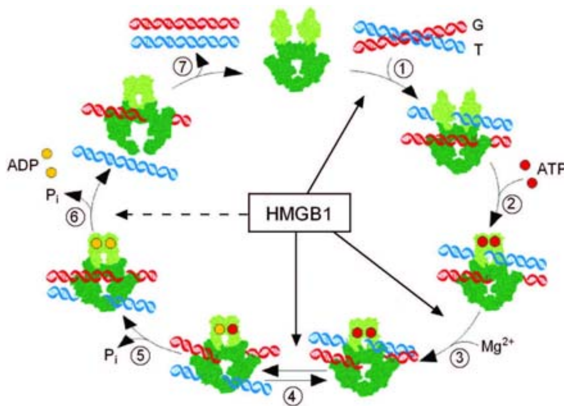
INSTITUTE OF BIOPHYSICS

ACADEMY OF SCIENCES OF THE CZECH REPUBLIC



RESEARCH REPORT 2007

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Introduction

In 2007, the production of impacted publications of the Institute substantially increased, as compared with the previous years. This statement applies to practically all departments and groups of the Institute. The total number of articles in WoS was 111 in 2007, as compared with 74 in 2005, when the evaluation started. The average impact factor (IF) of our publications was maintained, and consequently the cumulative IF also increased.

In order to further increase the performance of the Institute, the management together with the Council decided to continue with changes of the organizational structure. In accordance with the earlier introduced rules, those research teams whose results in the internal evaluation were below the agreed threshold were integrated with other - more productive - departments. In 2008, there are nine departments in the Institute with a high and stable production of impacted publications, a high citation index, an established international collaboration, broad pedagogical activities, an involvement in education of PhD students, a successful record of grant applications, and a favourable age distribution of the staff.

In 2007, the Institute converted into a public research organization with a newly elected Council, a newly appointed Director, and a new Supervisory Board. The management of the Institute cooperates very tightly with the Council, the result of which is optimal solutions to most problems of both internal and external origin. The new legal form of the Institute provides a higher flexibility, which should lead to a better performance.

The Institute participates in preparation of two Major projects, CEITEC and CESLAB, in close cooperation with the central management of the Academy of Sciences of the Czech Republic (AS CR). The Central European Institute of Technology (CEITEC) is a project of Brno universities and academic institutions, being prepared as the Centre of Excellence for the Operational Programme Research and Development for Innovations. The project is focused on advanced biology, biomedicine, and advanced materials and technologies. The Institute participates in preparation of the Mendel Research Centre (MRC), which will integrate biological research of the region. The MRC consists of four Research programmes: Structural biology and bioinformatics, Biochemistry and molecular biophysics, Cellular biology and biophysics, and Plant systems.

The Academy of Sciences proposes another major project: construction of an electron synchrotron (the Central European Synchrotron Laboratory, CESLAB), a medium size (3 GeV) accelerator, which is to be an ideal infrastructure for excellent research in the region of Brno. The investment costs will be € 264 million, which is relatively a small proportion of the Operational Programme, where more than € 2 billion is allocated. The operational costs will be less than €30 million per year, which is about 10% of the investment (much more favourable than in the case of other projects). CESLAB will cover a broad range of research and technological directions, being the top facility not only in the Czech Republic but also in the Central Europe. CESLAB will provide an instrument for development of that industry based on very advanced technologies and products. It will also help to raise the attractiveness of the Czech Republic, the level of the society education, and the public awareness of the importance of knowledge.

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Conformation of DNA GG intrastrand cross-link of antitumor oxaliplatin and its enantiomeric analog

Downstream processes that discriminate between DNA adducts of a third generation platinum antitumor drug oxaliplatin and conventional cisplatin are believed to be responsible for the differences in their biological effects. These different biological effects are explained by the ability of oxaliplatin to form DNA adducts more efficient in their biological effects. In this work conformation, recognition by HMG domain protein and DNA polymerization across the major 1,2-GG intrastrand cross-link formed by cisplatin and oxaliplatin in three sequence contexts were compared with the aid of biophysical and biochemical methods. The following major differences in the properties of the cross-links of oxaliplatin and cisplatin were found: i) the formation of the cross-link by oxaliplatin is more

deleterious energetically in all three sequence contexts; ii) the cross-link of oxaliplatin bends DNA slightly but systematically less in all sequence contexts tested; iii) the affinity of HMG domain protein to the cross-link of oxaliplatin is considerably lower independent of the sequence context; and iv) the Klenow fragment of DNA polymerase I pauses considerably more at the cross-link of oxaliplatin in all sequence contexts tested. We have also demonstrated that the chirality at the carrier ligand of oxaliplatin can affect its biological effects.

Mechanism of the formation of DNA-protein cross-links by antitumor cisplatin

DNA-protein cross-links are formed by various DNA-damaging agents including antitumor platinum drugs. The natures of these ternary DNA-Pt-protein complexes (DPCLs) can be inferred, yet much remains to be learned about their structures and mechanisms of formation. We investigated the origin of these DPCLs and their cellular processing on molecular level using gel electrophoresis shift assay. We show that in cell-free media cisplatin [cis-diamminedichloriplatinum(II)] forms DPCLs more effectively than ineffective transplatin [trans-diamminedichloriplatinum(II)]. Mechanisms of transformation of individual types of plain DNA adducts of the platinum complexes into the DPCLs in the presence of several DNA-binding proteins have been also investigated. The DPCLs are formed by the transformation of DNA monofunctional and intrastrand cross-links of cisplatin. In contrast, interstrand cross-links of cisplatin and monofunctional adducts of transplatin are stable in presence of the proteins. The DPCLs formed by cisplatin inhibit DNA polymerization or removal of these ternary lesions from DNA by nucleotide excision repair system more effectively than plain DNA intrastrand or monofunctional adducts. Thus, the bulky DNA-protein cross-links formed by cisplatin represent a more distinct and persisting structural motif recognized by the components of downstream cellular systems processing DNA damage considerably differently than the plain DNA adducts of this metallodrug.

Recognition of DNA three-way junctions by metallosupramolecular cylinders: Gel electrophoresis studies

The interaction of metallosupramolecular cylinders with DNA three-way junctions has been studied by gel electrophoresis. A recent X-ray crystal structure of a palindromic oligonucleotide forming part of a complex with such a cylinder revealed binding at the heart of a three-way junction structure. The studies reported herein confirm that this is not solely an

artefact of crystallisation and reveal that this is a potentially very powerful new mode of DNA recognition with wide scope. The cylinders are much more effective at stabilizing three-way junctions than simple magnesium dications or organic or metallo-organic tetracations, with the M cylinder enantiomer being more effective than P. The recognition is not restricted to three-way junctions formed from palindromic DNA with a central AT step at the junction; non-palindromic three-way junctions and those with GC steps are also stabilised. The cylinder is also revealed to stabilise other Y-shaped junctions, such as that formed at a fraying point in duplex DNA (for example, a replication fork), and other DNA three-way junction structures, such as those containing unpaired nucleotides, perhaps by opening up this structure to access the central cavity.

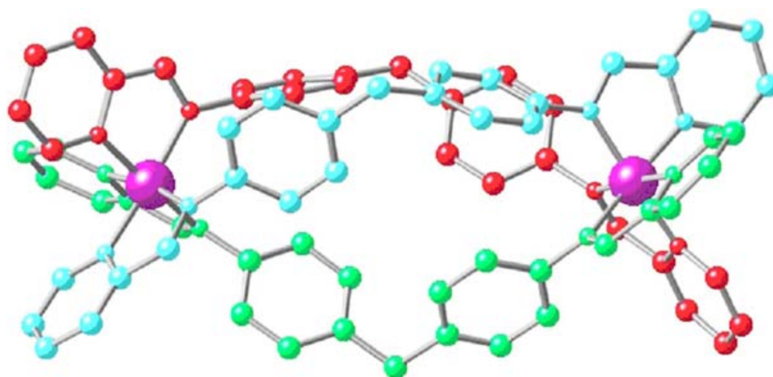

Fig. 1. Structure of the unsubstituted cylinder $[Fe_2L_3]^{4+}$ illustrating its three-dimensional structure.

Raman spectroscopy of DNA modified by intrastrand cross-links of antitumor cisplatin

Raman spectroscopy was employed to characterize the perturbations to DNA conformation induced in DNA by two different intrastrand adducts of antitumor cis-diamminedichloroplatinum(II) (cisplatin), namely by its 1,2-GG or 1,3-GTG intrastrand cross-links. We examined short deoxyribooligonucleotide duplexes containing single, site-specific cross-link by Raman spectroscopy and assigned the spectral alterations to conformational changes induced in DNA by 1,2-GG or 1,3-GTG intrastrand CLs determined earlier by other biochemical and biophysical methods. The

results confirmed significant perturbations to the B-form DNA backbone due to the intrastrand lesions and that several nucleotides changed their conformation from C2'-endo to C3'-endo. Evidence for a partial transition from B- to A-form was found in several regions of the Raman spectra as well. The spectra also confirmed the different and more extensive distortion induced in B-DNA by 1,3-GTG in comparison with 1,2-GG intrastrand CLs, consistent with their already known high resolution structures. The results of the present work demonstrate that Raman spectroscopy represents a suitable tool to provide insights into structural factors involved in the mechanisms underlying antitumor effects of platinum drugs.

1,2-GG intrastrand cross-link of antitumor dinuclear bifunctional platinum compound with spermidine linker inhibits DNA polymerization more effectively than the cross-link of conventional cisplatin

In order to learn more about the molecular basis for the inhibition of DNA replication produced by antitumor platinum drugs, we investigated DNA polymerization using DNA templates site-specifically modified with the 1,2-GG intrastrand cross-link of dinuclear bifunctional or conventional mononuclear cisplatin. These cross-links which have the same nature, but differ in the size and character of the conformational alteration induced in double-helical DNA, were analyzed for bypass ability with reverse transcriptase of human immunodeficiency virus type 1 and Klenow fragment of DNA polymerase I deficient in exonuclease activity. We found that the 1,2-GG intrastrand CL of BBR3571 inhibited DNA translesion synthesis markedly more than the same adduct of cisplatin. This result was explained by a larger size of the cross-link of BBR3571 and by a flexibility induced in DNA by this cross-link which can make the productive binding of this adduct at the polymerase site more difficult.

Dual triggering of DNA binding and fluorescence via photoactivation of a dinuclear ruthenium(II) arene complex

The dinuclear Ru^{II} arene complexes $[\{(\eta^6\text{-arene})RuCl\}^2(\mu\text{-}2,3\text{-dpp})](PF_6)_2$, arene = indan (1), benzene (2), p-cymene (3), or hexamethylbenzene (4) and 2,3-dpp = 2,3-bis(2-pyridyl)pyrazine, have been synthesized and characterized. Upon irradiation with UVA light, complexes 1 and 2 readily underwent arene loss, while complexes 3 and 4 did not. The photochemistry of 1 was studied in detail. In the X-ray structure of $[\{(\eta^6\text{-indan})RuCl\}_2(\mu\text{-}2,3\text{-dpp})](PF_6)_2$ (1), 2,3-dpp bridges two Ru^{II} centers 6.8529(6) Å apart. In water, aquation of 1 in the dark occurs with replacement of chloride with

biexponential kinetics and decay constants of 100 ± 1 min-1 and 580 ± 11 min-1. This aquation was suppressed by 0.1 M NaCl. UV or visible irradiation of 1 in aqueous or methanolic solution led to arene loss. The fluorescence of the unbound arene is ~40 times greater than when it is complexed. Irradiation of 1 also had a significant effect on its interactions with DNA. The DNA binding of 1 is increased after irradiation. The non-irradiated form of 1 preferentially formed DNA adducts that only weakly blocked RNA polymerase, while irradiation of 1 transformed the adducts into stronger blocks for RNA polymerase. The efficiency of irradiated 1 to form DNA interstrand cross-links was slightly greater than that of cisplatin in both 10 mM $NaClO_4$ and 0.1 M NaCl. In contrast, the interstrand cross-linking efficiency of non-irradiated 1 in 10 mM $NaClO_4$ was relatively low. An intermediate amount of cross-linking was observed when the sample of DNA already modified by non-irradiated 1 was irradiated. DNA unwinding measurements supported the conclusion that both mono- and bifunctional adducts with DNA can form. These results show that photoactivation of dinuclear Ru^{II} arene complexes can simultaneously produce a highly reactive ruthenium species that can bind to DNA and a fluorescent marker (the free arene). Importantly, the mechanism of photoreactivity is also independent of oxygen. These complexes, therefore, have the potential to combine both photoinduced cell death and fluorescence imaging of the location and efficiency of the photoactivation process.

