Resist. Updates, 8, 2005131-146.

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Hofr, C., Brabec, V.: *Thermal stability and energetics of 15-mer DNA duplex interstrand cross-linked by trans-diamminedichloroplatinum(II)*. Biopolymers, 77, 2005, 222-229.

Malina, J., Vojtíškova, M., Brabec, V., Diakos, C. I., Hambley, T. W.: *DNA adducts of the enantiomers of the Pt(II) complexes of the ahaz ligand (ahaz = 3-aminohexahydroazepine) and recognition of these adducts by HMG domain proteins*. Biochem. Biophys. Res. Commun., 332, 2005, 1034-1041.

Marini, V., Christofis, P., Nováková, O., Kašpárková, J., Farrell, N., Brabec, V.: *Conformation, protein recognition and repair of DNA interstrand and intrastrand cross-links of antitumor trans-[$PtCl_2(NH_3)$(thiazole)]*. Nucleic Acids Res., 33, 2005, 5819-5828.

Najajreh, Y., Kašpárkova, J., Marini, V., Gibbon, D., Brabec, V.: *Structural characterization and DNA interactions of new cytotoxic transplatin analogues containing one planar and one nonplanar heterocyclic amine ligand*. J. Biol. Inorg. Chem., 10, 2005, 722-731.

Najajreh, Y., Prilutski, D., Ardeli-Tzaraf, Y., Perez, J.-M., Khazanov, E., Barenholz, Y., Kašpárkova, J., Brabec, V., Gibbon, D.: *Structure and unique interactions with DNA of a cationic trans-platinum complex with the nonplanar bicyclic piperidinopiperidine ligand*. Angew. Chem. Int. Ed., 44, 2005, 2-4.

Novakova, O., Kašpárkova, J., Bursová, V., Hofr, C., Vojtíškova, M., Chen, H., Adler, P. J., Brabec, V.: *Conformation of DNA modified by monofunctional Ru(II) arene complexes: recognition by DNA-binding proteins and repair. Relationship to cytotoxicity*. Chem. Biol., 12, 2005, 121-129.

PhD thesis defended in 2005

Mgr. Radim Prokop, Molecular aspects of antitumor effects of selected platinum complexes – activation of trans geometry

MOLECULAR ANALYSIS OF PLANT DEVELOPMENT

HEAD

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pOp/LhGR system for stringent glucocorticoid-dependent transgene expression in tobacco

We developed pOp/LhGR, a dexamethasone-inducible derivative of the pOp/LhG4 transcription activation system, and tested its use in tobacco to regulate expression of *uidA* (encoding β-glucuronidase; GUS) and the cytokinin-biosynthetic gene *ipt*. The pOp/LhGR system exhibited stringent regulation and strong induced phenotypes in soil and tissue culture. In conjunction with an improved target promoter, pOp6, that carries six copies of an optimized *lac* operator sequence the pOp6/LhGR system directed induced GUS activities that exceeded those obtained with pOp/LhG4 or the CaMV 35S promoter but without increased uninduced activity. A single dose of dexamethasone was sufficient to direct cytotoxic levels of *ipt* expression in soil-grown plants although uninduced plants grew normally throughout a complete life cycle (Fig. 1).

In vitro, induced transcripts were detectable within an hour of dexamethasone application and 1 nM dexamethasone was sufficient for half maximal induction of GUS activity. Various methods of dexamethasone application were successfully applied under tissue culture and greenhouse conditions. We observed no inhibitory effects of dexamethasone or LhGR on plant development even with the highest concentrations of inducer,

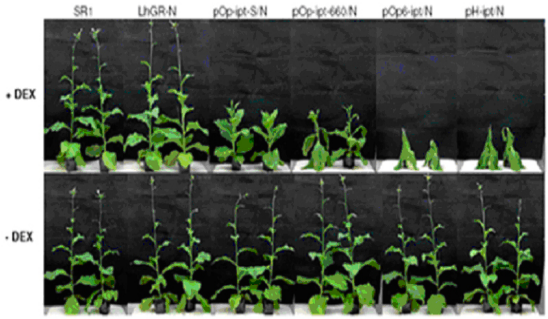

Fig. 1. Phenotypes of various *ipt* lines after treatment with dexamethasone. Wild type (SR1), LhGR-N, pOp-ipt-S/LhGR-N, pOp-ipt-660/LhGR-N, pOp6-ipt/LhGR-N, and pH-ipt/LhGR-N plants after 4 weeks of treatment with 50 ml of 20 μM dexamethasone solution (upper row) or 0.1% ethanol (lower row). Plants were induced after 3 weeks of growth in tissue culture and 3 weeks in soil.

although tobacco seedlings were adversely affected by ethanol used as a solvent for dexamethasone stock solutions. The pOp/LhGR system provides a highly sensitive, efficient, and tightly regulated chemically inducible transgene expression system for tobacco plants.

Granted projects

GA AS CR IAA600380507, Mechanisms maintaining hormonal homeostasis in plant cells. Principal co-investigator: B. Brzobohatý
2005 -5007

Publications

Šámalová, M., Brzobohatý, B., Moore, I.: *pOp6/LhGR: a stringently regulated and highly responsive dexamethasone-inducible gene expression system for tobacco*. Plant J., 41, 2005, 919-935.

PhD thesis defended in 2005

Mgr. Markéta Šámalová, Using a chemically inducible transcription activation system to study cytokinin metabolism and action in transgenic tobacco.

DNA-Molecular Complexes

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The main focus of the work in 2005 was to examine telomeres from an evolutionary perspective. 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. Furthermore, two different modes of initial elongation of the substrate primer were observed in Asparagales telomerases producing human-like repeats, which could be attributed to interactions between the telomerase RNA subunit (TR) and the substrate.

The evolutionary response was analysed of a protein complement of Asparagales telomeres to the evolutionary change of its sequence from Arabidopsis type to human type, and, to the final loss of human type-sequence. Nuclear protein extracts were tested from plants with human-type telomeres (e.g., *Scilla peruviana*) and from phylogenetically younger plants without any known minisatellite telomeres (*Allium cepa*) for a presence of proteins binding specifically the single-strand overhang of a G-rich strand of telomeric DNA. The G-rich overhang is a highly conserved telomere domain since it functions as a telomerase substrate and a target site of a number of regulatory proteins (for example, Cdc13 and Pot1). Using EMSA it was found that these extracts still contain proteins preferentially binding the ancestral *Arabidopsis*-type sequence. More detailed analyses have shown that, e.g., *Allium* extracts contain proteins of 30 and 38 kDa, which bind both Arabidopsis and human telomeric sequences, 18 kDa protein which associates only with *Arabidopsis*-type sequence and, finally, a 24 kDa protein which forms complexes only with human-type sequence.

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 IAA6000405, 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

Publications

Fajkus J., Sýkorová E., Leitch A.R.: *Techniques in plant telomere biology.* BioTechniques 38, 2005, 233-243

Neplechová K., Sýkorová E., Fajkus J.: *Comparison of different kinds of probes used for analysis of variant telomeric sequences*. Biophysical Chemistry 117, 2005, 225 – 231

Fajkus J., Sýkorová E., Leitch A.R.: *Telomeres in evolution and evolution of telomeres.* Chromosome Res. 13, 2005, 469-479

Kuchař M: *Plant telomere-binding proteins.* Biol. Plant. 50, 2006, 1-7

PhD thesis defended in 2005

Mgr. Milan Kuchař, Ph.D.: Plant telomere-associated proteins and their protein-protein interactions.

BIOPHYSICAL CHEMISTRY AND MOLECULAR ONCOLOGY

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Properties of nucleic acids and proteins at electrically charged surfaces and their applications in novel bioanalytical tools and electrochemical biosensors

Work was focused on electrochemistry of nucleic acids and electrochemical sensors for DNA hybridization and DNA damage as tools potentially applicable in molecular diagnostics and/or environmental monitoring:

Coupling of adsorptive transfer stripping and elimination voltammetry (AdTS EVLS) with linear scan was proposed for the resolution of reduction signals of cytosine (C) and adenine (A) residues in hetero-oligonucleotides. The AdTS EVLS was able to determine C/A ratio of oligonucleotides through the elimination function conserving the diffusion current

component and eliminating kinetic and charging current components. Optimal conditions of elimination experiments such as pH, time of adsorption and scan rate were found.

Amperometric biosensors were developed on the basis of stationary mercury-film and glassy-carbon electrodes and DNA or its fragments (ODNs), immobilized in a nitrocellulose matrix. Taking into account the high affinity of Cu(II) and Fe(III) ions to denatured DNA (19.1 ± 0.1) x 10^5 and (1.4 ± 0.3) x 10^5 L/mol, respectively, a procedure was proposed for the voltammetric determination of these ions in natural materials and blood serum at a level of 10^{-11} M. This procedure involves analyte pre-concentration on a DNA-containing biosensor. An ODN-containing biosensor (DNA probe) was used in the study of DNA hybridization for the highly specific determination of its nucleotide sequence.

The DNA-modified membrane electrode was prepared by casting a mixture of nitrocellulose (NC) with target DNA (tDNA) in organic solvent on glassy carbon electrode (GCE). Unlabeled polymerase chain reaction (PCR)-amplified human genomic sequence (628 bp) or synthetic oligodeoxynucleotides (ODNs) were used as tDNAs, creating a recognition layer. Biotinylated ODNs were used as hybridization probes to recognize specific nucleotide sequences. The hybridization events were detected via an enzyme-linked electrochemical assay involving binding of streptavidin-coupled alkaline phosphatase (SALP) to the biotin labels of the probe bound to tDNA. After the probe hybridization and SALP binding, the electrode was immersed into an electroinactive enzyme substrate (1-naphthyl phosphate). The alkaline phosphatase converted the inactive substrate into electroactive 1-naphthol that penetrated through the NC membrane to the GCE surface and was subsequently detected using an anodic voltammetric signal. The optimized method offered a good discrimination between complementary and nonspecific DNAs and yielded well-defined responses for both single-copy and repetitive tDNA sequences. In contrast to previously published methods using electrodes with mechanically attached membranes, the previously mentioned electrode is easily amenable to parallel DNA analysis.

Polished and mercury film-modified silver solid amalgam electrodes (p-AgSAE and MF-AgSAE, respectively) were used for the measurements of

intrinsic redox (faradaic) and tensammetric voltammetric signals of single- (ss) and double-stranded, linear (lin) or supercoiled (sc) DNAs, synthetic polynucleotides and free adenine base. At the MF-AgSAE, all of these species yielded signals similar to those previously obtained on the hanging mercury drop electrode (HMDE) or on solid silver amalgam electrode modified with mercury meniscus (m-AgSAE). Well measurable anodic peak G (due to guanine residues) and tensammetric signals of ssDNA were obtained also with the p-AgSAE (albeit the latter appeared at significantly less negative potentials than respective signals measured at HMDE). In contrast, the cathodic DNA peak CA (due to reduction of cytosine and adenine residues) was not detected at the polished electrode. For the free adenine base, at least an order of magnitude lower sensitivity (compared to HMDE or MF-AgSAE) was achieved when its reduction signal was measured. Double-stranded DNAs (including sc and linDNA) yielded no measurable tensammetric signals at the p-AgSAE. Similarly as with the HMDE and m-AgSAE, measurements at the MF-AgSAE allowed differentiation between sc and linDNA and were successfully applied for the detection of DNA strand breaks induced by ionizing radiation. The p-AgSAE could be used for detection of the DNA strand breaks too, but selective denaturation of the damaged DNA (converting it into ssDNA detectable at the polished electrode) was necessary prior to the voltammetric analysis. Our results suggest that the different behavior of DNA at the p-AgSAE and at the MF-AgSAE (HMDE) is probably related to a considerably weaker adsorption of the DNA molecules on the polished solid amalgam surface, compared to the surface of mercury (or liquid silver amalgam).

Electrochemical measurements at mercury or solid amalgam electrodes offer a highly sensitive detection of DNA strand breaks. On the other hand, electrochemical detection of damage to DNA bases at any electrode is usually much less sensitive. In this paper, we propose a new voltammetric method for the detection of the DNA base damage based on enzymatic conversion of the damaged DNA bases to single-strand breaks (ssb), single-stranded (ss) DNA regions, or both. Supercoiled DNA exposed to UV light was specifically cleaved by T4 endonuclease V, an enzyme recognizing pyrimidine dimers, the major products of photochemical DNA damage. Apurinic sites (formed in dimethyl sulfate-modified DNA) were determined

after treating the DNA with E. coli exonuclease III, an enzyme introducing ssb at the abasic sites and degrading one of the DNA strands. The ssb or ssDNA regions, or both, were detected by adsorptive transfer stripping alternating current voltammetry at the mercury electrode. This technique offers much better sensitivity and selectivity of DNA base damage detection than any other electrochemical method. It is not limited to DNA damage in vitro, but it can detect also DNA base damage induced in living bacterial cells.

We showed for the first time that thiol end-labeled oligodeoxynucleotides (ODN) produce specific voltammetric signals at mercury and solid amalgam electrodes in cobalt-containing solutions (usually used in polarographic and voltammetric analysis of peptides and proteins. Most of the measurements were performed by adsorptive transfer stripping (ex situ) method. 21-mer ODN, HS-$(CTT)_7$ was attached to the hanging mercury drop electrode (HMDE) and the DNA-modified electrode was immersed in the cobalt-containing empty background electrolyte. This ODN produced several differential pulse voltammetric signals not observed in absence of cobalt. The most negative signal (peak 3) of submicromolar HS-$(CTT)_7$ attained much larger heights than the catalytic (Brdicka) peaks of about 10-fold more concentrated cysteine-containing peptide. Peak 3 and a less negative peak 2 of the HS-ODN increased with increasing buffer concentration suggesting that, similarly to cysteine-containing peptides and proteins, for both SH-ODN peaks catalytic hydrogen evolution was responsible. Because peak 3 appeared at less negative potentials than the peptide Brdicka peaks it was possible to measure the ODN and peptide signals separately. This new possibility of measuring signals of thiolated DNA in the media, which proved suitable for protein analysis, can be utilized in the development of sensors for DNA-protein interactions, important in biomedicine and proteomics.

In addition to results attained in the above-mentioned area of electrochemical DNA sensors, considerable progress was reached in the field of electrochemistry of peptides and proteins, their complexes with heavy metals and application of new electrochemical techniques (biosensors) in environmental analysis:

The electroanalytical determination of avidin in solution, in a carbon paste,

and in a transgenic maize extract was performed in acidic medium at a carbon paste electrode (CPE). The oxidative voltammetric signal resulting from the presence of tyrosine and tryptophan in avidin was observed using square-wave voltammetry. In the case of the avidin-modified CPE, several parameters were studied in order to optimize the measurements, such as electrode accumulation time, composition of the avidin-modified CPE, and the elution time of avidin. In addition, the avidin-modified electrode was used to detect biotin in solution and to detect biotin in a pharmaceutical drug after various solvent extraction procedures. Comparable studies for the detection of biotin were developed using HPLC with diode array detection (HPLC-DAD) and flow injection analysis with electrochemical detection. The effects of applied potential, acetonitrile content and flow rate of the mobile phase on the FIA-ED signal were also studied.

Increasing concentration of heavy metals in the environment is a serious problem for human and animal health protection and production of foodstuffs in many countries around the world. Electrochemical biosensors have superior properties over other existing measurement systems because they can provide rapid, simple and low-cost on-field determination of many biological active species and a number of dangerous pollutants. We suggested a new heavy metal biosensor based on interaction of heavy metal ions (Cd(II) and Zn(II) with phytochelatin, which was adsorbed on the surface of the hanging mercury drop electrode, using adsorptive transfer stripping differential pulse voltammetry. We applied the suggested technique for the determination of heavy metals in a biological sample - human urine and platinum in a pharmaceutical drug. The detection limits (3 S/N) of Cd(II), Zn(II) and cis-platin were about 1.0, 13.3 and 1.9 pmole in 5 μl, respectively. The same approach was used with electrode modified by metallothionein. In this case we obtained detection limits of the selected heavy metals (cadmium and zinc) (which were analyzed in the presence of the basic electrolyte - 0.5 M NaCl, pH 6.4), as low as 250 fmol and 350 fmol in 5 μL drop, respectively. On the basis of the obtained results, we propose detection of heavy metals in environmental, biological and medical samples.

Structure and interaction of DNA and proteins in oncological research, especially with respect to the tumor suppressor protein p53

In this field, our attention was focused on structure and interactions of the tumor suppressor protein p53, particularly the structure-selective DNA binding of the protein and its ability to recognize damaged DNA.

Using electron microscopy, we analyzed the interaction of bacterially expressed full-length p53, p53(1-393), and its C-terminal fragment, p53(320-393), with long (approximately 3000 bp) dsDNA in linear and supercoiled (|DeltaLk| approximately 4-6) forms containing or lacking the p53 recognition sequence (p53CON). The main structural feature of the complexes formed by either protein was a DNA-protein filament, in which two DNA duplexes are linked (synapsed) via bound protein tetramers. The efficiency of the synapse, reflected in its length and the fraction of molecules exhibiting DNA-protein filaments, was significantly modulated by the molecular form of the protein and the topological state of the DNA. With linear DNA, the synapse yield promoted by the C-terminus fragment was very low, but the full-length protein was effective in linking noncontiguous duplexes, leading to the formation of intramolecular loops constrained at their bases by short regions of synapsed DNA duplexes. When the linear DNA contained p53CON, regions of preferential sequence, i.e., encompassing p53CON and probably p53CON-like sequences, were predominantly synapsed, indicating a sequence specificity of the p53 core domain. With scDNA, the synapse yield was significantly higher compared to the linear counterparts and was weakly dependent on the sign of superhelicity and presence or absence of p53CON. However, the full-length protein was more effective in promoting DNA synapses compared to the C-terminal fragment. The overall structure of the DNA-protein filaments was apparently similar for either protein form, although the apparent width differed slightly (approximately 7-9 nm and approximately 10-12 nm for p53(320-393) and p53(1-393), respectively). No distortion of the DNA helices involved in the synapse was found. We conclude that the structural similarity of DNA-protein filaments observed for both proteins is attributable mainly to the C-terminus, and that the yield is dictated by the specific and possibly nonspecific interactions of the core domain in combination with DNA topology. Possible implications for the sequestering of p53 in DNA-protein filaments are discussed.

In collaboration with the Laboratory of Molecular Biophysics and Pharmacology, we continued studies focused on p53 binding to DNA modified with antitumor agents (a paper on role of the protein p53 domains was accepted for publication at the end of 2005). Within the FP6 EU integrated project (No. 502983), research aimed to applications of electrochemical techniques and chemical probes in analysis of mutant p53 proteins was carried out.

In 2005, a book "Electrochemistry of nucleic acids and proteins. Towards electrochemical sensors for genomics and proteomics" edited by E. Palecek (together with F. Scheller and J. Wang) was published (Elsevier, Amsterdam, ISBN 0-444-5215-X). For almost 40 years electrochemistry of nucleic acids was a domain of a mere handful of laboratories in Europe. In the 1990's progress in genomics and particularly in the Human Genome Project greatly stimulated interest in new methods capable to unravel the genetic information stored in the nucleotide sequence of DNA. Development of DNA sensors and construction of DNA arrays (chips) with electrochemical detection (which is simpler and should be less expensive than optical detection) have become a booming field involving large number of laboratories all over the world. Similar increase in the interest in electrochemistry of proteins is foreseen in connection with the rapid development of proteomics. This book offers an up-to-date presentation of the interaction of nucleic acids and proteins with electrodes and about the development of sophisticated electrochemical biosensors for DNA hybridization and damage as well as about modern sensors for protein identification. In addition it summarizes the ways, which in the past century led to the present state of electrochemical analysis of nucleic acids and proteins. The book, written by renowned experts, represents the basic literature for those who already entered the field as well as for physicists, chemists and biologists, both professionals and students, who are interested in new biophysical-chemical methods and approaches useful in modern biotechnologies. The LBCMO members (E. Paleček, M. Fojta, F. Jelen and S. Billová) are authors or co-authors of 6 chapters of the book. Another invited review chapter was published by E. Paleček and M. Fojta in a monograph "Bioelectronics" (edited by I. Willner and E. Katz, Wiley-VCH, Weinheim).

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 KJB4004302, Application of chemical structural probes and electroanalytical methods in DNA damage detection. Development of DNA sensors. Principal investigator: L. Havran, 2003 - 2005

GA AS CR IBS5004355, Possibilities of electrochemical methods in genomics Basis for development of DNA sensors. Principal investigator: E. Paleček, 2003 - 2005

GA AS CR S5004107, Applications of biophysical methods in biotechnological and clinical praxis. Principal investigator: V. Vetterl, co-investigator: E. Paleček, 2001 - 2005

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

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

GACR 204/03/0566, Electrochemistry in protein analysis and in detection of DNA hybridization. Principal investigator: E. Paleček, 2003-2005

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

IGA MH NC/7574 - 3, Recognition of DNA damage by tumor suppressor proteins. Effects of anti-cancer drugs. Principal investigator: M. Fojta, 2003-2005

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

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

COST D21Work group, "Characterization of metalloproteins, key molecules for biological processes" Coordinator: Prof. M De Ley, Leuven, Belgium

COST OC D21.002, Characterization of metalloproteins important in cancer and their interactions with DNA. Principal investigator: E. Paleček (2002-2005)

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

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PhD theses defended in 2005

Mgr. Eva Brázdová-Jagelská, Influence of DNA topology on sequence-specific DNA binding of tumor suppressor protein p53

Mgr. Michal Masařík, Using electrochemical methods for protein analysis and their applications in proteomics, genomics and biomedicine

EXPERIMENTAL HEMATOLOGY

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In 2005, studies on hematopoiesis-modulating actions occuring after activation of adenosine membrane receptors continued. Effects of N^6-cyclopentyladenosine (CPA), the selective adenosine A_1 receptor agonist, on murine bone marrow hematopoietic progenitor cells for granulocytes and macrophages (GM-CFC) were investigated by utilizing the model of hematopoietic damage induced by 5-fluorouracil. A single injection of CPA at an optimum dose administered 22 h before a single injection of 5-fluorouracil protected GM-CFC against the cytotoxic damage as determined 4 days later. Because 5-fluorouracil is a cell cycle-specific drug damaging mainly cells in the S-phase, protective effects of CPA can be explained by its inhibitory action on the cell cycling. This interpretation was confirmed by experiments demonstrating that repeated administration of CPA in the hyperproliferation phase of the recovering hematopoiesis after 5-fluorouracil treatment inhibited transiently restoration of GM-CFC counts. These findings provided the first evidence for the inhibitory action of CPA on the cycling of hematopoietic progenitor cells.

In a co-operation with the joint-stock company Imunomedica, a study on hematopoiesis-stimulating effects of the immunomodulating drug Imunor® was performed. Imunor®, which is an ultrafiltered pig leukocyte extract, a heterogeneous mixture of low molecular weight (<10 kD) substances released from disintegrated pig leukocytes, was tested from the point of view of its hematopoiesis-modulating activities using experiments *in vitro* and *in vivo*. Attention was focused especially on evaluation of the contingent ability of Imunor® to potentiate the hematopoiesis-stimulating effects of recombinant human granulocyte colony-stimulating factor (G-CSF). Experiments *in vitro* revealed the capability of sera from mice administered Imunor® perorally (p.o.) to stimulate proliferation of progenitor cells for granulocytes and macrophages (GM-CFC) in cultures of normal bone marrow cells. In addition, Imunor®, as well as sera from mice given Imunor®, added to the cultures in combination with G-CSF enhanced the numbers of GM-CFC significantly over those induced by sera after administration of either of the preparations alone. In *in vivo* experiments, Imunor®was found to increase the counts of GM-CFC per femur and femoral bone marrow cellularity in sublethally irradiated mice when administered p.o. after irradiation in combination with G-CSF in comparison with the effects of G-CSF alone. These results indicate the possibility of using Imunor®, a commercially available preparation, for treatment of hematopoietic suppression of various etiology.

Our previous studies have shown that non-selective activation of adenosine membrane receptors induced by the combined administration of drugs elevating extracellular adenosine, i.e. dipyridamole (DP) and adenosine monophosphate (AMP), enhances murine hematopoiesis and potentiates the action of granulocyte colony-stimulating factor (G-CSF). In the experiments performed in 2005, colony stimulating activity (CSA) of blood serum of the mice treated with DP+AMP, G-CSF or all these drugs in combination, i.e. the ability of the sera to stimulate the growth of GM-CFC colonies *in vitro*, was assayed. Furthermore, the concentration of GM-CSF, G-CSF, IL-3, and IL-6 in sera was determined. Sera of mice given DP+AMP were found to enhance significantly serum CSA with a nearly constant effectiveness of the sera, which persisted till 24 hours after the last injection (Fig. 1A). Fig. 1B shows the mutually potentiating effects of DP+AMP and G-CSF on the serum CSA in early intervals after the administration of the drugs.

Furthermore, G-CSF and IL-6 levels were significantly elevated in the sera of mice which were administered DP+AMP either alone or in combination with G-CSF. The results show that the effects of DP+AMP are indirect, mediated through the induction of some cytokine(s) and/or growth factor(s) and that extracellular adenosine can act in co-operation with G-CSF. These findings contribute to the further elucidation of the role of adenosine in hematopoiesis.

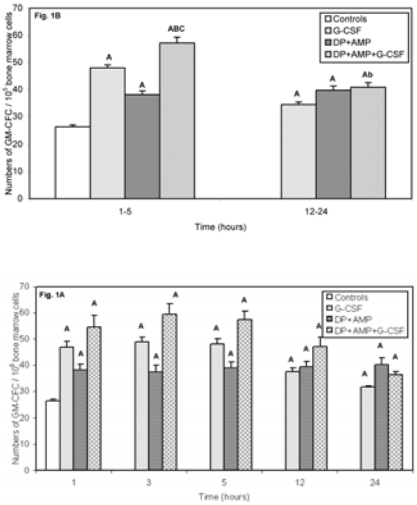

Fig. 1A, B. Numbers of GM-CFC per 10^5 bone marrow cells in cultures treated with serum of mice administered G-CSF, DP+AMP or a combination of DP+AMP+G-CSF in a single dose. Data are given as means ± SEM. Controls - sera of control mice. Time - time-interval between the last injection of the drugs and serum sampling (**Fig. 1A** – values in individual time intervals, **Fig. 1B** – values in grouped early and late intervals). Statistical significance: A, $p<0.01$, in comparison with controls. B and b, $p<0.01$ and $p<0.05$, respectively, in comparison with the cultures treated with serum of mice administered G-CSF alone. C, $p<0.01$, in comparison with the cultures treated with serum of mice administered DP+AMP.

Granted projects

Imunomedica, joint-stock company Contract of co-operation at studies on medical effectiveness of ultrafiltered preparation of peripheral blood leukocytes Imunor.® Principal investigator: M. Hofer, 2005

GA AS CR 305/03/D050, The role of adenosine signalling and its interaction with G-CSF in regulation of hematopoiesis. Principal investigator: L. Weiterová, 2003 - 2006

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MOLECULAR EPIGENETICS

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Studies of epigenetic regulation of ribosomal RNA genes expression

Epigenetic changes accompanying plant cell dedifferentiation and differentiation were found in 35S rDNA locus of tobacco. There was a reduction of CG and CNG methylation in both intergenic and genic regions of the rDNA cistron in fully dedifferentiated callus and root compared to leaf. The rDNA hypomethylation was not random, but targeted to particular rDNA gene families at units that are clustered within the tandem array. The process of hypomethylation was initiated as early as two weeks after the callus induction and established epigenetic patterns were stably maintained throughout prolonged culture. However, regenerated plants and their progeny showed partial and complete remethylation of units, respectively. Nuclear run-on assays revealed a two fold increase of primary (unprocessed) rRNA transcripts in callus compared to leaf tissue. However the abundance of mature transcripts in callus was elevated by only about 25%. FISH analysis of interphase nuclei showed high levels of rDNA chromatin condensation in both callus and leaf, with substantially less

decondensed rDNA than is observed in meristematic root tip cells. It is likely that the regions of the rDNA locus showing decondensation correspond to the clusters of hypomethylated units that occur in the tandem array at each locus. The data together indicate that the establishment of pluripotency and cell proliferation occurring with callus induction is associated with enhanced rRNA gene expression and overall rDNA hypomethylation, but is not associated with material enhanced relaxation of chromatin structure (decondensation) at rDNA loci.

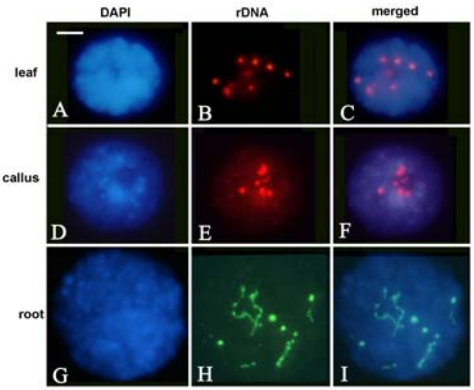

Fig. 1. Differential topology of rDNA chromatin in interphase nuclei of root, leaf and callus tissue. Fluorescence in situ hybridization using biotin- (red fluorescence) or digoxigenin- (green fluorescence) labeled probe against rDNA to interphase nuclei from mature leaf (A-C), fully dedifferentiated calli (D-F) and root-tip meristematic cells (G-I). Scale bar = 10 μm

Studies of plant allopolyploid nucleus formation

N. tabacum (tobacco, 2n = 4x = 48) is a natural allotetraploid combining two ancestral genomes closely related to modern *N. sylvestris* and *N. tomentosiformis*. Here we examine the immediate consequences of allopolyploidy on genome evolution using twenty S_4 generation plants derived from a single synthetic, S_0 plant made by Burk in 1973 (Th37). By molecular and cytogenetic methods, we analysed fourteen middle and highly repetitive sequences that together total about 4 % of the genome.