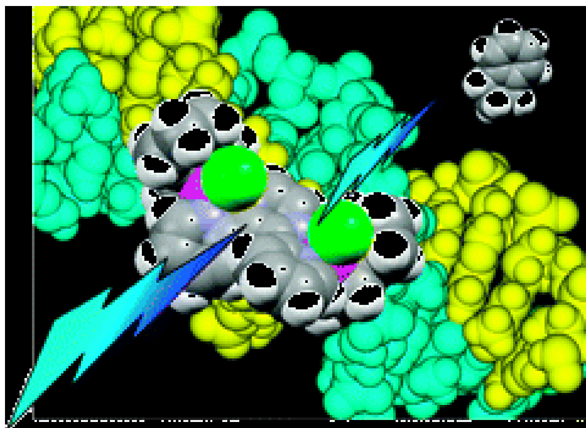

Fig. 2. Schematic representation of photoactivation of a dinuclear ruthenium(II) arene complex.

A potent cytotoxic photoactivated platinum complex

It has been shown by x-ray crystallography that the complex trans,trans,trans-$[Pt(N_3)_2(OH)_2(NH_3)(py)]$ (1) contains an octahedral Pt^{-IV} center with almost linear azido ligands. Complex 1 is remarkably stable in the dark, even in the presence of cellular reducing agents such as glutathione, but readily undergoes photoinduced ligand substitution and photoreduction reactions. When 1 is photoactivated in cells, it is highly toxic: 13-80 x more cytotoxic than the Pt^{II} anticancer drug cisplatin, and ca. 15 x more cytotoxic toward cisplatin-resistant human ovarian cancer cells. Cisplatin targets DNA, and DNA platination levels induced in HaCaT skin cells by 1 were similar to those of cisplatin. However, cisplatin forms mainly intrastrand cis diguanine cross-links on DNA between neighboring nucleotides, whereas photoactivated complex 1 rapidly forms unusual trans azido/guanine, and then trans diguanine Pt^{II} adducts, which are probably mainly intrastrand cross-links between two guanines separated by a third base. DNA interstrand and DNA-protein cross-links were also detected. Importantly, DNA repair synthesis on plasmid DNA platinated by photoactivated 1 was markedly lower than for cisplatin or its isomer transplatin (an inactive complex). Single-cell electrophoresis experiments also demonstrated that the DNA damage is different from that induced by cisplatin or transplatin. Cell death is not solely dependent on activation of the caspase 3 pathway, and, in contrast to cisplatin, p53 protein did not accumulate in cells after photosensitization of 1. The trans diazido Pt^{IV} complex 1 therefore has remarkable properties and is a candidate for use in photoactivated cancer chemotherapy.

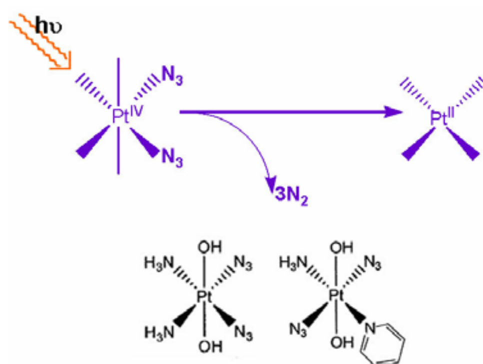

Fig. 3. Photoactivation of Pt^{IV} compounds. Potential for a new localised, less toxic therapy for cancer.

Biochemical studies of the thermal effects on DNA modifications by the antitumor cisplatin and their repair

Using biochemical methods, we have examined the effect of two factors that might play a role in the mechanism of the biological activity of cisplatin at elevated temperatures (>37°C). We show that increased temperatures result in distinct alterations in the modification of the target DNA by cisplatin, and in the repair of these modifications. Our in vitro results support the view that the enhanced DNA-cross-linking efficiency of cisplatin and the lower efficiency of native DNA repair mechanisms at higher temperature play at least a partial role in the potentiation of the antitumor effects of cisplatin under conditions of mild hyperthermia.

Bifunctional amine-tethered ruthenium(II) arene complexes form monofunctional adducts on DNA

The tethered Ru^{II} half-sandwich complexes $[\eta^6:\eta^1\text{-}C_6H_5(CH_2)nNH_2)RuCl_2]$ 1 (n = 3) and 2 (n = 2) have been synthesized as potential bifunctional anticancer complexes, and their X-ray crystal structures have been determined. They hydrolyze rapidly in aqueous solution to give predominantly mono-aqua mono-chlorido species. Mono-9EtG adducts, where 9EtG = 9-ethylguanine, form rapidly, but the second 9EtG binds more slowly and more weakly. In the X-ray crystal structure of the di-9EtG adduct $[(\eta^6\text{: }\eta^1\text{-}C_6H_5(CH_2)_3NH_2)Ru(9EtG)_2](CF_3SO_3)\ 2{\cdot}H_2O$ ($8{\cdot}H_2O$), one of the Ru-N7 bonds is significantly longer than the other (2.1588(18) vs 2.101(2) Å). The bound guanine bases adopt a head-to-head configuration, stabilized by tether NH_2 hydrogen bonding to C6O of 9EtG. The X-ray crystal structure of the dinitrato complex $[(\eta^6:\eta^1\text{-}C_6H_5(CH_2)_3NH_2)Ru(NO_3)_2]$ (3) showed both nitrates to be bound to ruthenium. This complex readily rutheniated calf thymus DNA but failed to produce stop sites on pSP73KB plasmid DNA during DNA transcription by an RNA polymerase. This suggested that only monofunctional DNA adducts formed, as did interstrand cross-linking assays. Also, the unwinding angle induced in negatively supercoiled DNA (9 ± 1°) was less than that induced by cisplatin (13°). These findings may explain why complexes such as 1 and 2 exhibited low cytotoxicities (IC_{50} values >100 μM) toward A2780 human ovarian cancer cells.

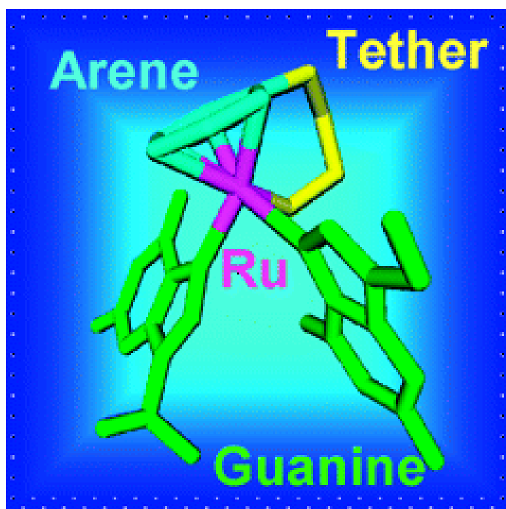

Fig. 4. Structure of bifunctional amine-tethered RuII arene complex coordinated to guanine residue.

DNA interactions of new cytotoxic tetrafunctional dinuclear platinum complex trans,trans-[{$PtCl_2(NH_3)$}$_2$(piperazine)]

A new tetrafunctional dinuclear platinum complex trans,trans-[{$PtCl_2(NH_3)$}$_2$(piperazine)] with sterically rigid linking group was designed, synthesized and characterized. In this novel molecule, the DNA-binding features of two classes of the platinum compounds with proven antitumor activity are combined, namely trans oriented bifunctional mononuclear platinum complexes with a heterocyclic ligand and polynuclear platinum complexes. DNA-binding mode of this new complex was analyzed by various methods of molecular biology and biophysics. The complex coordinates DNA in a unique way and interstrand and intrastrand cross-links are the predominant lesions formed in DNA in cell-free media and in absence of proteins. An intriguing aspect of trans,trans-[{$PtCl_2(NH_3)$}$_2$(piperazine)] is that, using a semi-rigid linker, interstrand cross-linking is diminished relative to other dinuclear platinum complexes with flexible linking groups and lesions that span several base pairs, such as tri- and tetrafunctional adducts, become unlikely. In addition, in contrast to the inability of trans,trans-[{$PtCl_2(NH_3)$}$_2$(piperazine)] to cross-link two DNA duplexes, the results of the present work convincingly demonstrate

that this dinuclear platinum complex forms specific DNA lesions which can efficiently cross-link proteins to DNA. The results substantiate the view that trans,trans-[{$PtCl_2(NH_3)$}$_2$(piperazine)] or its analogues could be used as a tool for studies of DNA properties and their interactions or as a potential antitumor agent. The latter view is also corroborated by the observation that trans,trans-[{$PtCl_2(NH_3)$}$_2$(piperazine)] is a more effective cytotoxic agent than cisplatin against human tumor ovarian cell lines.

NMR analysis of duplex d(CGCGATCGCG)$_2$ modified by Λ- and Δ-[Ru(bpy)$_2$(m-GHK)]Cl_2 and DNA photocleavage study

The interaction of the diastereomeric complexes Λ-[Ru(bpy)$_2$(m-GHK)]Cl_2 and Δ-[Ru(bpy)$_2$(m-GHK)]Cl_2 (bpy is 2,2‘-bipyridine, GHK is glycine–l-histidine–l-lysine) with the deoxynucleotide duplex d(5‘-CGCGATCGCG)2 was studied by means of 1H NMR spectroscopy. At a Δ-isomer to DNA ratio of 1:1, significant shifts for the metal complex are observed, whereas there is negligible effect on the oligonucleotide protons and only one intermolecular nuclear Overhauser effect (NOE) is present at the 2D nuclear Overhauser enhancement spectroscopy spectrum. The 1H NMR spectrum at ratio 2:1 is characterized by a slight shift for the Δ-isomer’s bpy aromatic protons as well as significant shifts for the decanucleotide G_4 H1‘ and H2‘‘, A_5 H2, G_{10} H1‘, T_6 NH and G_2 NH protons. Furthermore, at ratio 2:1, 11 intermolecular NOEs are observed. The majority of the NOEs involve the sugar H2‘ and H2‘‘ protons sited in the major groove of the decanucleotide. Increasing the Δ-isomer to d(CGCGATCGCG)$_2$ ratio to 5:1 results in noteworthy spectral changes. The Δ-isomer’s proton shifts are reduced, whereas significant shifts are observed for the decanucleotide protons, especially the sugar protons, as well as for the exchangeable protons. Interaction is characterized by the presence of only one intermolecular NOE. Furthermore, there is significant broadening of the imino proton signals as the ratio of the Δ-isomer to DNA increases, which is attributed to the opening of the two strands of the duplex. The Λ-isomer, on the other hand, approaches the minor groove of the oligonucleotide and interacts only weakly, possibly by electrostatic interactions. Photocleavage studies were also conducted with the plasmid pUC19 and a 158-bp restriction fragment, showing that both diastereomers cleave DNA with similar efficiency, attacking mainly the guanines of the sequence probably by generating active oxygen species.

Granted projects

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

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

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

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

GA CR 203/05/2032, Raman spectroscopy of DNA modified 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, Inhibition of telomerase by transition metal complexes. A new concept of antitumor drug design. Principal investigator: J. Kašpárková, 2005 - 2008

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

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

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

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

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

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PhD thesis defended in 2007

Mgr. Vlastimil Mašek, PhD., Characterization of DNA adducts of platinum complexes by Raman spectroscopy

Mgr. Vendula Bursová, PhD., Thermodynamic analysis of DNA modified by antitumor drugs

Ing. Pavla Heringová, PhD., Biophysical analysis of mechanism of antitumor effects of new metallodrugs

MOLECULAR ANALYSIS OF PLANT DEVELOPMENT

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Involvement of hormones and *KNOXI* genes in early Arabidopsis seedling development

Plant hormones control plant development by modulating the expression of regulatory genes, including homeobox-containing *KNOXI* genes. However, much remains to be elucidated about the interactions involved. Therefore, we investigated hormonal regulation of *KNOXI* gene expression using hormone applications and an inducible transgenic *ipt* expression system to increase endogenous cytokinin (CK) levels. Treatments by auxin, abscisic acid (ABA), cytokinins, ethylene and gibberellin (GA) did not result in ectopic expression of the *BP* gene. However, *BP* expression was strongly reduced by ABA, increased by auxin treatment (correlating with the initiation of lateral root meristems, which strongly express *BP*), and did not significantly respond to short-term treatments with the other hormones in whole seedlings.
Following short-term *ipt* activation, organ-specific differential regulation of *KNOXI* gene expression was observed. While several *KNOXI* genes were transiently up-regulated to low levels, STM was selectively repressed (especially at low light) in hypocotyls. In cotyledons, activation of CK-responsive genes preceded ipt induction, suggesting that CKs are transported more rapidly than the inducing agent (dexamethasone).

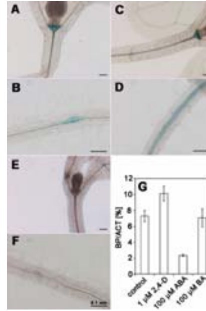

Fig. 1. Effects of exogenously applied hormones on *BP* expression. (A, B) p*BP*-GUS plants grown on MS agar, (C,D) 10 µM 2,4-D, (E,F) 100 µM ABA. Bars = 0.1 mm. (G) Q RT-PCR. *BP* transcript levels, normalized to *ACTIN* (%), were evaluated in intact plants treated/nontreated with 1 µM 2,4-D, 100 µM ABA and 100 µM BAP. Error bars represent SDs.