Two repeats related to endogenous geminiviruses (GRD5) and pararetroviruses (*Nto*EPRV) plus two classes of satellite repeats (NTRS, A1/A2) were partially or completely eliminated at variable frequency (25-60%). These sequences are all from the *N. tomentosiformis* parent. Genomic *in situ* hybridisation (GISH) revealed additivity in chromosome numbers in two plants (2n=48) while a third was aneuploid for an *N. tomentosiformis*-origin chromosome (2n = 49). Two plants had homozygous translocations between chromosomes of the S- and T- genomes. The data demonstrate that genetic changes in synthetic tobacco were fast, targeted to the paternal *N. tomenotosiformis*-donated genome and some of the changes showed concordance with changes that presumably occurred during evolution of natural tobacco.

Granted projects

GA CR 521/04/0775, Epigenetic regulation of gene expression in transgenic and endogenous loci of higher plants. Principal investigator: A. Kovařík, 2004-2006

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

GA CR 204/03/P104, The structure and expression of ribosomal RNA genes in allotetraploid and their parental genomes. Principal investigator: J. Fulneček, 2003-2005

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A.: *Dedifferentiation of tobacco cells is associated with rRNA genes hypomethylation, their increased transcription and chromatin alterations.* Plant Physiol. 139, 2005, 275-286.

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Kovarik, A., Pires, J.C., Leitch, A.R., Lim, K.Y., Sherwood, A., Matyasek, R., Rocca, J., Soltis, D.E., Soltis, P.S.: *Rapid concerted evolution of nuclear ribosomal DNA in two allopolyploids of recent and recurrent origin.* Genetics 169, 2005, 931-944.

Lim, K.Y., Matyasek, R., Fulnecek, J., Kovarik, A., Leitch, A.R.: *No evidence of intergenomic translocations or male-genome instability in the allopolyploid Nicotiana rustica.* Cytogenet. Genome Res. 109, 2005, 298-309.

MOLECULAR CYTOMETRY AND CYTOLOGY

HEAD

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HANA KŘIVÁNKOVÁ

Scientific results of the Laboratory are tightly related to the automated confocal microscopy. Technological achievements of the Laboratory in 2005 included implementation of a new Andor EM CCD camera with the possibility of signal on chip multiplication (up to 1000x). In addition to Andor software for image analysis and deconvolution, our own software has been developed for simultaneous control of the microscope x-y stage, Piezzo z-motion, filter exchange wheel (Sutter controller), AOTF excitation line selection (488, 568 or 647 nm) and EM CCD (time of exposition, selection of subimages, bining, etc).

The projects in 2005 were focused to epigenetics, i.e. histone modifications, HP1 proteins and chromatin/chromosome structural studies. A large number of different cell types have been used, from human fibroblasts and lymphocytes, to human tumor cells such as HT-29 or CML cells, to specially constructed lamin A/C deficient mouse embryonic fibroblasts, to human stem cells. Both fixed cell lines with preserved 3D structure and living cells have been used. The results contribute to better understanding of epigenetic control of cellular processes such as transcription, repair or cell transformation.

The effects of histone deacetylase inhibitors (HDACs) such as Trichostatin A (TSA) and sodium butyrate (NaBt) on histone epigenetic states were investigated. We have addressed the following histone modifications: di-methylation of histone H3 at the position of lysine 9 (H3-K9) and lysine 4 (H3-K4) as well as H3-K9 acetylation. Additionally we have analyzed nuclear rearrangements and levels of heterochromatin protein 1 (HP1). Very interesting changes were observed at the nuclear periphery (Fig. 1) that appeared as an important nuclear compartment undergoing several modifications induced by hyperacetylation processes. Taken together, inhibition of histone deacetylases stimulated dynamic reorganization of chromatin in parallel with changes in its epigenetic states. The results of these experiments were publishes in Bartová et al. (2005b).

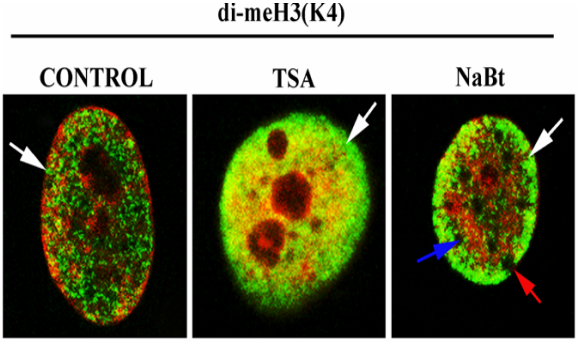

Fig. 1. Changes in the periphery of the cell nucleus induced by HDAC inhibitors. H3(K4) di-methylation (green) and total nuclear DNA (red) are shown. Arrows indicate changes of the nuclear structure that is observed on the nuclear periphery.

The nuclear topography and expression of the bcr/abl fusion genes were studied in Bártová et al. (2005a). Peripheral reposition of transcriptionaly inactive bcr/abl genes correlated with the differentiation-related heterochromatinization of amplified bcr/abl loci and with the changes in H3(K4) and H3(K9) di-methylations, that were studied using chromatin immunoprecipitation approaches (ChIP-PCR) at the major break point B3A2. In comparison with progenitor cells, differentiated population was characterized by specific arrangement of BCR/ABL and c-ABL proteins, that were located focally in cytoplasm while absence of these signals was

observed in apoptotic cells and cells influenced by Abl-RNA interference (Abl-RNAi) leading also to program cell death.

In order to study the c-myc gene expression in a single cell, we have addressed a new method, RNA-FISH that enables visualization of the c-myc gene transcript. In human colon carcinoma cells, single transcription site (Fig. 2 and 3) located in the region of primary sequences or released as a complex located in the inner part of cell nucleus was observed. The nuclear topography was studied for the c-myc transcription sites as well as for the c-myc coding sequences. The mentioned experiments and analyses of topographic data were performed during enterocytic cell differentiation of colon adenocarcinoma cells HT-29.

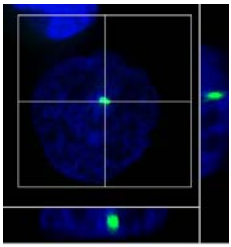

Fig. 2. The c-myc transcript (green signal) determined by RNA-FISH technique. Staining by TO-PRO-3 was used for visualization of individual interphase nuclei. Lateral x-z and y-z projections of interphase nuclei are shown.

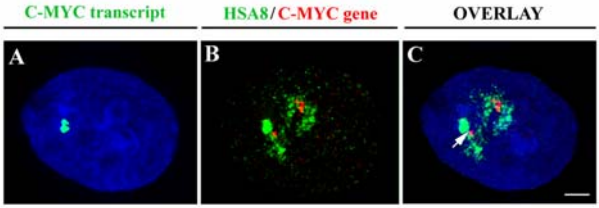

Fig. 3. (A) Human small lung carcinoma cell A549 stained by TO-PRO-3 (blue) and the c-myc transkript (green) visualized by RNA-FISH. In majority of cells we have observed a single transcription site. **(B)** Localization of original c-myc sequences (red) in the related chromosome territory (green) was determined using DNA-FISH. DNA-FISH conserved RNA-FISH signals. **(C)** Photoshop 5.0 software was used for image overlay. The arrow shows the c-myc transcript. Scale bar represents 1μm.

In the frame of the EU project LSHG-CT-2003-503441 we prepared DNA probes for the detection of gene-rich (RIDGE) and gene-poor (anti-RIDGE) regions of human chromosome 11. Nuclear topography of these regions was

analysed in cells undergoing enterocytic and megakaryocytic differentiation. We observed that RIDGE regions are located more peripherally while anti-RIDGE regions are positioned in the central parts of the interphase cell nuclei. More de-condensed RIDGE regions were positioned on the chromosome periphery while more condensed anti-RIDGE sequences were observed in the internal parts (Fig. 4).

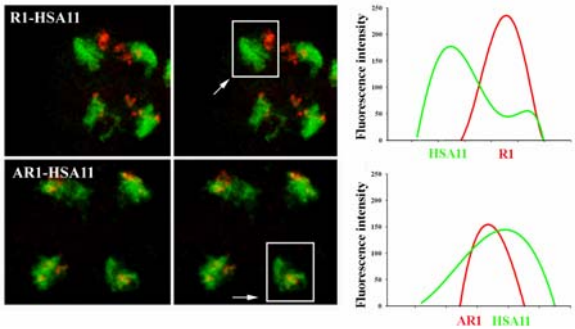

Fig. 4. Distributions of RIDGE a anti-RIDGE sequences (red signals) within nuclei of human chromosome 11 (green signals) in the cells of human adnocarcinoma HT29.

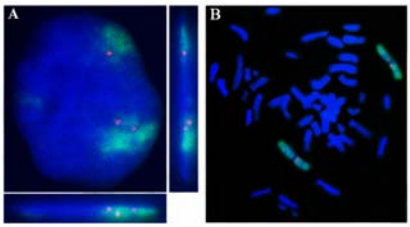

Fig. 5. Chromosomal region 1q21 (red signals) (A) in chromosome 1 territory (green signals) in the cell nucleus of HT-29 cells, (B) 1q21 arrangement within chromosome 1 in metaphase spreads of the cell with normal karyotype (lymphocytes).

Investigations of cytogenetics of multiple myeloma (MM) showed that there are no specific cytogenetic markers, however, the IgH, TP53, CCND1 and *c-myc* genes are frequently altered. For these loci, changes in the nuclear topography that could be associated with MM progression were studied. We have also addressed the effect of cytostatic treatments, used in MM therapy, and we analyzed the effect of gamma radiation on chromatin structure of interphase nuclei of human plasma cellular leukemia line ARH 77. We have also amplified the DNA from BAC clone, carrying the 1q21 insert that can

be used for FISH technique applied in clinical diagnostics (Fig. 5). The 1q21 fragment seems to be an important cytogenetic marker associated with poor patient prognosis and therefore, seems to be an important tool for clinical use.

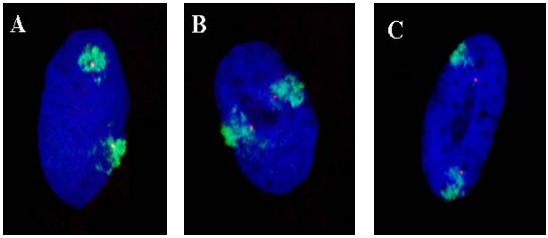

Fig. 6. Position of the CCND1 gene within human chromosome 11 territory. The gene was located either in the interior or periphery of chromosome territory. In some cases the locus was positioned on chromatin loops extended away from chromosome territory.

The effects of HDAC inhibitors and DNA de-methylating agents such as Trichostatin A and 5-aza-2-deoxycytidine in human, monkey and mouse primary fibroblasts was studied in order to confirm or disprove the theory of evolutionary conserved genome structure. After cell treatments by both inhibitors we have analyzed nuclear arrangement of selected genetic elements (Fig. 6). In the frame of the common project with Laboratory of gene expression, Charles University in Prague, we have studied the chromatin structure in lamin A/C deficient mouse embryonic fibroblasts. In these cells, the topography of nuclear speckles, splicing factors and foci with HP1 proteins were studied (Fig. 7).

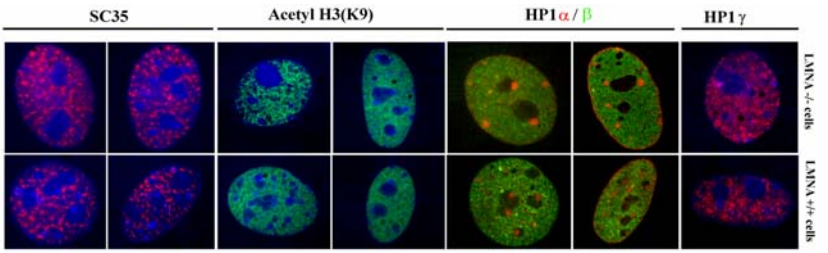

Fig. 7. Detection of the nuclear arrangement of nuclear speckles, involving several splicing factors (SC-35), nuclear pattern of acetylated histones H3K9) and all variants of heterochromatin protein 1 HP1 (α,β,γ) in mouse embryonic fibroblasts deficient in lamin A/C (LMNA -/- cells). For comparison, nuclear topography of selected structures was related to normal LMNA +/+ cells.

Chromatin structure, function and dynamics

Heterochromatin markers (methylated H3K9, HP1α, β, γ and MNEI) and their nuclear localization during the differentiation of stem cells of healthy and CML donors are studied. Our results show the disappearance of all HP1 proteins at day 12 of differentiation in both healthy and CML stem cells, increasing accumulation of MNEI (a serpine like protein) in the nucleus and its disappearance from the cytoplasm in terminally differentiated granulocytes (Fig. 8). These results show that HP1 proteins are replaced by MNEI during the granulocytic differentiation of progenitor cells. There were no differences in the process of *ex vivo* differentiation of progenitor cells coming from the bone morrow of these two different donors. The reason for the replacement of HP1 proteins in chromatin during the terminal differentiation of human granulocytes might be related to specific function of these cells.

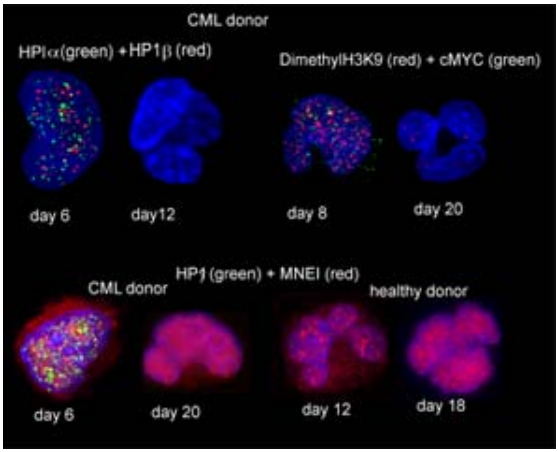

Fig. 8. Immunochemically detected proteins during the differentiation of progenitor $CD34^+$ cells from CML and healthy donors in IMDM medium supple-mented with rhG-CSF, recombinant human cytokines CC-100 and 10% fetal calf serum.

In living cell experiments, two different types of plasmid DNA movement in the cell nucleus were observed: Brownian and directional - to the nearest HP1 focus. Contact of plasmid DNA with heterochromatin led to strongly restricted Brownian movement. Our results also show interaction of plasmid DNA with DSB in chromatin of host cell detected by green immuno-fluorescence of phosphorylated H2AX (Fig. 9). DSB in host chromatin could facilitate plasmid DNA integration.

Changes of higher-order chromatin structure of genetic loci with high density of genes deregulated in acute promyelocytic leukemia (APL) patients by the oncogenic fusion protein PML/RARa were studied in PIR9 U937 cells, carrying PML/RARa fusion gene inserted under the control of Zn^{2+}-inducible promoter. The loci down regulated in cells expressing PML/RARa oncogene ("APL cells" = Zn^{2+}-treated cells) were identified by Affymetrix screenings (European Institute of Oncology, Milano). It was found that they significantly co- localize with PML/RARa microspeckles. The interaction of down regulated loci with PML/RARa fusion protein resulted in chromatin condensation, accompanied with gene silencing. The addition of *all-trans* retinoic acid (ATRA) or trichostatin (TSA) (histone deacetylases inhibitor) reverted chromatin structure close to the original state. In contrast, the loci non-regulated with PML/RARa did not co-localize with this fusion protein and preserved original chromatin structure.

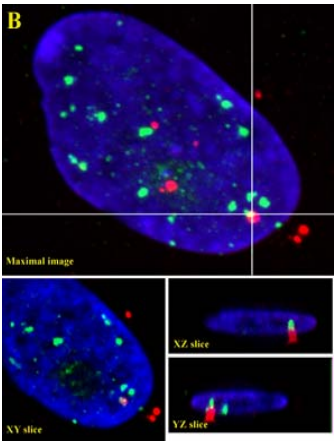

Fig. 9. Three-dimensional image of the irradiated nucleus of MCF 7 cells. Plasmid DNA (red) co-localizes on both figures with phosphorylated H2AX (green).

Studies of DNA double-strand breaks (DSBs), induced in human cells by γ-irradiation, showed movement of DSB in living cells, occasionally resulting in two or more break clusters (Fig. 10). Clustered DSBs represent hardly reparable lesions, persist in nuclei several days after irradiation and might pose a risk of chromosomal translocations. DSBs are not induced homogenously throughout chromatin but predominantly in active, decondensed euchromatin where DSB repair seems to be realized. It means that DSBs generated in heterochromatin have to protrude into low-dense chromatin. Despite of a low density of chromatin surrounding most of

DSBs, γ-H2A.X foci marking the breaks co-localize with dimethylated histone H3 at lysine 9 (dimet H3K9), HP1 and CENP-A proteins characteristic of heterochromatin; this indicates epigenetic silencing of non-repaired loci and/or participation of HP1 in reconstitution of chromatin structure after the DNA repair.

12 BAC clones for 11.3 Mb long RIDGE (region with increased gene expression) and 13 BACs for 12 Mb of ANTIRIDGE (region with low density of expressed genes) were used in different combinations (using repeated hybridization) to reveal higher order chromatin structure in these two regions. Individual BACs were separated by 0.5 to 1.2 Mb. Measurements of nuclear distances between them provide the possibility to find the differences in the compaction and arrangement of chromatin in both regions. Analysis of distances and angles among BACs are under way. Preliminary results show linear relationship between the mean-square of nuclear distance (d^2) between 2 BACs in the RIDGE region and their genomic separation (kb) indicating random walk polymer folding.

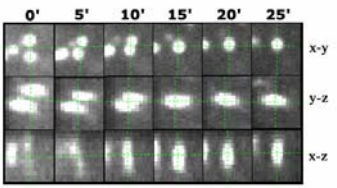

Fig. 10. Fusion of two DSBs marked by GFP-tagged NBS1 protein, presenting here the MRN repair complex. This observation was performed in living human MCF7 cell irradiated by 1.5 Gy of γ rays (^{60}Co).

Granted projects

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

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

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

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Kozubek S.: *Light microscopy in biological research* Biophysical Journal 88, 2005, 3741.

Stanislav Kozubek. *Spatial arrangement of the human genome and its possible functional role.* Atlas Genet Cytogenet Oncol Haematol. September 2005. URL : http://www.infobiogen.fr/services/chromcancer/Deep/SpatialArrangID20051.html

PhD thesis defended in 2005

Mgr. Eva Faltýsková, Expression profiles in colon cancer and normal epithelial tissue

CYTOKINETICS

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Laboratory of Cytokinetics focuses on the research of potential role of lipid membrane elements and their derivatives in cell signalling, and on the action of environmental substances, in particular 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. Interactions of membrane constituents with selected physiological regulators of cytokinetics (cytokines) are investigated in both tumor and non-tumor cell populations. 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.

Inhibitors of arachidonic acid metabolism in the effects of cytokines

Although it is known that regulators of hematopoietic and immune system, i.e. cytokines, polyunsaturated fatty acids (PUFAs) and their metabolites (eicosanoids) function in close collaboration, their effects are studied mostly separately. In the year 2005, the results of the project GACR No. 524/03/0766 were completed. Apart from leukemic cells, a complex comparative approach employing also embryonic stem cells and colon cell lines were used in this work. This may help to answer more general questions of 1) mechanisms of effects of n-3 and n-6 PUFAs and inhibitors of their metabolism; 2) the role of cytokines TGF-β1 and TNF-α in regulation of cytokinetics, especially in hematopoietic cells differentiating towards granulocytes and monocytes (Štika J. et al., Cancer Lett in press 2006, Vondráček J. et al. Leukemia Res. 30:81, 2006). This was achieved through multivariate analysis of cytokinetic data by the above mentioned factors and their combinations, and by analysis of data on a wide range of regulators of cell cycle, differentiation and apoptosis (changes in expression levels of cyclins, cyclin-dependent kinases, Bcl-2 family proteins, oxidative metabolism, etc.) (Souček K. et al. Leukemia Res. 20:607, 2006). It helped to achieve priority results improving the knowledge of causes of uncontrolled cell division. We have shown that both fatty acids and inhibitors of their metabolism significantly modulate the effects of cytokines studied and, consequently, cell proliferation, differentiation and apoptosis. These results indirectly imply the role of individual classes of eicosanoids in these processes, however, other mechanisms independent of these pathways are also suggested.

Interaction of fatty acids with endogenous apoptotic regulators of TNF family in colon epithelial cells

In the colon, many various agents (exogenous, endogenous) may operate together. Thus, signals from nutritional compounds (like polyunsaturated

fatty acids-PUFAs, and butyrate produced from fiber) and endogenous factors regulating cell growth, differentiation and apoptosis are integrated within the cell and have a substantial impact determining the final phenotype, metabolism and kinetics of colon epithelial cell popu-lation (see Fig. 1).

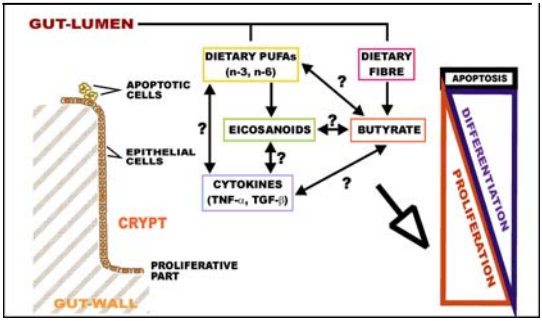

Fig. 1. Interactions of dietary factors and edogenous regulators supposed to affect cytokinetic of colonic epithelial cells

Using human colon epithelial cells in vitro, derived either from adenocarcinoma (HT-29) or normal fetal colon tissue (FHC), a/ the effects of PUFAs of ω-3 and ω-6 series and sodium butyrate (NaBt) and their mutual interactions, and b/ the role of PUFAs and/or butyrate in modulation of cell sensitivity to the effects of TNF family molecules (TRAIL, TNF-α, and anti-Fas antibody) have been investigated.

Our results showed that docosahexaenoic acid (DHA, ω-3) and arachidonic acid (AA, ω-6) are incorporated into cellular lipids and dose-dependently affect oxidative metabolism (production of reactive oxygen species-ROS, and lipid peroxidation-LP), cell proliferation (cell cycle and cell number) and death (% of floating cells, viability and apoptosis). The normal FHC cells appeared to be more sensitive to these effects than cancer HT-29 cells.

A different degree of response in HT-29 and FHC cells to TNF-α, anti-Fas antibody and TRAIL was also demostrated. Both cell lines did not respond to TNF-α, they expressed a limited sensitivity to anti-Fas antibody and different response to TRAIL. Sensitivity towards TRAIL-mediated apoptosis can be modulated at different levels in the TRAIL signalling pathway. Significant differences between HT-29 and FHC cells were

detected in the expression of DcR2, caspase activities, Apo2.7 protein expression, Bid and poly(ADP-ribose) polymerase (PARP) cleavage. The lower sensitivity of FHC cells to TRAIL could be attributed to a lower activity of mitochondrial pathway components followed by decreased caspase activities and PARP cleavage.

We suppose that the balance between pro-survival and apoptotic pathways may play an important role in regulation of the colon cancer cell sensitivity to the induction of apoptosis by TRAIL. TRAIL resistance at the basis of activation of „pro-survival kinases“, commonly found in many types of tumors, may limit the potential clinical effectiveness of TRAIL. Understanding the molecular events that are responsible for this resistance may provide new opportunities for cancer therapy. Combined treatments including TRAIL and ERK and/or Akt signalling pathway inhibitors could be an effective treatment strategy in epithelial-derived cancers, including colon cancer. Thus, we examined the role of the two most important pro-survival pathways, MAPK/ERK and PI3K/Akt, in regulation of the HT-29 cell sensitivity to TRAIL. Specific inhibitors of these pathways potentiated significantly the TRAIL-induced apoptosis, that was associated with significant changes at the level of mitochondria (decrease of mitochondrial membrane potential-MMP and changes of anti-apoptotic Mcl-1 protein level).

Our further results have shown that PUFAs may have beneficial effects in the colon enhancing apoptosis induced by other exogenous (NaBt) as well as endogenous (TNF family) compounds.

Pre-treatment of HT-29 cells with either AA or DHA attenuated cell cycle arrest caused by NaBt which is associated with modulation of $p27^{Kip1}$, but not $p21^{Cip1/WAF1}$ protein expression. On the other hand, PUFAs sensitised HT-29 cells to NaBt-induced apoptosis. Increased amount of floating cells and cells in subG_0/G_1 population was associated with increased ROS production and LP, decrease of MMP, activation of caspase-3 and -9, PARP cleavage, and decrease in the level of anti-apoptotic Mcl-1 protein. The observed effects were modulated by the addition of a protein synthesis inhibitor, cycloheximide, and partially reversed by the antioxidant Trolox (Hofmanová et al. 2005 a).

Pre-treatment with both types of PUFAs also modulated the proliferative and apoptotic response of HT-29 cells to TNF-α and anti-Fas antibody (CH-11). The higher amount of floating cells, cells in subG_0/G_1 population and apoptotic cells detected in pre-treated cells was potentiated by cycloheximide. The effects of CH-11 were associated with activation of caspase-8, -9, and -3, PARP cleavage, and decreased MMP, but these parameters were not significantly changed in PUFA pretreated cells (Hofmanová et al. 2005 b).

Cooperation of DHA with TRAIL in the induction of HT-29 cell death (changes in cell viability and adhesion, PARP cleavage and cells with nuclear fragmentation) was also demonstrated. These effects involved the caspase system (enhanced cleavage of caspase-8 and -3), ROS production, and mitochondrial pathway (cleavage of Bid protein and loss of MMP (Vaculová et al. 2005).

Differences in lipid metabolism of tumor and normal tissues also suggest a distinct response to supplied lipid compounds. In the study of the in vitro effects of five types of commercial parenteral lipid emulsions on HT-29 and FHC cells, the changes of fatty acid content in total cell lipids in both cell lines were detected. However, normal FHC cells responded more sensitively (increased % of floating cells and subG_0/G_1 population), probably due to their higher LP and ROS production. Co-treatment of cells with antioxidant Trolox reduced the effects observed. Our results imply that lipid emulsions can differently affect the response of colon cells of distinct origin (Hofmanová et al. 2005 c).

The studies of mechanism of the interaction between NaBt and TNF-□ using HT-29 and FHC cell models were completed. The changes of binding activity of NF-κB and PPAR-γ transcription factors were investigated during NaBt and TNF-α co-treatment. The studies on stable transfectants prepared by transfection with respective reporter construct indicated that NaBt-mediated PPAR-γ activity is potentiated by TNF-α, and TNF-induced activation of NF-κB is firstly enhanced and than suppressed by NaBt. The analysis of NF-κB activity was supplemented by detection of I-κB protein expression and by Electrophoretic Mobility Shift Assay (EMSA).

The system of non-adherent conditions for colonic cell lines cultivation was established in our laboratory in order to investigate the role of cell adhesion and detachment-induced apoptosis (anoikis) in the effects of agents studied.

We participated in the investigation of the role of receptor tyrosine kinase MET and Wnt signalling in transformation of colon epithelial cells performed at Department of Pathology, University of Amsterdam (Boon et al. 2005). Transfection of FHC cells with mutated MET led to activation of downstream PKB and MAPK, downregulation of E-cadherin and formation of tumors in mice comparing with cells transfected with wild-type MEK.

The effects of novel cytostatics

Generally, these studies were aimed to investigate the ability of novel Pt(IV) complex with adamantylamine, coded as LA-12, and/or its reduced counterpart with lower oxidation state Pt(II) - LA-9 to overcome acquired or intrinsic cisplatin resistance. Moreover, in the present studies the ability of the LA-12 and LA-9, to induce cytotoxicity, cell cycle perturbations, and cell death in SK-OV-3 ovarian cancer cell line with intrinsic cisplatin resistance has been characterized and compared both mutually and to clinically well established platinum drug cisplatin(II).

Our results showed that the LA-12 is characterized by significantly higher cytotoxicity than cisplatin in both parent cisplatin sensitive A2780 and cisplatin resistant A2780cis ovarian cancer cell lines and overcomes the acquired resistance to cisplatin in A2780cis cells (Kozubík et al. 2005). However, apoptosis is probably not the major type of cell death caused by LA-12 in doses around IC_{50} and IC_{90} in these cell lines, even though this platinum complex strongly increases the expression level of p53 protein. These facts could be associated with cell cycle perturbations. An important finding is the different dynamics of LA-12 effects on cell cycle and apoptosis compared to cisplatin.

Moreover, in our subsequent studies on SK-OV-3 ovarian cancer cells with intrinsic cisplatin resistance we observed strong differences between the effects of Pt(IV) complex - LA-12 and Pt(II) derivatives - LA-9 or cisplatin on cytokinetic parameters. Our results indicated that LA-12, is a compound active in a human ovarian cancer cell line that expressed LRP/MVP protein.

LA-12 displayed significantly higher cytotoxicity than cisplatin in these cells and overcame their intrinsic resistance to cisplatin and inhibited cell proliferation in a time- and dose-dependent manner. Similarly as for A2780, the major type of SK-OV-3 cell death caused by derivatives tested is probably not the apoptosis, although after 72 h, LA-12 in doses around IC_{50} caused significantly stronger apoptotic response than cisplatin or LA-9 (Horváth V. et al, Gynecologic Oncol, in press 2006). Our important finding of the different dynamics of cell cycle perturbation by LA-12 compared to Pt(II) derivatives cisplatin or LA-9 implies the necessity for further detailed studies, which should be undertaken for identification of possible preferential cell targets of LA-12 before its clinical use.