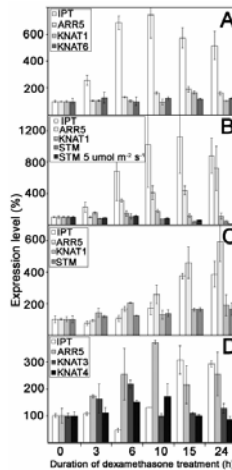

Fig. 2. mRNA levels in CaMV35S>GR>ipt plants after short-term DEX treatment. Seedlings (line11) were cultivated on MS medium and were then transferred to MS medium supplemented with 500 nM DEX for 3, 6, 10, 15 or 24 hours before the end of a 10-day growth period. (A) *Ipt, ARR5, BP* and *KNAT6* mRNA levels in roots. (B) *Ipt, ARR5*, *BP*, and *STM* mRNA levels in hypocotyls. The last set of columns shows STM mRNA levels established in plants cultivated under the 5 µmol m-2 s-1 light intensity regime. All other columns show results from plants grown under the 82 µmol m-2 s-1 regime. (C) *Ipt, ARR5*, *BP* and *STM* mRNA levels in apices. (D) *Ipt, ARR5, KNAT3*, and *KNAT4* mRNA levels in cotyledons.

Long-term increases in CK levels induced raised levels of several *KNOXI* transcripts in hypocotyls, correlating with the radial expansion of vascular tissues, the main domains of *KNOXI* gene expression, suggesting that CKs had little effect on *KNOXI* promoter activity. No alterations in hormone sensitivity were observed in a *bp* null mutant. Constitutive *BP* overexpression caused reductions in the length and number of lateral roots, while the primary root remained unaffected. The transgenic seedlings displayed weak, but significant, alterations in sensitivity to ABA, CK and ACC.

Cytokinin regulation of gene expression in the *AHP* gene family in *Arabidopsis thaliana*

In higher plants, histidine-aspartate phosphorelays are involved in hormone and stress signaling via two-component systems of signal transduction. In this system, a histidine-containing phosphotransmitter (HPt) mediates signal transmission from a sensory histidine kinase to a response regulator, providing integration and/or branching of several different signaling pathways. Five genes encoding HPts, *AHP1-5*, have been identified in Arabidopsis. Histidine-aspartate phosphorelays involving HPts have been at least partly implicated in cytokinin signaling. We analyzed the regulation by cytokinins of *AHP* gene expression. We compared the effects on steady state levels of *AHP* transcripts of a short-term treatment with an aromatic cytokinin and increase in endogenous isoprenoid cytokinin levels using an activable *ipt* system in 8-day-old *Arabidopsis* seedlings. Following *ipt* activation, a rapid and highly preferential increase in *trans*-zeatin-type cytokinins was observed while other isoprenoid-type cytokinins showed no or only marginal increases. The levels of cytokinin metabolites under long-term *ipt* activation suggest that the seedlings may have difficulties in efficiently down-regulating active forms of the hormone. Using real time RT-PCR, transient increases in steady-state levels of *AHP1-4* transcripts in response to both the short-term *N6*-benzyladenine treatment and the increase in endogenous *trans*-zeatin-type cytokinin levels were observed. In contrast, both the full and the alternatively spliced *AHP5* transcripts remained unaltered. On the contrary, increase in steady-state levels of *AHP1-4* transcripts observed in seedlings cultivated continuously in presence of exogenous *N6*-benzyladenine were not paralleled in seedlings with constitutively increased endogenous *trans*-zeatin-type cytokinins providing further indirect evidence for distinct functions of aromatic and isoprenoid cytokinins.

Granted projects

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

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

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

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

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DNA Molecular Complexes

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Analysis of telomere binding proteins in plants with human-type telomeres

As a part of our projects concerning telomere evolution in plants, we set to analyse telomere binding proteins in a group of plants within a plant order Asparagales, which synthesise human type of telomeres. First, we had to find and optimize a method for identification and characterization of telomere-binding proteins. Finally we used a relatively simple approach, in which proteins are eluted after Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE), then renatured and used for retardation analysis with labelled oligonucleotides corresponding to human and plant of telomeric sequences. We showed that this method is efficient to recover sequence-specific DNA-binding abilities of putative telomere-binding proteins.

This approach was then used to analyse proteins binding the single-stranded overhang of the G-rich strand of telomeric DNA in Asparagales plants. The 3’-overhangs of the G-rich strand represent a well-conserved feature of

telomeres synthesised by telomerase. These G-overhangs function as substrates for telomerase-mediated strand extension, and are critical for end-protection of telomeres. These functions and their regulations are mediated by specific G-overhang binding proteins. In species of the plant order Asparagales, telomere motifs have diverged from a type typical of the plant Arabidopsis thaliana (TTTAGGG)n to a type typical of human (TTAGGG)n. Presumably, this change in motif had an impact on the structure of the telomere and/or the binding of telomeric proteins, including the G-overhang binding proteins. Therefore, we analysed nucleoprotein complexes formed by protein extracts from plants possessing human-type telomeres (Muscari armeniacum and Scilla peruviana). Proteins were characterized that bind to the G-rich strand of both telomere motifs, or to the ancestral Arabidopsis-type motif alone, but none bound to double-stranded or C-rich complementary strand telomere motifs. We demonstrated the size, sequence-specificity and thermostability of these DNA-binding proteins. We also analysed the formation of complexes from renatured protein fractions after SDS-PAGE (sodium-dodecyl-sulphate polyacrylamide-gel-electrophoresis). A remarkable binding flexibility of the proteins, as observed in our studies, suggests interesting evolutionary consequences, namely their ability to act on both ancestral and present telomeric sequences. Of particular interest is that the ancestral repeat, which is thought not to form the present telomere, binds the proteins most strongly. These data were discussed in line with other known plant telomere-binding proteins and with the complex nature of the telomere in Asparagales carrying a human-type DNA sequence motif.

Granted projects

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

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

ME LC LC06004, Integrated research of plant genome. Principal investigator: B. Vyskot

Publications

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Biophysical Chemistry and Molecular Oncology

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In 2007 the Department of Biophysical Chemistry and Molecular Oncology pursued research concentrated to three main fields:

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

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

Field III: Structure and interaction of DNA and proteins in oncological research, especially with respect to the tumor suppressor protein p53 and its homologues

Research in the field I included systematic studies of the behavior of synthetic oligonucleotides (ODNs), chemically modified or damaged DNAs at electrodes, studies focused on novel techniques of electrochemical DNA labeling and development of new bioanalytical approaches applicable in practical DNA sensing.

Resolution of overlapped reduction signals in short hetero-oligonucleotides by elimination voltammetry

The resolution of overlapped reduction voltammetric signals of adenine (A) and cytosine (C) residues in short synthetic hetero-oligodeoxynucleotides (ODNs) with different sequences of A and C, but with the same ratio (A/C =1/2), was carried out by elimination voltammetry with linear scan (EVLS). The EVLS, as a mathematical transformation of voltammetric total currents measured at different scan rates, is capable to yield very sensitive resolution of potentially closed signals, which cannot be separated by common electrochemical methods. The best resolution of A and C signals was observed when the EVLS function eliminating the charging and kinetic currents (Ic, Ik) and conserving the diffusion current (Id) was combined with adsorption stripping technique (AdS). The resulting peak-counterpeak signal made it possible not only to separate the responses of A and C residues, but also to follow the dynamics of adsorption processes on mercury electrode surfaces. Together with the effect of the accumulation time on peak-counterpeak signal, the effect of pH and temperature on this signal was studied. The relation between elimination signals and the structure changes of ODNs was discussed.

Double voltammetry of short oligonucleotides

Elimination voltammetry with linear scan (EVLS) was applied to the resolution of reduction signals of adenine (A) and cytosine (C) residues in short synthetic hetero-ODNs with different sequences of A and C. The adsorptive stripping technique was combined with EVLS procedure, because for an adsorbed electroactive substance the EVLS function E4 eliminating charging and kinetic current components, and conserving the diffusion current component, yields specific and a very sensitive peak-counterpeak signal. Two homo-ODNs (dA9, dC9) and three selected hetero-

ODNs with different A and C sequences, i.e., 5´-CCC AAA CCC- 3´ (H3), 5´-CAC CAC CAC- 3´ (H4), and 5´-ACC CAC CCA- 3´ (H9), were studied on mercury electrode at various pH values and temperatures. The single E4 transformation, requiring three voltammetric curves measured at three different scan rates, showed that the best resolution of fully overlapped A and C signals of ODNs H3 and H4 was achieved at pH 6.34. However, for all three ODNs the separation at this pH was successful when double EVLS requiring voltammetric curves measured at five different scan rates was applied. As compared with simple EVLS, the current sensitivity of double EVLS is higher by more than one order of magnitude. The double EVLS, as a novel approach in the evaluation of voltammetric signals, in combination with the choice of pH and temperature offers a suitable tool for very sensitive electroanalysis of ODNs including the determination of base sequences.

Adsorption of the oligonucleotides A10, A25, A50 and A80 at the mercury/electrolyte interface

The adsorption of the oligonucleotides A10, A25, A50 and A80 at the mercury electrolyte solution interface is studied. The oligonucleotides are adsorbed at a potential close to -0.5 V (versus Ag/AgCl reference electrode) but they are desorbed at one or two potential ranges depending on the number of adenine molecules. The effect of the waiting time at the potential of maximum adsorption is studied and this time is closely related to the desorption peak heights. The adsorption/desorption is studied at two oligonucleotide concentrations and at two temperatures. The potentials of the desorption peaks of all oligonucleotides studied have almost the same value, indicating that the desorption process is almost the same. Capacity time curves following potential jumps from the adsorption region to desorption potentials show that the desorption is a very fast process but is usually followed by a reorientation/readsorption of the molecules at the interface depending on the number of adenines. Also, the capacity time curves show that the oligonucleotides are not fully desorbed at the desorption potential and stay very close to the interface.

Electrochemical sensing of chromium-induced DNA damage: DNA strand breakage by intermediates of chromium(vi) electrochemical reduction

DNA damage by Cr^{V} and/or Cr^{IV} intermediates of Cr^{VI} electrochemical reduction was detected using a supercoiled DNA-modified mercury electrode. A signal sensitive to formation of DNA strand breaks, AC

voltammetric DNA peak 3, increased due to incubation of the DNA-modified electrode in micromolar solutions of Cr^{VI} at potentials sufficiently negative for Cr^{VI} reduction. Damage to DNA in solutions containing Cr^{VI} and a chemical reductant (ascorbic acid, AA) was observed only at relatively high chromium concentrations (hundreds of μM). To eliminate interferences of excess Cr^{VI} in measurements of guanine electrochemical signals, a magnetoseparation double surface electro-chemical technique was introduced. Using this approach, DNA damage in solution was detected for 50 – 250 μM Cr^{VI} upon addition of 1 μM AA. Our results suggest more efficient DNA damage at the electrode surface due to continuous production of the reactive chromium species, compared to DNA exposure to chromium being reduced chemically in solution.

"Multicolor" electrochemical labeling of DNA hybridization probes with osmium tetroxide complexes

Labeling of oligonucleotide reporter probes (RP) with electroactive markers has frequently been utilized in electrochemical detection of DNA hybridization. Osmium tetroxide complexes with tertiary amines (Os,L) bind covalently to pyrimidine (predominantly thymine) bases in DNA, forming stable, electrochemically active adducts. We propose a technique of electrochemical "multicolor" DNA coding based on RP labeling with Os,L markers involving different nitrogenous ligands (such as 2,2'-bipyridine, 1,10-phenanthroline derivatives or N,N,N′,N′- tetramethylethylene-diamine). At carbon electrodes the Os,L-labeled RPs produce specific signals, with the potentials of these differing depending on the ligand type. When using Os,L markers providing sufficiently large differences in their peak potentials, parallel analysis of multiple target DNA sequences can easily be performed via DNA hybridization at magnetic beads followed by voltammetric detection at carbon electrodes. Os,L labeling of oligo-nucleotide probes comprising a segment complementary to target DNA and an oligo(T) tail (to be modified with the osmium complex) does not require any organic chemistry facilities and can be achieved in any molecular biological laboratory. We also for the first time show that this technology can be used for labeling of oligonucleotide probes hybridizing with target DNAs that contain both purine and pyrimidine bases.