Mechanisms of action of environmental organic pollutants

Diverse environmental organic pollutants are known or suspected carcinogens that have been reported to possess tumor-initiating and/or tumor-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 2005, we have continued our studies on both model and novel toxicants, aimed especially at the effects associated with activation of aryl hydrocarbon receptor (AhR) or estrogen receptors (ER) (Plíšková et al., 2005 a, Vondráček et al. 2005). In collaboration with Johannes Gutenberg-Universität (Mainz, Germany), we have demonstrated a functional role of AhR in disruption of contact inhibition induced by polycyclic aromatic hydrocarbons (PAHs) in rat liver epithelial progenitor WB-F344 cell line. Such an effect might have a profound role in the carcinogenic impact of PAHs on epithelial cells, a primary target of chemical carcinogens. We have also found distinct effects of PAHs on cyclin A expression, cyclin A/cdk2 activity, $p27^{Kip1}$ levels and pRB hyperphosphorylation that are related to effects of AhR ligands on cell proliferation and apoptosis. The proliferative effects and activation of AhR have been found to correlate also during analysis of a large series of methylated PAH derivatives, again confirming a crucial role for AhR in proliferative effects of PAHs. Using a model AhR antagonist 3'-methoxy-4'-nitroflavone, we have further characterized cellular events that might lead to enhanced cell proliferation. This compound has been found to

induce AhR nuclear translocation and transcription of genes associated with cell cycle progression (such as cyclin A), despite having an inhibitory effect on transcription of some model AhR-regulated targets, such as CYP1A1. We have further conducted studies aimed at characterization of possible interactions of environmental pollutants (methylated-PAHs, PCBs) with endogenous regulators such as arachidonic acid or tumor necrosis factor-□ (TNF). We have found that both groups of compounds induce a release of arachidonic acid in WB-F344 cells, in a time- and structure-dependent manner. Model AhR ligands have been also found to interact with TNF in regulation of xenobiotic-metabolizing enzymes expression and cell proliferation in contact-inhibited cells. These data suggest that important link may exist between impact of inflammatory mediators and toxic compound on liver progenitor cells. In collaboration, we have also participated in a range of studies on effects of chemopreventive compounds (silybin derivatives) on transcriptional activation of AhR and ER (Plíšková et al., 2005 b), and impact of PCB contamination on ER signaling (both in vitro and in vivo), within a large-scale epidemiological EU-supported study conducted in PCB-contaminated area in eastern Slovakia (Plíšková et al., 2005 c).

Granted projects

GA CR 524/03/0766, Modulation of proliferation, differentiation and apoptosis of hemopoietic cells – interactions of cytokines, drugs and lipid nutrition compounds. Principal investigator: A. Kozubík, 2003 - 2005

GA CR 525/03/1527, Chemical identification and in vitro screening of toxicity of aromatic contaminants in agricultural production environment. Co- principal investigator: J. Vondráček, 2003 - 2005

FRVŠ 2528/2005, High effectiveness of platinum(IV) complex with adamantylamine in overcoming resistance to cisplatin and suppressing proliferation of ovarian cancer cells in vitro. Principal investigator: V. Horváth, 2005

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 AV ČR 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

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RNDr. Zdeněk Andrysík, Cell cycle deregulation induced by polycyclic aromatic hydrocarbons in rat epithelial liver cells: the role of MAPK and AhR

Mgr. Jiří Štika, The role of arachidonic acid metabolism in the effects of cytokine TNF-α in the human myeloid leukemia cells

DNA Biophysics and Genome Bioinformatics

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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.

Some regions of the human genome expand and the expansions cause a number of serious, mostly neurodegenerative diseases. With the aid of our bioinformatic results we found that the pathological expansions could at least qualitatively be simulated by PCR of short synthetic DNA fragments occuring in the human genome. This inspired us to study the DNA expansion during PCR in a systematic way.

Under various conditions of PCR (the number of cycles, annealing and elongation temperatures, KCl and $MgCl_2$ concentrations in the PCR buffer, its pH, presence or absence of the particular deoxyribonucleotide triphosphates, use of various polymerases), we performed more than 3,500 reactions with more than 400 DNA fragments having 4-150 nucleotides in length. The products were separated by means of agarose or polyacrylamide

gel electrophoresis and visualized by various stains. Some of the products were also analyzed by CD spectroscopy, thermal melting by means of UV absorption spectroscopy and digestion by DNaseI, S1 and P1 nucleases. The results were summarized into a table containing the primary structure of the DNA fragment undergoing PCR, conditions of PCR and characterization of the PCR product including a semiquantitative estimate of its amount and length. The table is very extensive (over 100 pages of the A4 format). Its analysis has so far revealed the following information about regularities of DNA expansion in the course of PCR.

1. The result of PCR depends on the number of PCR cycles. Some fragments expand even after few cycles and further cycles do not lead to substantial changes in the length and amount of the PCR product. Other DNA fragments provide detectable amounts of the expanded DNA molecules only after much higher numbers of PCR cycles.

2. The expansion depends on the polymerase used.

3. The expansion products are most probably normal Watson-Crick duplexes. This is indicated by the observations that they do not arise in the absence of any of the four triphosphates, they stain with ethidium and are digested by DNase I, but not the single-strand DNA specific S1 and P1 nucleases. The PCR products also provide the characteristic CD spectroscopic, UV absorption and thermal melting properties.

4. The PCR products depend on the annealing temperature. They are generated at some annealing temperatures but not at others. The annealing temperatures leading to the expanded PCR products depend on the DNA fragment used. On the other hand, the PCR products do not significantly depend on the elongation temperature.

5. Tests of the influence of the concentrations of KCl and $MgCl_2$ and the pH value showed that maximum amounts of the products were produced at their values recommended by the polymerase producer.

6. The results of PCR depends on the length of the PCR fragments. We have examples when too short as well as too long fragments do not provide the expanded molecules whereas intermediate DNA fragments with the same primary structure provide the expanded molecules.

7. Thermal denaturation experiments showed that the DNA fragments were mostly, if not totally, denatured at the annealing temperatures leading to expansion. This means that the expansion canhardly result from the common primer effect unless the polymerase stabilizes the primer-template association.

8. We observe the expansion even with DNA fragments such as $(T)_n$ or $(CA)_n$ with which we have never observed homoduplex formation.

9. Some seemingly small changes of the primary structure of the DNA fragment lead to qualitative changes in the expansion. For example, A_8T_8 belongs among the most expanding fragments whereas T_8A_8 does not expand at all.

10. Some fragments containing only G and C do not expand, others do. Hence neither is the (G+C) content the decisive factor of the expansion.

11. We studied expansion of $(TTAGGG)_n$ fragments of various lengths, which occur at the ends of human telomeres and whose length relates to aging and cancer. These fragments do not expand in the course of PCR. This may be caused by guanine tetraplexes that these fragments form. The tetraplexes are probably too stable to serve as templates for the synthesis of the expanded DNA.

12. We have also studied $(TGG)_n$ and $(CCA)_n$ fragments that are the most length polymorphic microsatellites in the human genome. Both were found to expand a lot in the course of PCR. This is probably a consequence of the very extensive conformational polymorphism exhibited mainly by the $(TGG)_n$ strand.

13. We have discovered a unique expansion of the $(GAA)_{30}$ strand connected with Friedreich ataxia. $(GAA)_{29}$ and other related fragments do not expand.

14. Generally, we have found that some DNA fragments expand into kilobase lengths during PCR whereas others do not. Most of the expanding fragments have simple, repetitive primary structures characteristic for genomic microsatellites but not all such fragments expand in the course of PCR. This correlates with results of our bioinformatic studies of various genomes which suggest the same regularity.

15. Terminal nucleotides of the expanding DNA fragment are important for the expansion.

16. Fragments rich in (A+T) expand better than those rich in (G+C).

17. Fragments rich in pyrimidine nucleotides expand better than those rich in purine nucleotides.

18. A decisive role in the expansion is also played by a still unknown factor(s) characterizing the DNA molecule as a whole.

The above results have implications for the pathological microsatellite expansion in the human genome, to the appearance of the first kilobase DNA molecules as well as to the evolution of the modern organism genomes.

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

IGA MH CR NM 7634-3/2003, Development of a method for prediction of the pathological trinucleotide repeat expansion in the human genome.Principal investigator: Jaroslav Kypr, 2003-2005

GA AS CR FAA1004301, Conformational transitions in plasmid DNA. Principal investigator: Karel Nejedlý, 2003-2005

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Hanzálek, P., Kypr, J.: *DNA in phosphorus atom representation: the heteronomous double helices of poly(dA).poly(dT) and poly(dG).poly(dC) and simulation of the yeast genome and of a human chromosome DNA*. J. Theor. Biol., 232, 2005, 83-91.

FREE RADICAL PATHOPHYSIOLOGY

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Projects active in the lab within the year 2005 were associated mainly with: 1) the study of mechanisms leading to the generation of reactive oxygen metabolites by phagocytes including the interaction of phagocytes with other cell types and their mediators. The possible modulation od phagocyte metabolic activity using synthetic drugs like antihistamines and beta-adrenoceptor blockers was also studied; 2) the role of phagocyte-derived reactive oxygen metabolites and enzymes in ischemia/reperfusion and inflammation; 3) the response of antioxidative adaptive mechanisms on oxidative stress induced during these pathophysiological conditions. There were active collaborative links with other national and foreign research institutions in respect to phagocyte biology (University of Turku, Finland), pharmacological modulation of phagocyte activity (Institute of Experimental Pharmacology, SAS, Bratislava, Slovakia), modulation of oxidative stress using natural products (University of Barcelona, Spain; Institute of Organic Chemistry, BAS, Sofia, Bulgaria) or drugs (Instituto de Investigaciones Biomedicas, CSIC, Barcelona, Spain; National Institute of Traumatology, Budapest, Hungary), and electrochemical detection of nitric oxide (Palacky University, Olomouc, Czech Republic).

The effect of beta-adrenoceptor blocker carvedilol and H_1-antihistamines on respiratory burst of phagocytes and phagocyte-derived oxidants

It was found that, in the cell free system, carvedilol (the drug with beta-adrenoceptor blocking effect) dose dependently scavenged reactive oxygen metabolites in the following rank order: hydroxyl radical > hydrogen peroxide > superoxide anion. The inhibition of myeloperoxidase by carvedilol was also significant. Carvedilol in 0.01 mmol/L and higher concentrations significantly inhibited luminol-enhanced, phagocyte-derived chemiluminescence (CL) induced by both receptor-bypassing stimuli (calcium ionophore A23187, PMA)) and receptor-operating stimuli (fMLP, opsonized zymosan). In the presence of blood platelets, carvedilol potentiated the metabolic activity of phagocytes induced with A23187. This could be the result of the supportive effect of serotonin liberated from platelets by A23187.

In our study, effects of selected H_1-antihistamines of the 2nd generation on the respiratory burst of rat phagocytes were compared with Dithiaden, the representative of H_1-antihistamines of the 1st generation. Ketotifen-fumarate inhibited CL activity of leukocytes similarly as Dithiaden. Both drugs were effective in the concentrations higher than 0.01 mmol/L. The explanation could be found in high affinity of Ketotifen-fumarate not only for H_1-receptor but also for other receptors and in a similar chemical structure of both drugs. Astemizole was even more effective when only 0.001 mmol/L concentration did not affect the CL of leukocytes. In contrary, Loratadine was much less effective inhibitor of leukocyte–derived CL in comparison with Dithiaden. Loratadine inhibited CL in the highest concentration (0.5 mmol/L) only. The reason could be in higher selectivity but in lower affinity for H_1-receptor. No inhibition effects of acrivastine on CL were observed. Acrivastine shows high selectivity to H_1-receptor which supports an idea that the inhibition of leukocyte-derived CL induced by some antihistamines is caused at least partly via non H_1-receptor pathway.

As observed in further experiments, the decrease of CL observed for Astemizole, Dithiaden, Ketotifen-fumarate and Loratadine was not caused by direct scavenging properties of these antihistamines against superoxide anion, hydroxyl radical and peroxyl radical.

Based on our results, antihistamines which did not interfere with microbicidal mechanisms of leukocytes could be applied preferentially in situations where the organism has to cope with an infection. On the other hand, antihistamines inhibiting CL activity of leukocytes should be used preferentially under pathological conditions accompanied by the overproduction of reactive oxygen species.

Antiinflammatory and antioxidative properties of serotonin and its precursors

It was shown in our previous studies that serotonin inhibited the chemiluminescence response of neutrophils in human whole blood. The inhibition was partially due to the direct quenching activity of serotonin and due to the inhibition of myeloperoxidase activity. The hypothesis that the inhibitory activity of serotonin might be also receptor mediated was evaluated using various serotonin receptor agonists ((±)-8-Hydroxy-2-(di-*n*-propylamino)tetralin hydrobromide; (±)-DOI hydrochloride; 1-(3-Chlorophenyl)biguanide hydrochloride; 5-Carboxamidotryptamine maleate salt; Cisapride; *O*-Methylserotonin hydrochloride) and antagonists. None of the studied agonists exerted any direct antioxidative properties. Only (±)-DOI hydrochloride, a selective 5-HTR_2 agonist, exerted similar effects on phagocytic cells as serotonin.

The antioxidative properties of serotonin were compared to antioxidative properties of its precursors (L-tryptophan a 5-hydroxytryptophan) and selected metabolites (N-acetyl-5-hydroxytryptamine and melatonin). The total antioxidative capacity (TRAP) of studied compounds in a concentration range of 1 – 0.0001 mmol/L was determined using luminol-enhanced chemiluminescence as an ability to quench peroxyl radicals, generated by thermal decomposition of ABAP (2,2-azo-bis-2-amidinopropan hydrochloride). The antioxidative properties of studied compounds were further studied in various chemical systems generating individual reactive oxygen species: hypoxanthine/xanthine oxidase system (generates superoxide anion), hydrogen peroxide/ferrous sulphate system (generates hydroxyl radical) and hydrogen peroxide alone. Only two highest concentrations of serotonin exerted significant antioxidative properties against peroxyl radical. As for the other individual reactive oxygen species,

serotonin exerted the most potent antioxidative properties against hydroxyl radical and the least potent antioxidative properties against hydrogen peroxide. The other studied compounds exerted only a slight antioxidative capacity when compared to serotonin depending on the individual reactive oxygen species-generating systems. It can be concluded that serotonin exerted direct antioxidative properties depending on its unique chemical structure resulting from the type and location of substituents on the indole skeleton.

The role of myeloperoxidase in acute lung inflamation

Free radicals and enzymes released by polymorphonuclear neutrophils play a key role in inflammatory process, however the mechanisms remain incompletely characterized. Therefore, we suggested a new regulatory role of myeloperoxidase (MPO), an abundant hemoprotein of neutrophils, in the modulation of the course of acute lung inflammation by alternations of redox-sensitive signaling pathways controlling inflammatory processes. Our hypothesis was tested by various experimental approaches and following main results were obtained in cooperation with Dr. Eiserich at University of California in Davis, USA.