Voltammetry of osmium end-labeled oligodeoxynucleotides at carbon, mercury and gold electrodes

Voltammetric behavior of an ODN 5'-T40 (GAA)7-3' end-labeled with osmium tetroxide,2,2'-bipyridine [Os(VIII),bipy] was compared with

Os(VIII),bipy-base- and with Os(VI),bipy-sugar-modified thymine ribosides. Cyclic voltammograms of Os(VIII),bipy-modified ODN at mercury and carbon electrodes were similar but not identical to those of Os(VIII),bipy-modified thymine riboside. Treatment of the ODN with Os(VI),bipy did not result in the ODN modification, in agreement with the known specificity of the reagent to the sugar cis-diols. We show that in addition to mercury and carbon electrodes, the gold electrode can be used to detect Os(VIII),bipy-labeled ODN. Comparison of voltammetric behavior of end-labeled ODN using three types of electrodes most frequently used in DNA analysis may help to optimize electrochemical DNA sensors.

Covalent labeling of nucleosides with VIII- and VI-valent osmium complexes

Osmium tetroxide complexes with nitrogen ligands [Os(VIII),L] have been widely applied as probes of the DNA structure and as electroactive labels of DNA. Here we describe the electrochemical behavior of Os(VIII),bipy - base-labeled nucleosides. We show that electroactive label can be introduced also in the nucleoside ribose residues using six-valent osmium complex. Cyclic voltammograms of sugar-Os(VI)-modified ribosides are similar but not identical to those of the base-modified ribosides. Our results showing the electroactivity of sugar modified ribosides pave the way to facile end-labeling of RNA.

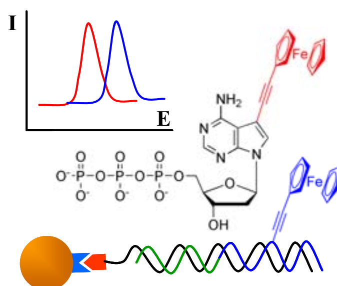
Purines bearing phenanthroline or bipyridine ligands and their RuII complexes in position 8 as model compounds for electrochemical DNA labeling – synthesis, crystal structure, electrochemistry, quantum chemical calculations, cytostatic and antiviral activity

A series of ethynyl- or (4-boronophenyl)bipyridines and -phenanthrolines were prepared as versatile building blocks for attachment of bidentate N-ligands to other molecules via cross-coupling reactions. 9-Benzyladenine derivatives bearing the bipyridine or phenanthroline complexes in position 8, attached via a conjugate acetylene or phenylene linker were prepared by cross-coupling reactions of the ethynyl- or 4-boronophenylbipyridines and phenanthrolines with 9-benzyl-8-bromoadenine. Complexation of these conjugates with $Ru(bipy)_2Cl_2$ afforded the corresponding Ru complexes as model compounds for electrochemical DNA labeling. The same compounds were also prepared directly by crosscoupling of 9-benzyl-8-bromoadenine with Ru complexes of the alkynes and boronic acids. Both approaches are compared in terms of potential applications for labeling of nucleic acids. The crystal structures of two Ru complexes were determined by X-ray

diffraction. The electrochemistry of the model purines bearing the phenanthroline or bipyridine ligands and the Ru complexes was studied by means of cyclic or square-wave voltammetry with carbon paste and mercury electrodes. The experimental redox potentials of the title compounds were compared with quantum chemical calculations. A very good agreement between experiment and theory was obtained, with a standard deviation of 0.13 V. It was shown that theoretical calculations can be of a limited predictive power for new RuII complexes, though it was difficult to reproduce differences smaller than 0.05 V. Several compounds of this series exhibited a considerable cytostatic effect and activity against the hepatitis C virus (HCV).

Ferrocenylethynyl derivatives of nucleoside triphosphates: synthesis, incorporation, electrochemistry, and bioanalytical applications

Modified dATP (2'-deoxyadenosine-5'-triphosphate) and dUTP (2'-deoxyuridine-5'-triphosphate) bearing ferrocene (Fc) labels linked via a conjugate acetylene spacer were prepared by single-step aqueous-phase cross coupling reactions of 7-iodo-7-deaza-dATP or 5-iodo-dUTP with ethynylferrocene. The Fc-labeled dNTPs were good substrates for DNA polymerases and were efficiently incorporated to DNA by primer extension (PEX). Electrochemical analysis of the 2'-deoxyribonucleoside triphosphates (dNTPs) and PEX products revealed significant differences in redox potentials of the Fc label bound either to U or to 7-deazaA and between isolated dNTPs and conjugates incorporated to DNA. Prospective bioanalytical applications have been outlined.



Magnetic beads as versatile tools for electrochemical DNA and protein biosensing

Magnetic beads (MBs) are versatile tools in the separation of nucleic acids, proteins and other biomacromolecules, their complexes and cells. In this article recent application of MBs in electrochemical biosensing and particularly in the development of DNA hybridization sensors is reviewed. In these sensors MBs serve not only for separation but also as a platform for optimized DNA hybridization. A hybridization event is detected separately at another surface, which is an electrode. The detection is based either on the intrinsic DNA electroactivity or on various kinds of DNA labeling, including chemical modification, enzyme tags, nanoparticles, electroactive beads etc., greatly amplifying the signals measured. In addition to DNA hybridization, other kinds of biosensing in combination with MBs, such as DNA-protein interactions, have been reviewed.

In the field II the work included basic studies of electrochemical behavior of peptides and proteins. Simple electrochemical techniques suitable for monitoring protein denaturation, aggregation, determination of redox state, as well as for studies of DNA-protein interactions, have been proposed.

Brdička-type processes of cysteine and cysteine-containing peptides on silver amalgam electrodes

Silver solid amalgam electrode (AgSAE) was used for differential pulse voltammetric (DPV) measurements of cysteine and cysteine-containing peptides, glutathione, Glu-Cys-Gly and phytochelatin $(Glu\text{-}Cys)_3$-Gly (PC3), in the presence of Co(II) ions. It had been established earlier that cysteine-containing peptides and proteins catalyze hydrogen evolution at mercury electrodes in presence of cobalt salts; these processes are known as the Brdička reaction. DPV signals measured with the AgSAE, the surfaces of which had been modified by mercury meniscus or mercury film, were qualitatively the same as those obtained with the hanging mercury drop electrode (HMDE). With these electrodes the number and the intensity of Brdička signals of cysteine, glutathione and PC3 differed, making a distinction among them possible. On the other hand, with the polished silver solid amalgam electrode (the surface of which was completely free of liquid mercury) all three compounds produced only one but strikingly intense peak in the region of Brdička reaction. Using this signal, cysteine, glutathione as well as PC3 could be determined at 10^{-8} M level, representing sensitivity up to 2 orders of magnitude better than attained with the mercury-modified AgSAEs or HMDE.

Native, denatured and reduced BSA. enhancement of chronopotentiometric peak H by guanidinium chloride

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

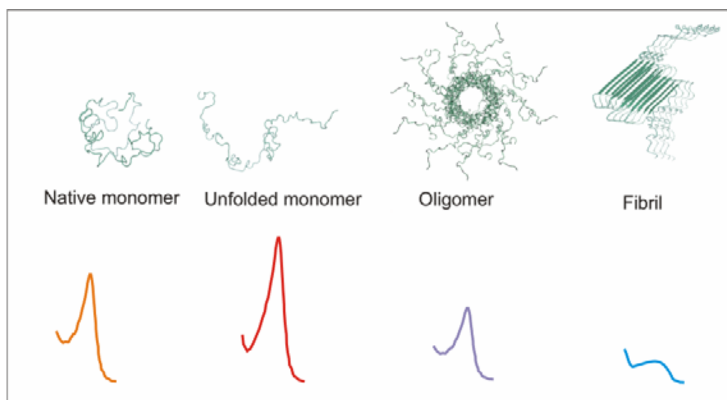
Chronopotentiometric determination of redox states of peptides

Reduced and oxidized forms of peptides were studied by different electrochemical methods at carbon electrodes and HMDE. Striking differences between the reduced and oxidized peptides were obtained by constant current chronopotentiometric stripping analysis (CPSA) at HMDE. Peptides yielded electrocatalytic peak H at highly negative potentials (ca. -1.75 V). Reduced peptides adsorbed at positively charged HMDE produced substantially higher peak H than the oxidized forms. Voltammetry reflected the peptide redox state less efficiently. Different orientation of reduced and oxidized molecules at the positively charged electrode and very fast potential changes in CPSA were probably responsible for the observed effects.

Changes in interfacial properties of α-synuclein preceding its aggregation

Parkinson disease (PD) is associated with the formation and deposition of amyloid fibrils of the protein α-synuclein (AS). It has been proposed that oligomeric intermediates on the pathway to fibrilization rather than the fibrils themselves are the pathogenic agents of PD, but efficient methods for their detection are lacking. We have studied the interfacial properties of wild type AS and the course of its aggregation in vitro using electrochemical analysis and dynamic light scattering. The oxidation signals of tyrosine residues of AS at carbon electrodes and the ability of fibrils to adsorb and catalyze hydrogen evolution at HMDE decreased during incubation. HMDE was particularly sensitive to pre-aggregation changes in AS. Already after 1 h of a standard aggregation assay in vitro (stirring at

37 ^{o}C), the electrocatalytic peak H increased greatly and shifted to less negative potentials. Between 3 and 9 h of incubation, an interval during which dynamic light scattering indicated AS oligomerization, peak H diminished and shifted to more negative potentials, and AS adsorbability decreased. We tentatively attribute the very early changes in the interfacial behavior of the protein after the first few hours of incubation to protein destabilization with disruption of long-range interactions. The subsequent changes can be related to the onset of oligomerization. Our results demonstrate the utility of electrochemical methods as new and simple tools for the investigation of amyloid formation.



Label-Free Voltammetric Detection of Single-Nucleotide Mismatches Recognized by the Protein MutS.

MutS, a protein involved in DNA mismatch repair, recognizes mispaired and unpaired bases in duplex DNA. We have previously used MutS in an electrochemical double-surface technique (DST) for in-vitro detection of point mutations in DNA. The DST involved binding of unlabeled MutS to DNA heteroduplexes at the surface of magnetic beads followed by a highly sensitive electrochemical determination of the protein by measurement of a catalytic protein signal (peak H) at mercury electrodes. Detection of MutS using a peak resulting from oxidation of tyrosine and tryptophan residues of the protein at a carbonpaste electrode (CPE) was also possible but was approximately three orders of magnitude less sensitive. In this work we present an optimized technique for ex-situ voltammetric determination of MutS at a CPE. Choice of optimum experimental conditions (pH of

supporting electrolyte, square-wave voltammetry settings etc.) resulted in substantial improvement of the sensitivity of the assay, enabling detection of approximately 140 pg (1.6 fmol protein monomer) of MutS in a 5-µL sample. The sensitivity was increased further by acid hydrolysis of the protein before measurement. The hydrolyzed protein was detectable down to 5 pg (approx. 56 amol) of MutS in 5 µL solution. By using the DST combined with determination of the bound unlabeled MutS at the CPE we demonstrated selective interactions of the protein with single-base mismatches and discrimination among different base mispairs in 30-mer or 95-mer DNA duplexes. In agreement with previous studies, binding of the protein to the 30-mer substrates followed the trend G:T>>C:A>A:A>C:T>homoduplex. The electrochemical data were confirmed by use of an independent technique - quartz-crystal microbalance for real-time monitoring of MutS interactions with DNA duplexes containing different base mispairs. By using the electrochemical DST, a G:T mismatch was detectable in up to 1000-fold excess of homoduplex DNA.

Electroactivity of nonconjugated proteins and peptides. Towards electro-analysis of all proteins

Present proteomics and biomedicine require sensitive analytical methods for all proteins. Recent progress in electrochemical analysis of peptides and proteins based on their intrinsic electroactivity has been reviewed. Tyrosine and/or tryptophan-containing proteins are oxidizable at carbon electrodes. At mercury electrodes all peptides and proteins (about 13 peptides and >25 proteins were tested) produce chronopotentiometric peak H at nanomolar concentrations. This peak is sensitive to changes in protein structure. Microliter sample volumes are sufficient for the analysis. Electrochemical methods can be used in studies of nucleic acid-protein interactions and can be applied in biomedicine. Examples of such applications in neurogenerative diseases and cancer have been outlined in this review.

In the field III, the studies on structure and interactions of the tumor suppressor protein p53, its mutants and homologues were continued. A number of new data concerning sequence-specific and structure-selective DNA binding by wild type and mutant p53 proteins, p53 homologues such as the p73 protein, as well as other proteins playing important roles in cell cycle control and cancerogenesis, have been obtained. These studies are in progress and the results will be published during the next year.