Molecular mechanisms governing the binding of MPO to ECM proteins, and its effect on MPO enzymatic functions were characterized. It was demonstrated that MPO avidly binds to extracellular matrix proteins by a mechanism dependent upon glycosaminoglycans enhancing the enzymatic activity of MPO. These results provide novel insights into the biological compartmentalization and activity of MPO during acute and chronic inflammation.

Next, role of MPO in acute pulmonary inflammation in mice was evaluated. Direct role of MPO in acute pulmonary inflammation induced by lipopolysaccharide was evaluated with taking advantage of MPO deficient mice. Significantly higher lung neutrophilia in MPO deficient mice was connected with higher levels of chemotactic cytokine RANTES and delayed onset of apoptosis in MPO deficient neutrophils. It suggests that MPO modulates the course of acute lung inflammation by alternation of neutrophil apoptosis and their clearance from the site of inflammation.

To test an ability of MPO, either directly or indirectly, to modulate the balance of pro- and anti-inflammatory lipid mediators during acute inflammation a comprehensive array of enzymatic- and free radical-derived linoleic and arachidonic acid oxidation products in mice after induction of acute inflammation was evaluated. MPO deficiency significantly decreased levels of epoxides of these fatty acids with known anti-inflammatory properties and, in contrast, significantly increased levels of pro-inflammatory leukotrienes. These data suggest that MPO can directly function as a fatty acid epoxygenase, and this may be responsible for leukotoxin synthesis by neutrophils. In contrast, MPO can contribute to the oxidative destruction of leukotrienes by MPO. Our results reveal that MPO, either directly or indirectly, modulates the balance of pro- and anti-inflammatory lipid mediators during acute inflammation.

Protective effect of ischemic preconditioning

Changes in small molecular antioxidants were followed up in a model of small intestinal ischemia in Wistar rats to evaluate their possible role in ischemic preconditioning. The superior mesenteric artery (SMA) was occluded as follows: Group I - SMA was occluded for 60 minutes. Group P1 - 15 minutes preconditioning and 5 min reperfusion and 60 min SMA ischemia. Group P2 - 15+15minutes preconditioning with 5 minute reperfusion periods interposed + 60 minutes SMA ischemia. Group P3 - 15+15+15 preconditioning with 5 minute reperfusion periods interposed + 60minutes SMA ischemia. Total antioxidant capacity in serum, serum antioxidants (uric acid, ascorbic acid, bilirubin), and the thiobarbituric acid reactive substances in both serum and mucosa were measured. The 60-minute ischemia alone (group I) induced a significant increase in TRAP when compared to the intact control (group C). Short ischemia periods foregoing the main ischemia prevented the increase in TRAP observed in group I and the group P2 reached the level of non-significance when compared to intact controls. The concentration of uric acid was significantly increased in group I, when compared to the group C. This increase was prevented significantly in groups P1 and P3, while no significance was observed in group P2. Ascorbic acid serum concentration was increased in all experimental groups when compared to that in intact controls. For

bilirubin, a significant decrease was observed in its concentration in group I in comparison to group C. Short intermittent ischemia periods foregoing 60 minute-ischemia prevented this decrease The correlation between TRAP and ascorbic acid and uric acid concentration was R = 0.67 and R = 0.52, respectively. Lipid peroxidation in serum samples was significantly higher when compared to intact controls and lipid peroxidation in mucosa samples slightly decreased depending on the duration of intermittent ischemia when compared to group I. In short, the small molecular antioxidants measured did not contribute to the phenomenon of ischemic preconditioning.

The role of small molecular antioxidants in ischemia and reperfusion

Changes in small molecular antioxidants were followed up in a model of small intestinal ischemia in Wistar rats to evaluate their contribution to the plasma total peroxyl radical-trapping antioxidant capacity (TRAP). Ischemia of anaesthetised Wistar rat small intestine was induced by occluding superior mesenteric artery for 45 minutes. Blood samples were taken via heart puncture after 2 and 4 hours of reperfusion. TRAP of plasma was determined by luminol-enhanced chemiluminescence. Individual plasma antioxidants (uric acid, albumin, SH-groups) and TBARS, an index of lipoperoxidation, were determined spectrophotometrically. TRAP of plasma increased significantly after reperfusion in a time-dependent manner. From the examined antioxidants, only uric acid contributed significantly to TRAP increase after reperfusion and a high correlation between these two parameters was proved (R = 0,93). However, even the high increase in uric acid concentration accompanied by increase in TRAP value was not sufficient to prevent the lipoperoxidation in plasma – interestingly, there was found a significant correlation between TRAP and TBARS (R = 0,76). We came to conclusion that the increase in TRAP after reperfusion is more an indicator of injury than of targeted mobilisation of protective antioxidant mechanisms. This view is supported by two observations. First, the high increase in plasma concentration of uric acid was probably caused by shock and circulatory failure that lead to decreased renal excretion and to decreased degradation of this compound by hepatic urate oxidase. Second, TRAP can be strongly influenced by the antioxidants released from destructed cells. Even the mild hemolysis can significantly

increase TRAP value as confirmed by additional experiments. It is very plausible that the cell destruction contributed to TRAP increase in animals exposed to ischemia/reperfusion since there was found a remarkable disproportion between plasma concentration of SH groups and concentration of albumin, the main donor of plasma SH groups. In the animals that underwent 4 hour reperfusion the concentration of plasma SH groups was on control level whereas albumin concentration was decreased by 20 %. Albumin contributes to the total plasma pool of SH groups by 80 %; thus, a decrease of 20 % should manifest as a significant decrease in the level of SH groups. It is possible that the recovery of SH groups to the control level in this group of animals might be caused by releasing protein and non-protein substances containing SH groups from the intracellular milieu.

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

Philip Morris External Research Program, Modulation of Acute Pulmonary Inflammatory Responses by Myeloperoxidase. Principal Investigator: L. Kubala 2005

MEYS - Kontakt 131, The effects of blood platelets on the oxidative burst of neutrophils. Principal investigator: Antonín Lojek, 2004 - 2005

MEYS - Kontakt MD_45_1, Changes in granulocyte functions following severe tissue injury. Principal investigator: Antonín Lojek, 2004 - 2005

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MVDr. Ivana Papežíková, Antioxidant adaptive mechanisms in organ ischemia and reperfusion.

Mgr. Martina Pavelková, The relation of reactive oxygen and nitrogen species to the functional properties of normal and transformed cells

STRUCTURE AND DYNAMICS OF NUCLEIC ACIDS

HEAD

JIŘÍ ŠPONER

SCIENTISTS

JUDIT ŠPONEROVÁ, NAĎA ŠPAČKOVÁ, KAMILA RÉBLOVÁ

GRADUATE STUDENTS

FILIP RÁZGA, MARYNA KRASOVSKÁ

Principles of base pairing in complex functional RNAs are strikingly different from those in DNA molecules. Due to the presence of the 2'-OH hydroxyl group of ribose, RNA molecules utilize an astonishing variability of non-Watson-Crick base pairing patterns to build up their structures and perform the biological functions. We have carried out a systematic quantum chemical analysis of RNA base pairing with the aim to complement the structural information known from crystallographic studies with the energetics of interactions and other electronic properties that are not accessible by the experiments. Advanced electron correlation calculations provided a detailed structural and energetic characterization for the cis- and trans- Watson-Crick/Sugar Edge as well as cis- and trans-Sugar Edge/Sugar Edge base pair families. We also predicted structures and energies of many RNA base pairs there have not yet been observed by the atomic resolution experiments.

We found sound correlation of the intrinsic structures of key RNA base pairs with their crystallographic geometries for the Watson-Crick/Sugar Edge and the cis-Sugar Edge/Sugar Edge base pairs. This revealed that the principle of isosteric substitutions in RNA originates in the intrinsic structural properties of the isolated base pairs. Occasionally, deviations were observed between the computed and crystal structures in the trans-Sugar Edge/Sugar Edge base pair family, which was explained by the fact that these RNA interactions often participate in more complex interaction patterns, such as the crucial tertiary interaction called A-minor motif.

The calculations further show that the relative importance and absolute value of the dispersion energy in the RNA base pairs are enhanced compared to the standard (canonical) base pairs. Eventually, dispersion is the dominating stabilizing force in the Sugar Edge/Sugar Edge interaction patterns. This likely is one of the reasons why the A-minor motif is the single most prominent type of tertiary interactions in functional RNAs. We also found that the distribution of the total interaction energy over the sugar-base and base-base contributions is determined by the cis-trans isomerism. Further, the calculations demonstrated that the Cornell et al. force field commonly used in molecular modeling and simulations of nucleic acids provides satisfactory description of the non-Watson-Crick RNA base pairs.

Ab initio computational modeling was used to investigate the mechanism of action of anticancer titanocene derivatives. We considered a series of drug candidates with variable antitumor activities, and for each compound we computed the thermodynamic driving force of the hydrolysis and protolysis reactions. The calculations were carried out at the Density Functional Theory level and the solvent effects were included with the COSMO polarized continuum solvent model. The computed thermodynamic data obtained for the protolysis reaction showed a sound correlation with the anticancer activity of the studied titanocene derivatives while no correlation was observed for the hydrolysis reaction. Thus, we concluded that the different anticancer activity of the substituted titanocene derivatives cannot be directly related to the change of the metal-aromatic ring interaction caused by addition of pendant arms to the cyclopentadienyl rings. Instead, we suggested that proton induced loss of the aromatic rings may account for the experimental observations. Thus, titanocene might dissociate before it reaches its primary target, i.e., the DNA.

The Hepatitis Delta Virus (HDV) ribozyme (Fig. 1) is an RNA enzyme found in the RNA genome of a human pathogen, the Hepatitis Delta Virus. The ribozyme catalyzes a site-specific transesterification reaction generating 5'-hydroxyl and 2',3'–cyclic phosphate termini, and plays essential role in formation of antigenomic and genomic strands during the viral replication of multimeric intermediates. The HDV ribozyme was the first RNA enzyme for which direct involvement of its own nucleobase C75 in catalysis was suggested.

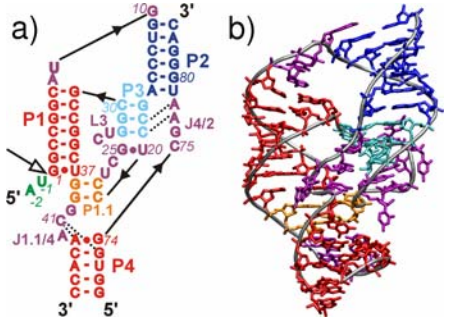

Fig. 1. a) Sequence and secondary structure of the simulated HDV ribozyme, the cleavage site is highlighted by an open arrow. b) 3D structure of the product form.

Recent crystal structures of the precursor and product of self-cleavage, together with detailed kinetic analyses, have led to hypotheses on the catalytic strategies employed by the HDV ribozyme. The crystallographic data suggest that C75 is well poised to act as the general base to deprotonate the nucleophilic 2'-OH at the cleavage site. On the other hand, the mechanistic data suggest that C75 may act as the general acid to protonate the leaving group, which requires C75 to be N3-protonated just before cleavage.

We have carried out molecular dynamics (MD) simulations to test the plausibility that specific conformational rearrangements are involved in catalysis. A precursor simulation with unprotonated C75 revealed a rather weak dynamic binding of C75 in the catalytic pocket with spontaneous, transient formation of a H-bond between U-1(O2') and C75(N3). This H-bond would be required for C75 to act as the general base. Upon protonation in the precursor, $C75H^+$ has a tendency to move deeper towards loop L3 (resembling its product location) and establish a firm H-bonding network within the catalytic pocket. However, a $C75H^+$(N3)-G1(O5') H-bond, which would be expected if C75 acted as a general acid catalyst, is not observed on the present simulation timescale. The simulations confirmed that loop L3 is relatively dynamic and may serve as a flexible structural element, possibly gated by the closing U20.G25 base pair, to facilitate a conformational switch induced by a protonated $C75H^+$. Loop L3 also controls the electrostatic environment of the catalytic pocket, which in

turn may modulate C75 base strength and metal ion binding.

Kink-turn (K-turn) motifs are asymmetric internal loops found at conserved positions in diverse RNAs (recurrently observed in ribosomes and showing high degree of sequence conservation), with sharp bends in sugar-phosphate backbones producing "V"-shaped structures. This unique 3D architecture is stabilized by the A-minor interaction between the helical stems (see Fig.2). Explicit-solvent MD simulations were carried out for selected K-turns from 23S rRNA (Kt-38, Kt-42, Kt-58) and for K-turn of human U4 snRNA (Kt-U4). The MD simulations reveal hinge-like K-turn motions on the nanosecond time-scale and thus indicate that K-turns are dynamically flexible and capable of regulating significant inter-segmental motions. The nanosecond dynamics of isolated K-turns can be qualitatively considered as a hinge-like motion of two rigid helical stems controlled by a very flexible internal loop. The overall behavior of the simulations suggests that the sampled geometries are essentially isoenergetic and separated by minimal energy barriers. This internal dynamics of K-turns is strikingly different for example from the bacterial 5S rRNA Loop E motif or BWYV frameshifting pseudoknot which appear to be rigid in the same type of simulations. Bistability and flexibility of K-turns were also suggested by several recent biochemical studies.

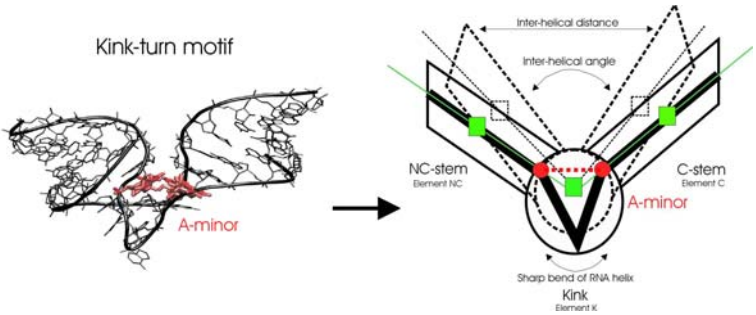

Fig. 2. Kink - turn motif.

The first conserved A-minor interaction between the K-turn stems is entirely stable in all simulations. The angle between the helical arms of Kt-

38 and Kt-42 is regulated by local variations of the second A-minor (type I) interaction between the stems (see Fig. 2). Its variability ranges from closed geometries to open ones stabilized by insertion of long-residency waters between adenine and cytosine. The simulated A-minor geometries fully agree with x-ray data. Kt-58 and Kt-U4 exhibit similar elbow-like motions caused by conformational change of the unpaired base from the nominally unpaired region instead of A-minor dynamics. Despite the observed substantial dynamics of K-turns, the key tertiary interactions are stable and no sign of unfolding is seen. The presence of K-turns at key functional sites in the ribosome suggests that they confer flexibility to RNA protuberances that regulate the traversal of tRNAs from one binding site to another across the interface between the small and the large subunit during protein synthesis cycle. Specifically, Kt-42 is suggested to allow large scale motions of the factor binding domain (seen in Cryo-EM) in all three kingdoms while Kt-38 (when present) may be essential for the dynamics of the A-finger regulating the access of tRNA from A-site to P-site. Thus, while the whole ribosomal assembly superficially resembles a sophisticated LEGO toy, K-turns are well poised to act as major recurrent elbow-like dynamical ribosomal building blocks.

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 mechanism of action. 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

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F. Barone, F. Lankaš, N. Špačková, J. Šponer, P. Karran, M. Bignami and F. Mazzei: *Structural and dynamic effects of single 7-hydro-8-oxoguanine bases located in a frameshift target DNA sequence.* Biophysical Chemistry 118, 2005, 31-41.

PhD thesis defended in 2005

Mgr. Kamila Réblová. Molecular Dynamics Study of Functional RNA Molecules

ANALYSIS OF CHROMOSOMAL PROTEINS

HEAD

MICHAL ŠTROS

RESEARCH FELLOWS

ALENA BAČÍKOVÁ, TEREZIE BARIČÁKOVÁ

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EVA POLANSKÁ

We have found that transient expression of retinoblastoma protein pRb in osteosarcoma Saos-2 cells significantly reduces the transactivation potential of HMGB1 protein. These results may explain our previous findings demonstrating a distinct effect of HMGB1 on transactivation of p53/p73-responsive gene promoters in cells containing (H1299, K562, MCF-7) or lacking functional pRb (Saos-2). We have also determined amino acids of the evolutionarily conserved HMG-box domain B involved in DNA bending and recognition of bent DNA. These results helped us to understand mechanism of binding of HMGB1 to hemicatenated DNA loops (Jaouen et al.: Determinants of specific binding of HMGB1 to hemicatenated DNA loops. *J. Mol. Biol.* **353**, 822-837, 2005).

Topoisomerase IIα (topo IIα) is a target of a number of clinical effective anticancer drugs such as etoposide and doxorubicin. We have discovered that HMGB1 could stimulate catalytical activities of human topo IIα and also regulate the activity of the *topo IIα* gene promoter. Our preliminary results also suggest that the expression of the *topo IIα* gene promoter is further modulated by pRb.

We have also proposed a model depicting a possible involvement of HMGB1 and tumor suppressor proteins p53 and pRb in modulation of activities of *the topo IIα* gene and its product, topoisomerase IIα (Fig. 1).

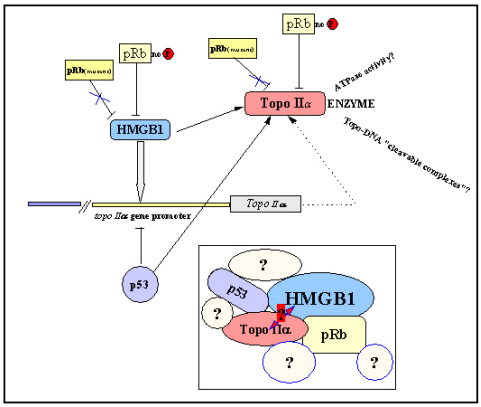

Fig. 1. Proposed mechanism of involvement of HMGB1 and tumor suppressor proteins in modulation of activity of topoisomerase IIα. HMGB1 protein stimulates the activity of topo IIα. Activity of topo IIα is also stimulated by p53 but inhibited by (dephosphorylated, active form) of pRb. HMGB1 can up-regulate the activity of the *topo IIα* gene, but p53 and possibly also pRb inhibit the activity of the *topo IIα* gene (Štros et al., paper in preparation). Inset: possible interactions of HMGB1 with selected proteins involved in response (or resistance) of cells to anticancer drugs specifically inhibiting topo IIα.

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

Publications

Jaouen, S., de Konnig, L., Gaillard, C., Muselíková-Polanská, E., Štros, M., Strauss, F.: *Determinants of specific binding of HMGB1 protein to hemicatenated DNA loops.* J. Mol. Biol., 353, 2006, 822-837.

BIOMACROMOLECULE PHYSICS

HEAD

VLADIMÍR VETTERL

SCIENTISTS

LUDĚK STRAŠÁK, STANISLAV HASOŇ

GRADUATE STUDENTS

LUKÁŠ FOJT, PETRA ŠTEFKOVÁ, JAKUB DVOŘÁK

UNDERGRADUATE STUDENTS

JAN NOVÁK, LUCIE VETTERLOVÁ, DANA OHLÍDALOVÁ

TECHNICAL ASSISTANTS

JAN IGNÁC, LIBUŠE GANDELOVÁ

Microanalysis of oligonucleotides by cathodic stripping voltammetry at amalgam-alloy surfaces in the presence of copper ions

We proposed a new method to the: (a) detection of different homopurine oligonucleotide (ODN) lengths at a picomolar concentrations (related to the monomer), and (b) determination of the number of purine (adenine + guanine) units within the ODN samples containing a random sequence segments involving both the purine and pyrimidine units in connection with the Hg-modified graphite electrodes and solid amalgam-alloy ones. This electrochemical method is based on the electrochemical accumulation of the complex of the purine base residues released from ODN-chains during acid hydrolysis of ODNs with copper ions Cu(I) (ahODN-Cu(I) complex) at the potential of reduction of copper ions Cu(II) followed the cathodic stripping of this complex from the electrode surface. We showed that: (a) the intensity of the cathodic stripping current peak of the electrochemically accumulated ahODN-Cu(I) complexes increased linearly with increasing number of purine units in the ODN-chains used, and (b) the percentage

content of purine (adenine + guanine) units to the whole length of different ODNs (each of them has a same length) correlate to the percentage content of the intensity of the cathodic stripping current peak of the electrochemically accumulated ahODN-Cu(I) complexes. We also showed that a sensitive analysis of acid-treated ODN in the presence of copper can be performed under a three-electrode electric circuit in "inverted" microliter electrochemical cell (20-µl volumes) at the amalgam-alloys.

Sensitive determination of oligonucleotides by anodic adsorptive stripping voltammetry at surface-roughened glassy carbon electrodes in the presence of copper

We drown a procedure, which is increased the sensitivity of the detection of the acid hydrolyzed ODN samples containing the purine units within the ODN-chains in the presence of copper at the surface-roughened glassy carbon electrode (GCE). The surface-roughening of the GCE cause the increasing numbers of active sites for electrodeposition of the ahODN-Cu(I) complex. The detection of ODNs is based on the enhancement of the oxidation peaks of purine bases after anodic stripping of the electrochemically accumulated complex of Cu(I) with a purine base residues released from ODN-chain during acid hydrolysis of the ODNs (ahODN-Cu(I) complex) from the carbon electrode surface. The optimisation of the measurement showed that the most sensitive detection of the ODN samples can be performed at the GCE mechanically polished by abrasion particles with diameter of about 15 µm. The 15-µm GCE has the root-mean-square function (RMS) of about 296 nm. The heights of the ODN signals at the GCE polished with 1 µm diamond paste (1 µm-GCE) were about 22-times less than on the 15 µm-GCE.

Effect of electromagnetic fields on biological objects

The effect of extremely low frequency (ELF) magnetic fields (f = 50 Hz, amplitude of magnetic field induction B_m = 10 mT, t = 25 ^{o} C) and high frequency electromagnetic fields (GSM band, f = 900 MHz, output power P = 1W.cm^{-2}) on bacteria, yeat and human small lung carcinoma cells was studied.

ELF fields – bacteria. The changes in growth curves of different bacterial strains – rod shaped (*E. coli, L. adecarboxylata)* and spherical (*P. denitrificans, S. aureus, S. paucimobilis, R. erythropolis)* as well as the decrease in viability of all bacteria after magnetic field exposure during the time interval 48 min. were observed. The decrease was bigger for rod-shaped bacteria.

ELF fields – yeasts. A 50 Hz magnetic field effect on the growth of yeasts *Saccharomyces cerevisae* was studied. The cylindrical coil induced magnetic fields with inductions up to 10mT. Duration of exposure varied up to 24min. Exposure took place at laboratory temperature (24-26°C) and the air ventilator maintained the temperature at the place of sample. We measured growth curves of yeasts in broth and we calculated the number of CFU (colony forming units) on solid soil. We found that magnetic field decreases the number of yeasts, and slowed their growth down.

ELF fields - small lung carcinoma cells A549. We studied low-frequency magnetic field effect on cytoskeleton and on the structure of chromatin in human cells. We used cell line of small lung carcinoma (A549) and the effects of magnetic field on cytoskeleton and higher-order chromatin structure were analyzed 96 hours after magnetic field exposure. In such affected and control cells the F-actin was estimated using FITC-conjugated Phalloidin and mitochondria were studied using MitoTracker (Molecular Probes). Images of cytoskeleton and genetic loci were acquired using confocal microscopy and analysis was performed by FISH 2.0 software. Slight morphological changes of F-actin filaments and mitochondria were observed in affected cells and nuclear condensation was found. These effects could be related to the process of cell death apoptosis probably induced by magnetic field. The studies aimed at centromeric heterochromatin (9cen) did not show statistically significant changes.

High-frequency fields. In co-operation with Faculty of Medicine, Charles University of Plzeň we measured the viability of bacteria *E.coli* and yeast *S. cerevisae* after their exposure to high frequency electromagnetic field (900 MHz, P = 1 $W.cm^{-2}$). We have found that the viability is decreased by the electromagnetic field, the decrease of viability is lower for yeasts.

Electromagnetic fields in public transportation

We measured magnetic fields in tramways, trolleys and buses in Brno city, tramways, trolley and buses in Ostrava city and subway and tramways in Prague. In addition the magnetic fields in intercity trains were measured. Our results stated that the measured values do not exceed the ICNIRPs limit.

Monitoring of the growth of yeast Saccharomyces cerevisiae on mercury electrode

The growth of yeast culture *S.cerevisiae* was measured using mercury electrode. We measured the differential capacity of the electrode double layer of the mercury electrode immersed in the yeast culture from the beginning of the growth during 8 hours. The capacity maximum observed at potential –0.4V was higher for longer time of cultivation. Differential capacity depends on the number of cells in the electrolyte. We were able to make the calibration curves and we estimated the number of cells in solutions in the range 10^3-10^6 cells/ml. From the time dependence of the differential capacity the formation of biofilm at the mercury electrode can be followed

Viability of osteoblasts in activated platelet-reach plasma PTP

We started to measure the proliferation of osteoblasts in PTP-modified medium activated by tissucol. We compared different concentrations of PTP in medium and the effect of its activation.

Granted projects

GA AS CR S5004107, Aplication of biophysical methods in biotechnological and clinical praxis. Principal investigator: V.Vetterl, 2001 – 2005

ME, KONTAKT 685, Development of methods and construction of sensors for the detection of interaction of DNA with drugs. Principal investigator: V.Vetterl, 2003 - 2005

GA AS CR A4004404, Interaction of biopolymers with ligands and detection of their conformation changes at phase boundaries with the aid of

electrochemical and optical methods. Principal investigator: V. Vetterl, 2004-2006.

GA AS CR KJB 4004305, Chemical modified electrodes in electrochemical analysis of nucleic acids and their components. Principal investigator: S. Hasoň, 2003-2005.

ME, FRVŠ 2541/2005/F4/a Aplication of electrochemical and optical methods by the proposal of chemical sensors and biosensors. Principal investigator: V. Vetterl, 2005

ME, Bilateral Czech-Greek scientific-technical cooperation. RC_3_24 Development of methods and construction of sensors for the detection of interaction of DNA with drugs. Principal investigator: V.Vetterl, 2003 - 2005.

ME, Bilateral Czech-Slovac scientific-technical cooperation. 94 Study of DNA hybridization at surfaces. Principal investigator: V. Vetterl, 2004 - 2005.

IGA MH CR, 1M0021622409, Stomatological research center. Co-investigator: V. Vetterl, 2005 - 2009

Publications

Hasoň, S., Jelen, F., Fojt, L., Vetterl, V.: *Determination of picogram quantities of oligodeoxynucleotides by stripping voltammetry at mercury modified graphite electrode surfaces.* J. Electroanal. Chem. 577, 2005, 263-272. ISSN: 0022-0728

Vetterl, V., Hasoň, S.: *Electrochemical properties of nucleic acid components*. In : Perspectives in Bioanalysis Vol.1. Electrochemistry of nucleic acids and proteins. Towards electrochemical sensors for genomics and proteomics. (Editors E.Paleček, F.Scheller and J.Wang), Chapter 2, Elsevier, Amsterdam 2005, p. 18 - 69. ISBN: 0-444-52150-X ISSN: 1871 - 0069

Hasoň, S., Vetterl, V.: *Microanalysis of oligodeoxynucleotides by cathodic stripping voltammetry at amalgam-alloy surfaces in the presence of copper ions.* Talanta, in press 2006.

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Strašák, L., Vetterl, V., Fojt, L.: *Effects of 50Hz Magnetic Fields on the Viability of Different Bacterial strains.* Electromagnetic Biology and Medicine 24, 2005, 293-300.

Kroupová, J., Bártová, E., Fojt, L., Strašák, L., Kozubek, S., Vetterl, V.: *Low-frequency magnetic field effect on cytoskeleton and chromatin.* Bioelectrochemistry, BES-ISE Symposium 2005 special issue, in press.

CD Spectroscopy of Nucleic Acids

Head

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Research fellows

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We have used CD spectroscopy and polyacrylamide gel electrophoresis to study conformational properties of DNA fragments containing $(CCA)_n$ and $(TGG)_n$ repeats, which are the most length-polymorphic microsatellite sequences of the human genome. The $(CCA)_n$ fragments are random single strands at neutral pH but they fold into intramolecular intercalated cytosine tetraplexes at mildly acid pH values. More acid values stabilize intermolecular tetraplex formation. The behavior of $(TGG)_n$ repeats is more complex. They form hairpins and antiparallel homoduplexes in low salt solutions which, however, are transformed into parallel-stranded guanine tetraplexes at physiological KCl concentrations. Their molecularity depends on the repeat number: $(TGG)_8$ strands associate into tetramolecular complexes, $(TGG)_4$ form octaplexes. Only the $(TGG)_n$ with an odd repeat numbers (5, 7 and 9) are capable to form a hairpin loop to generate, in addition to tetramolecular, also bimolecular parallel tetraplexes. The only $(TGG)_7$ can fold into an intramolecular tetraplex with three hairpin loops. This tetraplex is formed at low KCl concentrations, and is antiparallel. Moreover, the $(TGG)_n$ fragments, namely those containing an even number of repeats, provide various mutually slipped conformers whose population increases with salt concentration and with the increasing repeat number. The slippage includes single bases as well as the whole triplet and,

obviously, is a consequence of a possibility of both, G*G, T*T and T*G pairing. Tetraplex arrangements of the $(TGG)_n$ motif are very stable and neither temperature up to 100 ^oC can dissociate them. We suppose that the extreme conformational variability of $(TGG)_n$ strands stands behind the length polymorphism which the $(CCA)_n/(TGG)_n$ repeats exhibit in the human genome.