Granted projects

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

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

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

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

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

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

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

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

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

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

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

GA CR 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

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

GA CR 203/05/0043, Conjugates of nucleobases with metal complexes as electroactive markers. Application of labeled oligonucleotides in electrochemical DNA sensors. Principal investigator: M. Hocek (IOCB Prague), Co-investigator: L. Havran, 2005 - 2007

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 - June 2007

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

ME 7-2006-19, Application of biophysical methods in biotechnology and biomedicine. Principal investigator: V. Vetterl, 2005 - 2007

ME 1 M0528, Stomatological Research Centre. Principal investigator: J. Vaněk (MU Brno), Co-investigator and guarantor at IBP: V. Vetterl, 2005 - 2009

MIT 1H-PK/42, Research and development of a new-type of electro-chemical biosensor for the detection of nucleotide sequences and genotoxic agents in the environment. Project leader: M. Fojta, guarantor: E. Paleček, May 2004 - June 2007

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

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

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Brázdilová, P., Vrábel, M., Pohl, R., Pivoňková, H., Havran, L., Hocek, M., and Fojta, M.: *Ferrocenylethynyl derivatives of nucleoside triphosphates: synthesis, incorporation, electrochemistry, and bioanalytical applications.* Chem. Eur. J. 13, 2007, 9527-9533.

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PhD thesis defended in 2007

Mgr. Lukáš Fojt, PhD., Effect of electromagnetic fields on biological systems

Mgr. Pavel Kostečka, PhD., Osmium tetroxide complexes as electroactive markers for DNA and their analytical utilization

EXPERIMENTAL HEMATOLOGY

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The research performed in the Laboratory of Experimental Hematology has been aimed in 2007 at continuation of our endeavors to obtain more detailed knowledge on the regulatory roles of various factors in mouse hematopoiesis and on the possibilities to favorably influence the course of regeneration of suppressed hematopoiesis.

A study was performed targeted at defining the optimum conditions of the stimulatory action of the adenosine A3 receptor agonist, IB-MECA, on bone marrow hematopoiesis in mice. Morphological criteria were used to define the proliferation state of the granulocytic and erythroid cell systems. Significant negative correlation between the control proliferation state and the increase of cell proliferation after IB-MECA treatment irrespective of the cell lineage was found. The results suggest the homeostatic character of the induced stimulatory effects and the need to respect the functional state of the target tissue when investigating effects of adenosine receptor agonists under in vivo conditions.

Dipyridamole (DP) and adenosine monophosphate (AMP), which elevate extracellular adenosine and thus non-selectively activate adenosine receptors, administered to mice, were found to induce serum colony-stimulating activity, i.e. the ability of the serum to stimulate the growth of colonies from granulocyte/macrophage progenitor cells, and to elevate serum interleukin-6 levels. These results suggest that the effects of DP + AMP are indirect, mediated through the induction of some cytokine(s) and/or growth factor(s). The findings contribute to the further elucidation of

the role of adenosine in hematopoiesis.

Our studies on the role of adenosine in the growth of tumor cells continued in 2007 by a study on the effects of the drug on leukemia cell line MOLT-4. It was found out that adenosine suppressed the growth of the MOLT-4 cells in vitro. Since dipyridamole, a drug preserving adenosine in the extracellular space and preventing it from cellular uptake, reversed the adenosine-induced growth suppression, it might be deduced that the action of adenosine on the MOLT-4 cells comprised its cellular uptake and intracellular operation. This mechanism of adenosine action differs from previously published mechanism of adenosine's suppressive, receptor-mediated effects on fibrosarcoma cells.

IMUNOR®, a low-molecular weight ultrafiltered pig leukocyte extract, was previously found to have significant stimulatory effects on murine hematopoiesis suppressed by ionizing radiation or cytotoxic drugs. Studies performed in 2007 supplied data on the mechanisms of these effects. IMUNOR® was found to elevate serum levels of granulocyte colony-stimulating factor and interleukin-6. It may be hypothesized that the hematopoiesis-stimulating effects of IMUNOR® are mediated by inducing increased production of these growth factors. On the other hand, IMUNOR® was observed to decrease nitric oxide formation and hematopoiesis-stimulating cytokine production in lipopolysaccharide-stimulated RAW 264.7 macrophages. These findings suggest that a homeostatic role of IMUNOR® may be postulated consisting in stimulation of impaired immune and hematopoietic systems but also in cutting back the production of proinflammatory mediators in cases of overstimulation which threats with undesirable consequences.

Granted projects

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

Publications

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MOLECULAR EPIGENETICS

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Evolution of rDNA in Nicotiana allopolyploids: a potential link between rDNA homogenization and epigenetics

The evolution and biology of rDNA have interested biologists for many years, in part because of two intriguing processes: (i) nucleolar dominance and (ii) sequence homogenization. We review patterns of evolution in rDNA in the angiosperm genus Nicotiana to determine consequences of allopolyploidy on these processes. Allopolyploids of Nicotiana are ideal for studying rDNA evolution because phylogenetic reconstruction of DNA sequences has revealed patterns of species divergence and likely ancestral parents. From these studies we also know that polyploids formed over widely different time frames (thousands to millions of years) enabling comparative and temporal studies of rDNA structure, activity and chromosomal distribution. In addition, studies on synthetic polyploids enable the consequences of de novo polyploidy on rDNA activity to be determined. We propose that rDNA epigenetic expression patterns established even in F1 hybrids have a material influence on the likely patterns of divergence of rDNA. It is the active rDNA units that are

vulnerable to homogenization, which probably acts to reduce mutational load across the active array. Those rDNA units that are epigenetically silenced may be less vulnerable to sequence homogenization. Selection will not act against these silenced genes and they are likely to accumulate mutations and eventually be eliminated from the genome. It is likely that whole silenced arrays will be deleted in polyploids of 1 million years old and older.

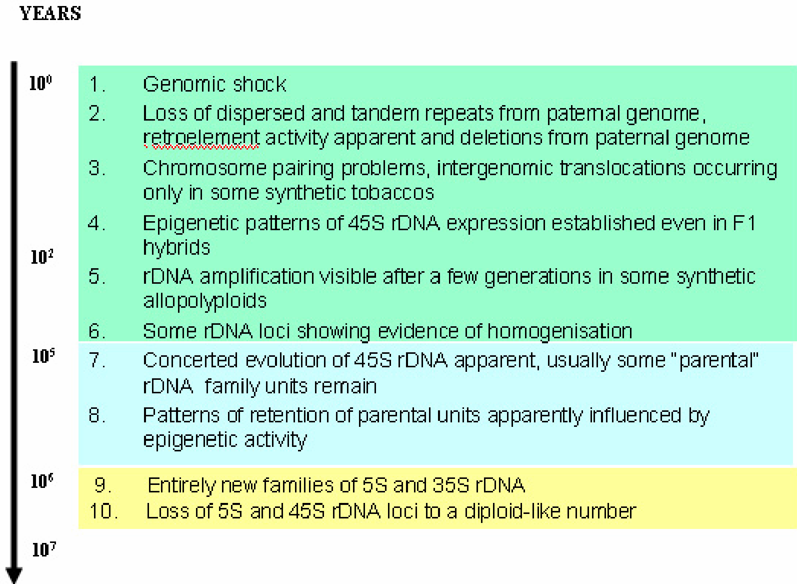

Fig. 1. Summary of rDNA evolution and other genetic events occurring after the formation of Nicotiana allopolyploids. The background colours separate the allopolyploids according to the categories established here: green background – de novo polyploids (from synthetic polyploids, few generations); blue background - young polyploids (<200,000 years old); brown background – old polyploids (c. 1-5 million years old).

Low abundant spacer 5S rDNA transcripts are frequently poly-adenylated in Nicotiana

In plants, 5S rRNA genes (5S rDNA) encoding 120-nt structural RNA molecules of ribosomes are organized in tandem arrays comprising thousands of units. Failour to correctly terminate transcription would generate longer inaccurately processed transcripts interfering with ribosome biogenesis. Hence multiple termination signals occur immediately after the

5S rRNA coding sequence. To obtain information about the efficiency of termination of 5S rDNA transcription in plants we analyzed 5S rRNA pools in three Nicotiana species, N. sylvestris, N. tomentosiformis and N. tabacum. In addition to highly abundant 120-nt 5S rRNA transcripts, we also detected RNA species composed of a genic region and variable lengths of intergenic sequences. These genic-intergenic RNA molecules occur at a frequency several fold lower than the mature 120-nt transcripts, and are posttranscriptionally modified by polyadenylation at their 3' end in contrast to 120-nt transcripts. An absence of 5S small RNAs (smRNA) argue against a dominant role for the smRNA biosynthesis pathway in the degradation of aberrant 5S RNA in Nicotiana. This work is the first description of polyadenylated 5S rRNA species in higher eukaryotes originating from a read-through transcription into the intergenic spacer. We propose that polyadenylation may function in a "quality control" pathway ensuring that only correctly processed molecules enter the ribosome biogenesis.

Concerted evolution of rDNA in recently formed Tragopogon allotetraploids is typically associated with an inverse correlation between gene copy number and expression

We analyzed nuclear ribosomal DNA (rDNA) transcription and chromatin condensation in individuals from several populations of Tragopogon mirus and T. miscellus, allotetraploids that have formed repeatedly within only the last 80 years from T. dubius and T. porrifolius and T. dubius and T. pratensis, respectively. We identified populations with no (2), partial (2) and complete (4) nucleolar dominance. It is probable that epigenetic regulation following allopolyploidization varies between populations, with a tendency towards nucleolar dominance by one parental homeologue. Dominant rDNA loci are largely decondensed at interphase while silent loci formed condensed heterochromatic regions excluded from nucleoli. Those populations where nucleolar dominance is fixed are epigenetically more stable than those with partial or incomplete dominance. Previous studies indicated that concerted evolution has partially homogenized thousands of parental rDNA units typically reducing the copy numbers of those derived from the T. dubius diploid parent. Paradoxically, despite their low copy number, repeats of T. dubius origin dominate rDNA transcription in most populations studied, i.e. rDNA units that are genetic losers (copy numbers) are epigenetic winners (high expression).

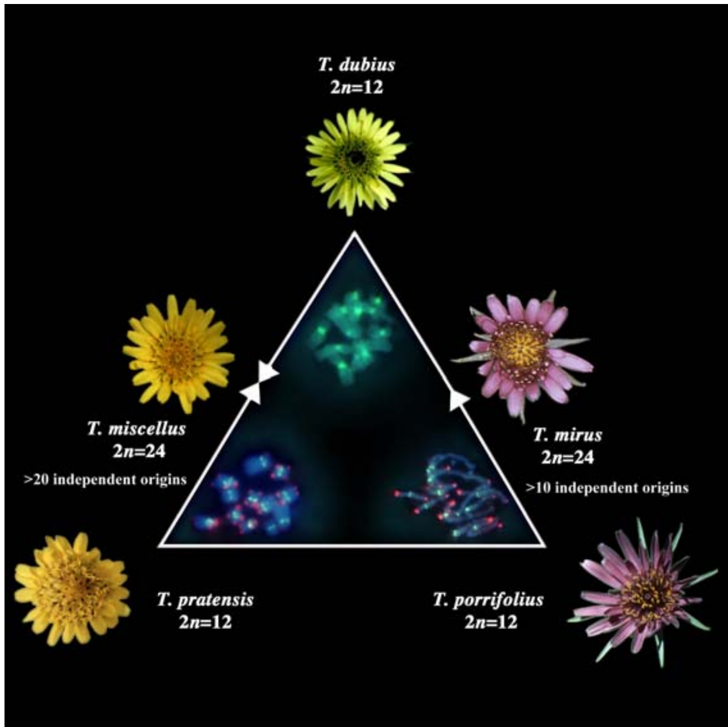

Fig. 2. The origin of recently formed Tragopogon mirus and T. miscellus allotetrapoloid species. In the edges of the triangle the parental diploid genome donors are shown. An interspecies hybridization of T. dubius x T. pratensis resulted in formation of T. miscellus; hybridization of T. dubius and T. porrifolius resulted in formation of T. mirus. Arrows indicate direction of interspecific crosses (from mother to father).

Granted projects

GA CR 521/04/0075, Epigenetic regulation of gene expression in transgenic and endogenous loci of higher plants. Principal investigator: A. Kovařík

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

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

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

Publications

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Molecular Cytology and Cytometry

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Nuclear topography and transcriptionalactivity of the c-myc gene during differentiation

Laboratory is focused on the studies on chromatin architecture in the view of gene expression that undergoes remarkable changes during various differentiation pathways. We tried to make correlations between observed structural phenomena and changes in epigenetic profiles such as histone acetylation and methylation. The highly conserved core histones H2A, H2B, H3 and H4, and their epigenetic modifications are thought to influence the coding and genetic potential of DNA. Epigenetics refers to heritable changes in the phenotype that occur irrespective of alterations in the DNA sequences. Therefore, the role of histone modification in gene silencing and/or X chromosome inactivation can be considered as an epigenetic process. Both euchromatin and heterochromatin are characterized by specific epigenetic patters and experiments related to structural and

functional characteristics of these genomic regions are performed in our laboratory.

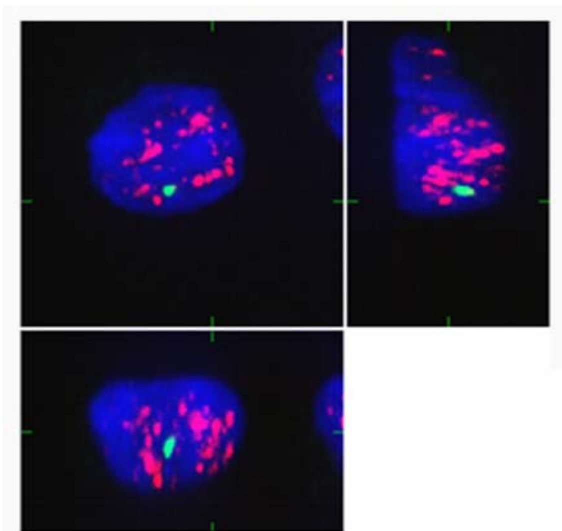

Fig. 1. 3D-projection of interphase nucleus of HT29 cell. Red regions are SC-35 domains involving splicing factors and green signals represent 3D-projection of c-myc transcription site.

Majority of our analyses were focused on nuclear location of the c-myc gene, and c-myc transcription sites in human adenocarcinoma HT29 cells. We have observed that the c-myc genes and its transcription sites were located non-randomly within the interphase nucleus, and c-myc RNA signals associated with the periphery of the centrally located nucleoli. Up-regulation of the APC gene reduced both the level of the c-MYC protein and the number of c-myc transcription sites. This correlated well with the decreased number of cells in the late G1- and S-phase of the cell cycle, during which the c-myc gene is mainly transcribed. A single transcription site of the c-myc gene co-localized from ~80% with the RNAP II region “transcription factories”. However, in ~20% of cells, the c-myc transcriptional complex (green signal in enclosed figure) was released from the site of synthesis and did not co-localized with transcription factories. Partial association of c-myc transcripts with SC-35 domains “nuclear speckles” (red regions in enclosed figure) was observed. In majority of cells (~65% of nuclei), the c-myc RNA signals were located in close proximity to SC-35 regions, but promyelocytic leukaemia (PML) bodies adjoined to

transcription sites in only ~20% of nuclei. We suggest that c-myc gene transcription and c-myc pre-mRNA processing take place in close proximity to nucleoli, with the participation of factors contained in those SC-35 domains located in the most internal parts of the cell nucleus.

C-myc (8q24.21) nuclear arrangement was also studied in human embryonic stem cells (hES) that are unique in their pluripotency and capacity for self-renewal. Additionally, nuclear radial arrangement of Oct3/4 gene (6p21.33), responsible for hESC pluripotency, was analyzed. Unlike differentiated hES cells, pluripotent hES cell populations were characterized by a high level of decondensation for the territories of both chromosomes 6 (HSA6) and 8 (HSA8). The Oct3/4 genes were located on greatly extended chromatin loops in pluripotent hES cell nuclei, outside their respective chromosome territories (Fig. 2). However, this phenomenon was not observed for the Oct3/4 gene in differentiated hES cells and for the C-myc gene in the cell types studied. The high level of chromatin de-condensation in hES cells also influenced the nuclear distribution of all the variants of HP1 protein, particularly HP1β, which did not form distinct foci, as usually observed in most other cell types. Our experiments showed that unlike C-myc, the Oct3/4 gene and HP1 proteins undergo a high level of de-condensation in hES cells. Therefore, these structures seem to be primarily responsible for hES cell pluripotency due to their accessibility to regulatory molecules. Differentiated hES cells were characterized by a significantly different nuclear arrangement of the structures studied.

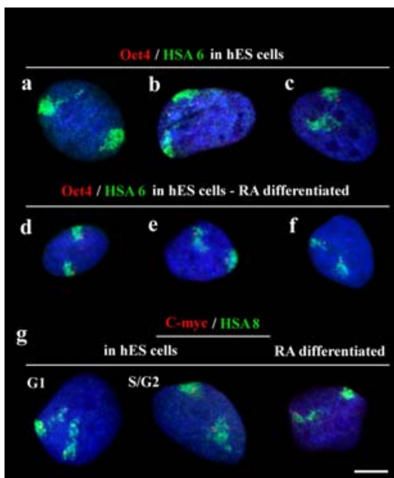

Fig. 2. Nuclear positionong of Oct3/4 (red) and c-myc (red) genes within interphase nuclei (blue) of pluripotent and RA-differentiated hESCs. Arrangement of selected genes was also analyzed in relationship to relevant chromosome territories (diffuse green) (Bártová et al., Differentiation, 2008). Bar shows 6 microm.

Studies on multiple myeloma

Part of our working group is engaged in the studies on multiple myeloma genetics and epigenetics. Chromosomal rearrangements can be frequently observed in tumour cells and multiple myeloma is not exception. Karyotypic abnormalities of MM are accompanied by changes in the epigenome, which preferentially involves aberrant DNA/histone methylation and histone acetylation, as well. We focused on analysis of epigenetic patterns in CD138+ and CD138- cells isolated from the bone marrow of patients with diagnosed MM. Additionally, we have studied the epigenetic changes in myeloma cells treated by clinically used cytostatics. In our experiments, chromatin immunoprecipitation (ChIP) in combination with polymerase chain reaction (PCR) enable us to analyze epigenetic profiles such as H3K9 acetylation and H3K9 di-methylation at promoters and coding regions of the genes which are important in pathogenesis of MM. In selected patients with diagnosed MM we observed different H3K9 acetylation and H3K9 dimethylation at c-myc and CCND1 sequences. Epigenetic changes in the c-myc and CCND1 genes were also studied in MM cells treated by melphalan, used in MM theraphy. ChIP-on-chip microarray analysis of H3K9 acetylation at promoter regions of hundreds of genes showed an increased H3K9 acetylation at promoters of many genes after melphalan treatment. This type of epigenetic modification is associated with an increased gene expression. Our data documented results of basic research, but on the other hand, we showed original ChIP-PCR and ChIP-on-chip analyses in clinical samples. Furthermore, we provided a useful tool how assess therapeutic potential of cytostatics, which significantly influence histone code.

Lamin A/C deficiency and chromatin structure

Diploma work of Gabriela Galiová is aimed at the study of lamin A/C deficiency and its effect on nuclear architecture. In article, accepted for publication in EJCB, we have shown that lamin A/C deficiency caused condensation of chromosome territories, which was compensated or even more pronounced by HDAC inhibition. Both lamin A/C deficiency and HDACi induced nuclear reorganization of centromeric heterochromatin, which was accompanied by the appearance of a chain-like morphology of HP1βfoci. Our observations lead to the suggestion that lamin A/C function could be connected to the reorganization of both hetero- and euchromatin as well as of some chromatin associated domains such as the HP1βfoci. Additionally, TSA can compensate the lamin A/C dependent chromatin changes, which support the statement that interaction between lamins and

specifically modified histones dictates nuclear architecture.

Chromatin structure influences the sensitivity of DNA to γ-radiation

The major advancement of this study is the direct evidence that ionizing radiation induces most double-strand breaks in genetically active, gene-dense regions of the human genome. Therefore, the most active regions of the human genome (structurally characterized by an open chromatin conformation) are subjected to the highest risk of radiation damage. On the other hand, we demonstrate the protective role of condensed chromatin, containing a low density of genes with low expression (functionally usually equivalent to heterochromatin). Higher sensitivity of open chromatin to damage is accompanied by more efficient DSB repair compared with the condensed chromatin. However, contrary to physiological heterochromatin, hypercondensed chromatin experimentally induced by hyperosmotic medium does not shield DNA against the damage by γ-radiation. Hypercondensed chromatin is probably formed by simple contraction of relaxed chromatin provoked by a decrease in the negative charge of the DNA in high concentration of salts without participation of additional chromatin-binding proteins, and therefore its sensitivity to ionizing radiation remains the same as that in cells with normal chromatin organization. To evaluate the radiosensitivity in functionally and structurally different chromatin domains we used ImmunoFISH technique, enabling the concomitant detection of DSBs as phosphorylated H2AX foci together with specific chromosomal regions. The most direct proofs follows from the comparison of DSB induction and repair in two regions of the same length (11 Mbp), located on chromosome 11 (separated by 12 Mbp) but markedly differing in the amount of highly expressed genes and therefore also in their chromatin structure. While the region RIDGE (region of increased gene expression) contains extremely high density of highly expressed genes and thus open chromatin structure, the anti-RIDGE has very low density of genes, mainly with low expression, and its chromatin is of 40 % more condensed than in RIDGE. The number of DSBs induced in the anti-RIDGE was of 76% lower and the repair was two times less effective compared the RIDGE. The same conclusions also follow from studies of DSB induction and distribution inside of individual chromosomal territories (CTs). Higher sensitivity to radiation damage was observed for chromosomes containing high density of genes (HSA19 and HSA11) contrary to those with low gene densities (HSA4, HSA18 and HSA2). This difference was, however, not so great (about 50%), probably because neither group of chromosomes contains only condensed or decondensed chromatin, unlike the RIDGE and anti-RIDGE.

The organization and structure of chromosome territories is maintained by nuclear lamina and polymeric actin

The role of lamina and nuclear actin in organization of chromosome territories (CT) was studied in cells of mammary carcinoma MCF7 by the damage of nuclear networks of these proteins either by inhibition of lamin A/C transcription or by preclusion of actin polymerization. The control of the efficiency of lamin A/C down regulation was pursued by RT-PCR, Western-blotting and immunodetection of the protein in individual nuclei. The actin filaments were detected by faloidin conjugated with rhodamine. CTs of HSA1, HSA2 and HSA13 were visualized by FISH using the painting probes conjugated with Cy3. The selected chromosomes differ in their length and in gene density but all of them are in contact with the nuclear membrane. All images obtained by confocal microscopy were deconvolved with Andor IQ 1.7 software (Andor Technology), using measured point-spread functions from Tetraspeck fluorescent beads (Molecular Probes-Invitrogen) and the Jansson-van Cittert deconvolution algorithm. To identify and quantitatively analyze FISH-labeled areas, deconvolved 3D images were treated with a bandpass filter and subsequently segmented using a range of thresholds in Huygens Essential software (Scientific Volume Imaging BV).

The disruption of the lamina in cells with silenced lamin A/C resulted in a decrease of the volume and surface area of chromosome territories, especially for chromosomes with low gene density and thus with high content of heterochromatin. The volume of CT2 and CT13 decreased of about 40%, while that of CT1 only of 17 %. Inhibition of actin polymerization led to significant enlargement of volumes and surface areas of chromosome territories. This increase was in correlation with gene density of chromosomes. The largest increase (150%) was observed for CT1, followed by that for CT2 (100%) and CT13 (70%).

These results show that lamina and nuclear polymeric actin, both participate on the maintaining of chromosome territories structure by different ways and independently each other. However changes induced in the size of CTs by disruption of one or another protein network are dependent on gene density determining the level of open chromatin structure of a particular chromosome.

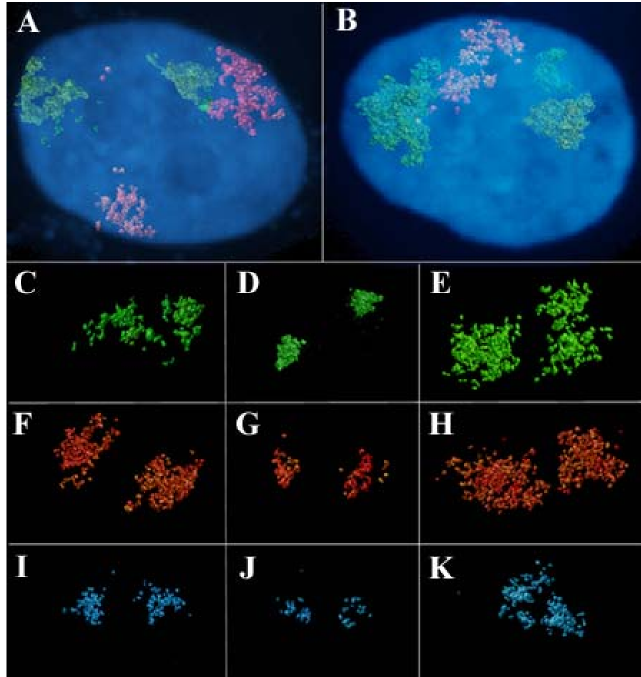

Fig. 3. Images of CTs after deconvolution and segmentation. CTs of chromosomes 1 (green), 2 and 13 (red) touch the nuclear periphery (A, B). The row of green images (C, D, and E) demonstrates representative structural changes of CTs of chromosome 1 after lamin A/C silencing (D) and cytochalasin D treatment (E) compared with a control cell (C). Similarly, the row of red images (F, G, and H) shows changes of chromosome 2 CTs and the row of blue images (I, J, and K) shows changes of chromosome 13 CTs.