Secondary structures of the G-rich strand of human telomere DNA fragments $G_3(TTAG_3)_n$, n=1-16, have been studied in solutions of physiological potassium cation concentrations. It has been found that folding of these fragments into tetraplexes as well as tetraplex thermostabilities and enthalpy values depend on the number of the $TTAG_3$ repeats. The proposed topologies include, for example, antiparallel and parallel bimolecular tetraplexes, a tetraplex consisting of three parallel chains and one antiparallel chain, an intramolecular antiparallel tetraplex, a poorly stable parallel intramolecular tetraplex with three propeller-like ($TTAG_3$) side loops, and both parallel and also antiparallel tetramolecular tetraplexes. $G_3(TTAG_3)_3$ folds into a single, stable, and very compact intramolecular antiparallel tetraplex. With an increasing repeat number, the fragment tetraplexes surprisingly are ever less thermostable and their migration and enthalpy decrease indicates increasing irregularities or splitting of the structure into more domains. Sharp enthalpy changes with a periodicity of 4n lead to an imagination that the single stranded human telomere overhang has a nucleosome-like architecture of beads on a string. The basic unit - bead is represented by the intramolecular antiparallel tetraplex formed by three guanine tetrads. These properties may contribute to elucidation of stepwise telomere shortening.

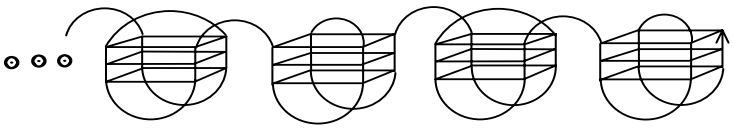

Fig. 1. Proposed arrangement of the single-stranded human telomere DNA

Guanine tetraplexes are effectively stabilized by potassium as well as by other cations as e.g. Sr^{2+}, Pb^{2+}, Mg^{2+} or Na^{+}. It is thought that the cations present in the cavity between consecutive guanine tetrads are an integral part of tetraplexes. We have shown using CD spectroscopy that ethanol induces the guanine tetraplexes like or even better than potassium cations. We present 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 a further 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 enviroment than the B-form, which is stabilized by water. Hence perhaps the lowered water activity and enhanced base-solvent interactions stand behind the stabilization of the three non-B structures by ethanol.

Use of ethanol will permit studies of guanine tetraplexes that cannot be induced by potassium cations or other tetraplex-promoting agents. Our results demonstrate that still a broader spectrum of nucleotide sequences can fold into guanine tetraplexes than it has so far been thought. It is to be noted that aqueous ethanol may better simulate conditions existing in vivo than the aqueous solutions.

Granted projects

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

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PhD thesis defended in 2005

Mgr. Michal Zemánek, Conformational Properties of Trinucleotide Motifs $(XGG)_n$.

PLANT DEVELOPMENTAL GENETICS

HEAD

BORIS VYSKOT

RESEARCH FELLOWS

ROMAN HOBZA, BOHUSLAV JANOUŠEK, EDUARD KEJNOVSKÝ, JIŘÍ ŠIROKÝ, JITKA ŽLŮVOVÁ

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ZDENĚK KUBÁT, MICHAELA MARKOVÁ, ELLENI MICHU, PETR MOKROŠ, JAROSLAV MRÁČEK, JAN VRBSKÝ

UNDERGRADUATE STUDENTS

PETRA BULÁNKOVÁ, TOMÁŠ ČERMÁK, MARTINA HRUBÁ, HANA KUBEKOVÁ, DANA PAULÍČKOVÁ, MARTINA TALIÁNOVÁ, JIŘÍ ŽÁK

TECHNICAL ASSISTANT

MARTINA KAŠÍKOVÁ

The research realised in this lab is focused on genomic structure and function in model plant species. We especially study gene function(s) controlling developmental processes and their impact in evolution. The majority of our research efforts are spent on the model dioecious plant *Silene latifolia*, which offers a unique opportunity to study evolutionary young sex chromosomes. The basic questions of nuclear stucture and dynamics are studied on *Arabidopsis thaliana*.

Structural genomics of sex chromosomes is realised in collaboration with U of North Carolina, Osaka U, and IEB Olomouc. We study the structure of sex chromosomes and processes of genetic degeneration of the Y chromosome of *Silene latifolia*, namely accumulation of repetitive DNA sequences in non-recombining parts of the Y chromosome as proposed by theoretical models. In these studies, a number of sophisticated techniques and tools of structural genomics are used including separation of sex

chromosomes by flow sorting and laser microdissection, construction of sex chromosome-specific libraries, construction of BAC libraries, BAC-FISH mapping, mapping of genes by PCR on sorted chromosomes, and chromosome painting. Bioinformatics tools are also used in analysing DNA sequence data obtained. The main results include:

(i) Chloroplast DNA sequences are preferentially accumulated on the Y chromosome. A screening of the Y-chromosome-specific library showed that genic chloroplast sequences located on the Y chromosome are more degenerated than their homologues on the X chromosome.

(ii) MADS box gene *SlAP3* was duplicatively transferred from an autosome onto the Y chromosome. It demonstrates that processes of Y-accumulation of genes advantageous for male gender is accompanied by genetic degeneration of the Y chromosome in plants.

(iii) We isolated and characterised a tandem repeat DNA accumulated on the Y chromosome. A remarkable feature of this tandem repeat is its palindrome structure located in the central part. These elements contribute to the large size of the Y chromosome and formally resemble analogous structures described in the human Y chromosome.

(iv) We isolated and characterised a new gypsy-like retrotransposon, named Retand, which harbours a tandem repeat. This tandem repeat was more amplified in a non-autonomous Retand element (3.7 kb) than in a longer autonomous element (11.2 kb), which possessed all genes and other features typical for retrotransposones. Retand is transcriptionally active in all tested plant parts, and it is localised at subtelomeres of all chromosomes. This phenomenon could represent a new mechanism of tandem repeat spreading in the genomes via retrotransposition.

Evolution of sex chromosome-linked genes and their functions is studied in collaboration with ENS Lyon, U of Edinburgh, and Sofia U. Sex chromosomes are supposed to evolve from a regular pair of autosomes. The process of sex chromosome evolution started with the appearance of sex-determining gene(s). The presence of sex-determining locus (loci) on the Y chromosome is expected to serve as an "attractant" for genes benefit for male sex, which were probably preferentially recruited to the proximity of the sex-determining gene(s). To strengthen the linkage disequilibrium

between the sex-determining and -benefit loci, a recombination arrest may occurred. Because of the recruitment of novel genes to the proximity of the non-recombining region, the non-recombining region is expected to expand along the sex chromosomes. The aim of our research is to better understand most of the above-mentioned processes involved in the Y chromosome evolution. Working on the model *Silene latifolia* species we have shown:

(i) Most of the genes essential for male reproduction program present on the Y chromosome are clustered around the male-promoting sex determining gene. It means that the sex determining gene serves as an attractant of male-benefit genes under natural selection conditions.

(ii) The X chromosome contains regions with similar synonymous substitutions divergence between the X and Y DNA sequences. These regions are orderly distributed with the newest (least diverged) being the closest to the pseudoautosomal region and the oldest (most diverged) being the furthest from the pseudoautosomal region. These results indicate that the process of recombination restriction between the sex chromosomes in *S. latifolia* was gradual (see the Figure attached).

(iii) The Y chromosome harbours one large inversion, which took place after the recombination restriction with the X chromosome. It also harbours locus/loci responsible for the pairing arrest between the sex chromosomes. Both these data highly support recent theories on an inversion-independent mechanism of recombination arrest between the sex chromosomes.

(iv) The Y chromosome contains several genes indispensable for male reproduction. Two of these genes diverged form their progenitors to such extent that their absence is not complemented by their orthologues from a related hermaphroditic species. It means that evolution of male-reproduction-indispensable genes on the Y chromosome is very rapid.

(v) A gynoecium-suppressing sex determining gene influences expression of *CUP-SHAPED COTYLEDONS*-directed signalling pathway. For the first time in the dioecious plants we have identified a signalling pathway involved in sex determination. A search for upstream regulatory genes should lead to the cloning of sex-determining gene.

Stability and integrity of nuclear genome are studied in collaboration with GMI Vienna and TAMU 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. As a consequence of the nuclear DNA impairment, a cascade of repair processes is started leading to the restoration of the structure and function of DNA or, alternatively, to the programmed cell death. On the model plant, *Arabidopsis thaliana*, we study the stability of plant genome in the absence of some essential proteins responsible for both the recognition of DNA damage and repair.

(i) In plants possessing extremely shortened telomeres caused by a knock-out mutation in telomerase we detected massive genome rearrangements including structural and numeric chromosome aberrations. Chromosomes fused at their ends giving rise dicentric chromosomes which were manifested as anaphase bridges during somatic cell divisions.

(ii) We showed that nucleolar organizer regions (NORs) in the telomerase-mutant plants fused more frequently when compared to other chromosomal loci as a possible outcome of functional and spatial organizational proximity of these respective loci in nucleoli. Moreover, we described mechanisms leading to the formation of additional nucleoli in somatic cell nuclei by mitotic nondisjunction.

(iii) *In vitro* cell cultures derived from the telomerase deficient plants were capable of long-term proliferation (>3 years) despite the absence of telomeric DNA sequences. Karyological analysis revealed dramatic genomic rearrangements, a large variation in ploidy, and an extremely high percentage of anaphase bridges. We suppose that apparent absence of checkpoint pathways that respond to telomere dysfunction provided the cultures with a mechanism to overcome the end-replication problem: cell survival through aneuploidy or polyploidy.

(iv) *Arabidopsis* mutants defective in the protein complex Mre11, responsible for a number of genome stabilizing functions such as DNA checkpoint, double-strand break repair, control of proper meiotic divisons,

and telomere maintenance, exhibited abundant dicentric chromosomes and chromosomal fragments in somatic cells.

(v) Cytogenetic examination of pollen mother cells in the *Arabidopsis mre11* mutant revealed massive fragmentation of meiotic chromosomes and the absence of synapsis of homologous chromosomes. We conclude that the Mre11 complex plays a role in early stages of meiotic homologous recombination. The nuclear fragmentation was substantially suppressed by a mutation in the protein responsible for the induction of double-strand breaks during meiotic recombination: *Spo11*. This result indicates that Mre11 protein is required for repair rather than for the induction of meiotic DNA breaks in *Arabidopsis*.

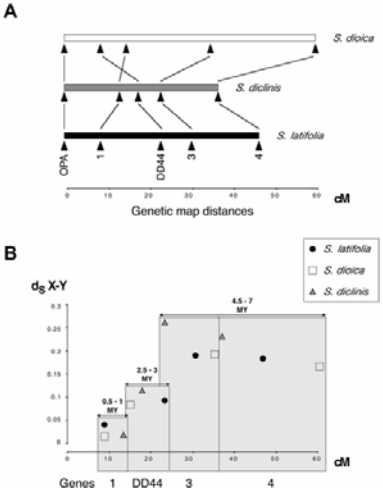

Fig. 1. X-genetic maps in the three dioecious species versus plot of synonymous divergence. (A) Gene orders and the map positions of the genes. (B) Plot of synonymous divergence between X and Y pairs against the map position using the gene order in S. latifolia and S. diclinis.

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

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Nicolas M., Marais G., Hykelová V., Janoušek B., Laporte V., Vyskot B., Mouchiroud D., Negrutiu I., Charlesworth D., Moneger F. *A gradual and ongoing process of recombination restriction in the evolutionary history of the sex chromosomes in dioecious plants.* Public Library of Science, Biology 3, 2005, 47-56.

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Matsunaga S., Lebel-Hardenack S., Kejnovsky E., Vyskot B., Grant S.R., Kawano S. *An anther- and petal-specific gene SlMF1 is a multicopy gene with homologous sequences on sex chromosomes.* Genes and Genetic Systems, 80, 2005, 395-401.

Prestigious International Projects

6. FP EU, LSHG-CT-2003-502983, Mutant p53 as a target for improved cancer treatment. 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

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

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

HHMI, INTNL 55000313, Basis for new structure-pharmacological relationship of platinum antitumor drugs Principal investigator: J. Kašpárková, 2001 - 2005

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: E. Paleček (started 1.1.2006)

ME, LC06004, Center of Basic Research, Integrated research of the plan genome. Principal investigator: B. Vyskot (started 1.1.2006)

ME, LC06030, Center of Basic Research, Biomolecular Center. Co-principal investigators: V. Brabec, J. Šponer (started 1.1.2006)

ME, LC06027, Center of Basic Research for Monoclonac Gamapathy and Multiple Myeloma. Co-principal investigator: E. Bártová (started 1.1.2006)

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CENTER OF INFORMATION TECHNOLOGIES

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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 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) runníng under UNIX, MS Windows NT/2000/XP or MS Windows 95/98/ME. CIT also provides consulting services for individual scientists.

Fig. 1. The central file-server with 6.8 TB disk capacity (RAID 5).

Main attention of CIT was devoted to the security issues. Security patches were installed in time and antivirus databases were regulary updated. All e-mails are monitored at the server by two independent virus scanners with 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.

The central RACK of LAN was rebuilt in order to be compatible with the additional equipment connected to the computer network. Cabling was reinstalled to the new RACK. The central fileserver with 6.8 TB disk capacity (RAID 5) and the tape library equipped with two Ultrium960 tape drives was installed. Each night the data from disk arrays are backed up to magnetic tapes. The fileserver is used by scientist as safe data storage.

RECONSTRUCTION OF LABORATORIES

Approximately a half of the laboratories of the Institute have already been upgraded. In 2005 the reconstruction of laboratories continued.

The new premises of the Laboratory of Molecular Cytology and Cytometry (on the top). **Iverse Leica microscope with Andor EM CCD camera**, automated **confocal microscope** (Leica DMRXA, CSU-10, CoolSnap CCD, Piezzo, the chamber is required to keep the temperature ±0.1°C) and **Inova Ar/Kr Laser with AOTF**.

AWARDS AND HONORS

Jana Šlotová, director of the Institute of Biophysics, Academy of Sciences (her term ended on June 30, 2005) received the Jan Patočka Memorial Award for the results of her lifelong work in science.

Viktor Brabec, head of the Laboratory of Molecular Biophysics and Pharmacology received the Prize of the Chairman of Grant Agency of the Czech Republic for outstanding results of the project „Oligonucleotides modified by complexes of platinum for selective modulation of gene expression; the relation to "antisense" strategy in the developement of new pharmaceuticals“.

Michal Štros, Alena Bačíková, Eva Muselíková-Polanská a Božena Krönerová (Laboratory of Chromosomal Proteins) received the Prize of the Academy of Sciences for outstanding scientific results obtained during the accomplishment of the research projects.

Stanislav Hasoň, received the Otto Wichterle Award for young scientists.

50TH ANNIVERSARY OF THE FOUNDATION OF IBP AS CR

Christmas Party

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