Changes in differentiation of granulocytes in acute myeloid leukemia

Our earlier results show that human terminally differentiated neutrophils of peripheral blood do not have HP1 proteins even if their nuclear chromatin is highly condensed. The high chromatin condensation of these cells is accompanied by extremely high level of dimethylated histone H3 on lysine 9 (H3K9), however contrary to other terminally differentiated cells this methylated lysine is not accessible for immunodetection. In chronic phase of chronicle myeloid leukemia (CML), the characteristics of blood neutrophils are similar as that of healthy neutrophils; there is the absence of HP1 proteins and contrary to healthy neutrophils, a very low level of

immunodetected dimethylated H3K9 could be detected, indicating some small changes in chromatin condensation. However, as the disease accelerates and proceeds to the blast crises, the immunodetected level of dimethylated H3K9 became high together with appearance of the HP1 proteins, especially HP1 β and γ. The monitoring of the process of blood stem cells myeloid differentiation ex vivo in the presence of cytokines and Granulocyte Colony-Stimulating Factor (G-CSF) showed that HP1 proteins are eliminated from heterochromatin of stem cells soon after the cessation of their proliferation. This changeover is accompanied also by decrease of H3K9 accessibility for immunodetection and in the end of this process neither HP1 nor dimethylated H3K9 are detected in final differentiated neutrophils.

In collaboration with the Hemato-oncological Clinic of the Faculty Hospital in Olomouc we started to study the status of HP1 proteins and dimethylated H3K9 also in neutrophils of acute myeloid leukemia (AML) with normal karyotype. It is a recently find form of AML provoked by mutations in the gene for nucleophosmin (NPM1). The favorable prognosis of this disease is dependent on mutations in additional genes. The most frequent collateral mutations arise in the FLT3 (fms-like-tyrosinkinase) gene and they aggravate the prognosis. Our results show that neutrophils of all patients with some mutation in NPM1 gene, analyzed until now, have small amount of HP1β and immunodetected dimethylated H3K9 (exceptionally HP1γ but never HP1α) in their chromatin regardless mutations in additional genes. It follows from this finding that terminal differentiation of neutrophils of these patients is impaired resulting in incomplete condensation of their chromatin. We suppose that in the case of the successful treatment of this disease, the terminal differentiation of neutrophils will be complete which will be manifested by disappearance of HP1 proteins from their chromatin.

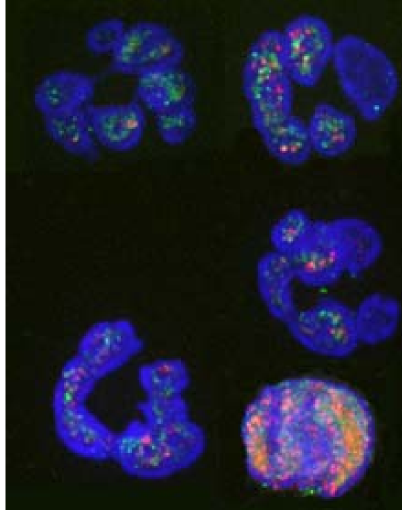

Fig. 4. Neutrophils of an AML patient having mutations in NPM1 and FLT3 genes. Immunodetection shows the presence of HP1β (red) and HP1γ (green) proteins. Much higher level of these proteins could be seen in a lymphocyte on the bottom of the image.

Chromatin structure in two regions of chromosome 11 differing fundamentally in the content of highly expressed genes

The transcriptom map of Caron et al. (Science 291,2001,1289-1292) shows significant clustering of highly expressed genes with patterns that are remarkably similar among many cell or tissue types. The most highly expressed genes tend to be clustered in genomic regions called RIDGEs (regions of increased gene expression). ANTI-RIDGEs regions have low gene density and contain mainly weakly expressed genes.

In this work we tried to find the nuclear structure and arrangement of chromatin in one RIDGE and one ANTI-RIDGE of chromosome 11 by the measuments of distances between probes in both regions visualized by 3D FISH in G1 nuclei of human fibroblasts. To get the most precise information about the structure of chromatin in these regions, the statistic analysis of large number of measurements was performed owing to the variable character of chromatin in individual cells.

The measurements of nuclear distances between the high number of DNA

probes in these regions show that chromatin in both regions exhibits the random walk behavior typical of linear flexible molecular chain folded in a random manner. The relationship between mean-square interphase distances and genomic distances between probes has two linear phases with transition at ~ 2 Mbp showing the existence of two levels of chromatin organization in both regions. One, at lower genomic distances is looser and that at distances up to ~ 2 Mbp is more tight. However the chromatin compaction in both levels of its organization is of about 40 % lower in the region rich on highly expressed genes – RIDGE. Thus, the results show that the three dimensional structure of interphase chromosome underlie to the same rules regardless the local distribution and content of highly expressed genes. However the distribution and content of highly expressed genes determine the degree of chromatin compaction in the chromosome region being more open in the region of clusters of highly expressed genes.

Granted projects

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

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

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

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

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

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

ME, LC 535, Center of Basic Research, Dynamics and organization of chromosomes during the cell cycle. principal investigator: I. Raška, Co-principal investigator: S. Kozubek, 2005-2009

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Laboratory of Cytokinetics focuses on the research in the field of cellular signalling and physiology relevant to cancer and potential role of lipid membrane elements and their derivatives in these processes. In particular, the effects of environmental substances, such as lipid nutrition components (essential polyunsaturated fatty acids and butyrate) and xenobiotics (cytostatics and environmental organic pollutants) on regulation of cytokinetics, i. e. cell proliferation, differentiation and apoptosis are studied. Using both tumor and non-tumorigenic cells, new types of interactions of lipid dietary components, anticancer drugs (non-steroidal anti-inflammatory drugs-NSAIDs, cytostatics) or selected environmental pollutants (polycyclic aromatic hydrocarbons, PCBs, dioxins) with physiological regulators of cytokinetics are being investigated. Attention is being paid especially to tumor necrosis factor (TNF) family, tumor growth factor (TGF) family, fibroblast growth factor (FGF) and Wnt/beta-

catenin pathway signalling. The results are exploited in cancer prevention/therapy and in ecotoxicology.

Cellular and molecular physiology of lipids

Among lipid nutrients, ω-6 and ω-3 essential polyunsaturated fatty acids (PUFAs) and short-chain fatty acid produced from dietary fiber - butyrate (NaBt) are required for a proper functioning of colon epithelium and they play a role in the colon carcinogenesis. Our previous studies have documented that lipid dietary components may interact both mutually and with endogenous regulators operating in the colon, such as apoptotic inducers of tumor necrosis factor (TNF) family. Moreover, we suggest that the response of colon cells is altered during colon cancer development.
Using human colonic cell lines derived from fetal tissue (FHC) and colon adenocarcinoma (HT-29, HCT-116) our attention is focused on interaction of i/ NaBt with arachidonic (AA) and docosahexaenoic acid (DHA), ii/ NaBt with TNF family molecules, and iii/ DHA with TRAIL. Different effects of NaBt, PUFAs, and their combination on FHC and adenocarcinoma cell lines.

We showed significant differences in cytokinetic response between these cell lines. While in FHC and HT-29 cells NaBt induced G0/G1 arrest, differentiation and low level of apoptosis, in HCT-116 cells G2/M arrest, no differentiation and high level of apoptosis were detected. Moreover, in FHC (and partially in HT-29 cells), a significant potentiation of apoptosis accompanied with an increased cell cycle arrest, cell detachment, and decreased differentiation were detected after combined treatment with NaBt and PUFAs, especially with DHA.

Using previously established models of non-adherent cellular cultivation of colon epithelial cells, we studied the effects of NaBt on the expression of proteins mediating cell-ECM and cell-cell interactions during both adherent and non-adherent cultivation of FHC and HT-29 cells. We have found changes in the expression and activation of two key protein kinases (focal adhesion kinase – FAK and integrin-linked kinase – ILK) involved in cell adhesion. NaBt induced the expression of both cell-ECM and cell-cell interaction molecules, but decreased the activation of the kinases involved in cell adhesion.

Mechanisms of TRAIL effects

TRAIL is a promising inducer of tumor-specific cell death, but in some cases it can also stimulate signalling pathways leading to proliferation and survival of cancer cells of different origin. We detected higher but limited sensitivity of cancer HT-29 then fetal FHC cells to TRAIL treatment. The TRAIL-induced apoptosis of HT-29, but not of FHC cells, was significantly enhanced by U0126, which inhibits MEK/ERK pathway. The most significant differences

between these two cell lines were observed with regard to the involvement of the mitochondrial apoptotic pathway.

In our previous work we showed that TRAIL is capable of triggering a response leading to significant early transient ERK-mediated transcriptional up-regulation of anti-apoptotic Mcl-1 mRNA and protein in colon cancer cells responsive to its apoptotic effects, but not in TRAIL-resistant colon cells. In 2007 we continued our studies focused especially on the anti-apoptotic action of Mcl-1 protein. Using an siRNA approach, we demonstrated that the up-regulation of Mcl-1 protein exerted by TRAIL could be an important mechanism attenuating/delaying its apoptosis-inducing effects.

Furthermore, we found an increase in FAK activation and Akt phosphorylation during non-adherent cell cultivation compared to adherent cultivation. Based on this, we have proposed an association between adhesion kinase and PI3/Akt pro-survival pathways, which might be responsible for the decreased sensitivity of the colon cells to the TRAIL action during the non-adherent type of cell cultivation.

Growth factors in cancer cell signalling

Pathophysiological conditions reflected in deregulation of differentiation, proliferation and apoptosis modify homeostasis and function of prostate epithelia and can lead to diseases such as benign hyperplasia and cancer. Transforming growth factor-beta, interleukin-6 (IL-6) and Wnt family members represent highly biologically active molecules, secreted to the tissue microenvironment, where they can induce different signalling pathways in paracrine and/or autocrine manner. These autocrine/paracrine factors have been shown to change microenvironment in prostate gland and modulate growth and survival of cancer cells. Defects in functions of components of these pathways have been observed in various human cancers. In 2007 we continued with studies focused on (1) functional role of GDF-15 (member of transforming growth beta family) in the effects of cyclooxygenase inhibitors (non-steroidal anti-inflammatory drugs (NSAIDs), (2) role of PUFAs in modulation of signalling pathway of pro-inflammatory cytokine IL-6, (3) modulation of neuroendocrine differentiation (NED) of prostate cancer.

Studies investigating anti-cancer effects of NSAIDs have shown modulation of the PI3K/Akt pathway. We observed significant differences in the sensitivity of prostate and colon cancer cell lines to antiproliferative effects of NSAIDs. The prostate cancer cell line LNCaP, which is PTEN and SHIP2 negative, was the most sensitive to these effects. Knockdown of SHIP2 by RNA interference in PTEN negative prostate and colon cancer cell lines resulted in higher sensitivity to antiproliferative effects of NSAIDs, which was assessed by analyzing the cell

cycle profile and expression of cell cycle regulating proteins. Using RNAi we have also disclosed that NSAIDs-induced early cell cycle arrest in LNCaP cells is not dependent on increased expression of GDF-15. Our data suggest that multiple defects in negative regulation of the PI3K/Akt pathway may contribute to increased sensitivity to chemopreventive effects of widely used drugs. The mechanisms driving this process are currently under investigation.

Neuroendocrine differentiation of epithelial prostate cancer cells is a phenomenon clearly associated with cancer progression. However, mechanisms controlling differentiation of prostate epithelial cells have remained poorly characterized. We have established experimental model of neuroendocrine differentiation of prostate epithelial cells in our laboratory. This model is currently used for studies focused on describing of mechanism driving neuroendocrine differentiation. The successful completion of these studies will reveal innovative strategies for treating prostate cancer in terminal stages.

Molecular mechanisms of Wnt signalling

Wnt signalling represents one of the most conserved means of intercellular communications, which regulates both development and diseases, such as cancer. Although it is generally accepted that Wnts are crucial regulators of both homeostatic and disease conditions, it is unknown how Wnt signal is modulated and transduced in the cytoplasm via phosphoprotein Dishevelled (Dvl).

Our results showed that all Wnts can induce activation of casein kinase 1, which in turn phosphorylates Dvl. These data identified a possible common module, which can be utilized by both canonical and non-canonical Wnt signalling (REF: JCS). When we studied in detail the effects of endocytosis on cytoplasmic Wnt signal transduction, we have surprisingly discovered that Dvl is unstable protein, which can be quickly and potently degraded when endocytosis is blocked (REF: Acta). These data suggested that level of Dvl is physiologically regulated and decides the sensitivity of cell to Wnt ligand. One of the possible partners of Dvl in endocytotic process is beta-arrestin. When we studied the interaction of these two proteins in detail we found out that beta-arrestin is required component for canonical Wnt signal transduction and may be a missing link between Dvl and downstream effectors such as axin (REF: PNAS). We have also critically reviewed this and other recently published data in the summarizing article about signal transduction between Frizzled receptors and downstream effectors such as Dvl.

Mechanisms of fibroblast growth factor 3 (FGFR3) signalling

In 2007 our main focus was on mechanisms of FGFR3 signalling in cartilage.

We have discovered several novel features of FGFR3 signalling in chondrocyte environment that are briefly outlined bellow.

(1) Description and analysis of FGFR3-mediated matrix metalloproteinase (MMP) induction and activation that underlines the novel phenotype of FGFR3 signalling in cartilage, i.e. the loss of chondrocyte extracellular matrix.

(2) Molecular analysis, for the first time, of the Frs2, Shc and Gab1 adaptor complexes proximal to FGFR3 and their contribution to the FGFR3-mediated sustained activation of Erk MAP kinase pathway in chondrocytes.

(3) Discovery of a critical role of protein kinase C in FGFR3-mediated activation of Erk MAP kinase pathway in chondrocytes.

(4) Development of the chondrocyte-based high-throughput screening assay for identification of novel pathways of FGFR3 signalling in chondrocyte environment.

(5) Exclusion of STAT1 and STAT3 transcription factors from their direct participation in FGFR3-mediated chondrocyte growth arrest.

We have also studied the FGF/FGFR signalling in B-cell chronic lymphoid leukemia (BCLL), resulting in a discovery of marked upregulation of a potential novel oncogene in BCLL, the apoptosis-inhibitor 5 (Api5).

Cellular and Molecular Toxicology

Diverse environmental organic pollutants are known interfere with physiological regulatory mechanisms controlling cell proliferation, apoptosis or cell-to-cell communication, thus leading to disruption of homeostasis and development of diseases, including cancer. The principal aim of our studies is to contribute to understanding of their effects at molecular and cellular level, which might be linked to carcinogenesis, reproductive or developmental impairment. In 2007, our work concentrated on the AhR-dependent gene transcription, deregulation of cell proliferation and cell-to-cell communication in cells affected by various group of organic contaminants, including polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and dioxins. The results published in 2007 can be divided into three principal groups. First, we characterized the impact of some less-known groups of PAHs on model liver cells, including methylated derivatives of phenanthrene, anthracene, naphthalene and benz[a]anthracene, or PAHs with higher molecular weight, including dibenzoanthracenes and benzochrysenes. We have characterized some structural determinants of their AhR-mediated activity and/or ability to inhibit gap-junctional intercellular communication. We found that given their environmental levels, many of these compounds should be considered to be important contributors to toxic effects related to carcinogenesis, including activation of AhR.

Second, we have further characterized rat liver progenitor cells as a valuable in vitro model, which enables insight into toxic modes of action of environmental carcinogens. We have shown that rat liver ‘stem-like’ cells are a useful tools for studies on genotoxic effects of PAHs, harboring high levels of inducible enzymes involved in metabolic activation of PAHs and providing material for analysis of formation of DNA adducts and related genotoxic events. Moreover, we have used this cellular model to characterize impact of different classes of AhR ligands (agonists vs. partial antagonists) on deregulation of cell proliferation or expression of xenobiotic-metabolizing enzymes.

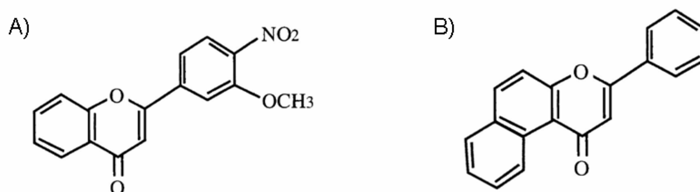

Fig. 1. Chemical structures of model flavones. A) 3´-methoxy-4´-nitroflavone was designed as AhR antagonist with basic flavone structure; B) beta-naphthoflavone, so-called 5,6-benzoflavone, was shown to act as potent AhR agonist.

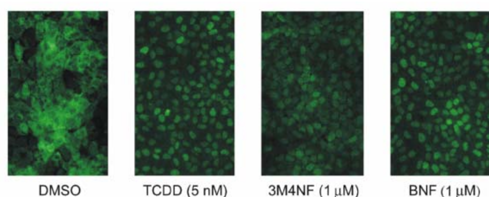

Fig. 2. Cellular localization of AhR. Localization of AhR was assessed by indirect immunofluorescence using staining with anti-AhR antibody in confluent WB-F344 cells treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 3´-methoxy-4´-nitroflavone (3M4NF), beta-naphthoflavone (BNF); DMSO (0.1%) was used as a control.

Finally, we have analyzed interactive effects of AhR ligands and a principle

proinflammatory cytokine, tumor necrosis factor- alpha (TNF). We have observed that TNF is able to synergistically enhance the ability of dioxins or PCBs to disrupt contact inhibition in rat liver epithelial cells, which associated with enhanced induction of cyclin A expression. Moreover, TNF- alpha was found to differentially affect expression of cytochrome P450 (CYP) enzymes involved in metabolic activation of promutagens, such as benzo[a]pyrene, leading to enhanced expression of CYP1B1, increased formation of DNA adducts and related genotoxic events. These results suggest that conditions of chronic inflammation might potentiate toxic effects of environmental pollutants related to both tumor initiation and promotion. Our present results thus have implications for understanding of the process of chemical carcinogenesis and evaluation of risks associated with exposure to carcinogenic compounds.

The effects of cytostatic compounds

LA-12 is a novel platinum(IV) compound with adamantylamine, which is able to overcome intrinsic and acquired resistance to widely used platinum compound cisplatin in ovarian cancer cell lines. We intensively examin the mechanisms of LA-12 effects and compare its effects with cisplatin and oxaliplatin (III. generation of platinum drug). Modulation of cell cycle machinery reflects complexity of the cellular response to platinum-compound caused DNA damage and leads to the cell cycle arrest and/or apoptosis. Analysis of the cell cycle of ovarian cancer cell line A2780 revealed that equitoxic concentration of cisplatin and LA-12 caused accumulation of cells in S-phase of the cell cycle, but only in case of LA-12, persistent arrest was achieved. Furthermore, various components of cell cycle machinery were studied with regard to regulation of passage through the S-phase to G2-phase, e.g. associated kinase activities and protein-protein interactions between CDK inhibitor p21 and cyclin depentent kinase 2 (Cdk2), cyclins A and B1. Additionally, expressions of various proteins involved in DNA-damage-responsive signalling were assesed. Simultaneously, LA-12 toxicity was characterized in colorectal cancer cell lines HCT116 and HT-29. Compared to oxaliplatin, LA-12 associated cytotoxicity was superior to oxaliplatin in both cell lines tested.

In cooperation with UPJS Košice, Slovak Republic we have developed the study of hypericin photocytotoxicity mediated by photodynamic therapy (PDT) in colon cancer cells.

Hypericin, one of the promising photosensitisers, is known to induce apoptosis with high efficiency in various cell lines. However, we reported the prevalence of necrosis accompanied by suppression of caspase-3 activation in colon adenocarcinoma HT-29 cells exposed to an extensive range of PDT doses evoked by various hypericin concentrations and light doses. Introduction of Bcl-

2 into HT-29 cells invoked caspase-3 activation, changed the Bcl-XL expression pattern, increased apoptosis and arrested cells in G2/M phase of the cell cycle.

We evaluated the effectiveness of a combined modality approach using pretreatment of HT-29 cells with various inhibitors of lipoxygenase (LOX), cyclooxygenase (COX) and cytochrome P450-monooxygenase pathways followed by hypericin-mediated PDT. We demonstrated that pretreatment of cells with 5-LOX inhibitor MK-886 as well as 5-, 12-LOX and 12-LOX inhibitors (esculetin and baicalein, respectively) significantly and dose-dependently altered hypericin-mediated PDT effects on cell proliferation and viability. Pretreatment of cells with various COX inhibitors promoted PDT therapy, but these effects are probably COX independent. These results imply that some of inhibitors used could be considered for potentiation of PDT.

Granted projects

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

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

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

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

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

GA CR 524/06/0517, Mechanisms of disruption of cell-to-cell communi-cation

and regulation of cell proliferation in liver cells. Principal investigator: J. Vondráček, 2006 - 2008

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

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

European Social Fund (ESF) - Ministry of Education, Youth and Sports
Improvement of qualification and flexibility of Ph.D. students of the Faculty of medicine, UP. Principal co-investigator: A. Kozubík, 2006 - 2007

Programme KONTAKT, International Scientific and Technological Cooperation (Czech Republic – Slovak Republic). The effects of COX-2 inhibition in both tumour and non-tumour colon epithelial cells exposed to phototoxic hypericin effects. Principal investigator: A. Kozubík, 2006 - 2007

MU Rector's Programme for Students´ Creative Activity Support 20061431C0007, Effects of cytostatics and xenobiotics on deregulation of cell proliferation and apoptosis. Principal investigator: J. Zatloukalová, 2006 - 2007

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DNA BIOPHYSICS AND GENOME BIOINFORMATICS

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The laboratory studies regularities of primary, secondary and tertiary structures of genomic molecules of DNA, RNA, 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, how they undergo changes. A particular attention is focused on the role of DNA unusual structures in the human genome.

In the year of 2007, we mainly achieved results in the field of photochemical probing of DNA unusual structures in genomic molecules of DNA and their use in the studies of DNA B-A transition and guanine quadruplex formation.

The (Gn).(Cn) blocks are the known to be the most prone to adopt A-form. Therefore we expected that their B-A transition should take place at low negative superhelics. A-form is much more UV resistant than B-form. That is only we detected the photoproducts by restrictases cleaving DNA in the (Gn).(Cn) blocks. These occur in the polylinker of pUC19.

We constructed two recombinant plasmids that have longer (Gn).(Cn) blocks introduced in the polylinker. These plasmids include pKN9G (block G9) and pKN12G (block G12) distant from C3G4 block by 9 bp. Using topoisomerase and EtBr we prepared topoizomers of the recombinant pUC19 having low (-0.008, -0.016) and high (-0.070) superhelical densities. Relaxed DNA, linearized DNA, and native density DNA (superhelical densities -0.055 and -0.066) are used as control samples.

Samples of DNA (0.2 - 0.25 μg in 20 μl) dissolved in 10mM Tris.HCl, pH 6.4, 0.1 mM EDTA were irradiated by the dose of 15 kJm-2 at -1°C. After drying, the DNA was linearized by CaiI or AlwNI restrictases. The samples were checked for complete digestion. The damage was detected by restrictases extending into the G4 (pUC19), i.e. Eco 88I and BamHI, G9 (pKN9G) or G12 (pKN12G), i.e. Bam HI. Detection restrictases cleaving the DNA outside the polylinker region, included Bsa XI (CGCGG4), Bse GI (G5) or Eci I (C6). The damage was quantified as a ratio of uncleaved DNA divided by the sum of cleaved and uncleaved DNA.

The restriction target site of Eco 88I contains a guanine trimer. In the B-form it is sensitive to UV light whereas in the A-form it is almost totally resistant. We studied dependence of cleavage by this restrictase on the plasmid superhelical density and found negligible effects even at the highest superhelical densities. Hence we conclude that the UV damage of the restriction sites does not depend on the superhelical density. Hence superhelical density does not induce the A-form in DNA, which is surprising.

Next we performed an analogous study with the plasmid pKN9G where we used Eco 88I and Bam HI restrictases for the detection of UV damage. Eco 88I cleaves within the G4 block, Bam HI within the G9 block. The two targets are separated by mere 9 base pairs. Neither did their study revealed results indicating a superhelical-induced B-A transition.

Finally, we did a similar study with the pKN12G plasmid containing a 12 bp G12.C12 block. The results were the same again. Hence the studies showed that negative superhelical density, even the very high one, does not induce the B-A transition at least within the Gn.Cn blocks which are known to be the most prone to this transition. This is a surprising and very interesting result.

Granted projects

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

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

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Free Radical Pathophysiology

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Carvedilol and adrenergic agonists suppress the lipopolysaccharide-induced NO production in RAW 264.7 macrophages via the adrenergic receptor activation

The interaction of adrenergic agonists and/or antagonists with the adrenergic receptors expressed on immunologically active cells including macrophages plays an important role in regulation of inflammatory responses. Our study investigated the effects of carvedilol, a unique vasodilating β-adrenergic antagonist, on the production of nitric oxide (NO) by lipopolysaccharide-stimulated murine macrophage cell line RAW 264.7. NO production was determined as the concentration of nitrites in cell supernatants (Griess reaction) and inducible nitric oxide synthase (iNOS) protein expression (Western blot analysis). Scavenging properties against NO were measured electrochemically. Carvedilol (in a concentration range of 1, 5, 10 and 25 μM) inhibited iNOS protein expression and decreased the nitrite concentration in cell supernatants. Adrenergic agonists adrenaline, noradrenaline or dopamine inhibited the iNOS protein expression and the nitrite accumulation. Blockers of α- and β-adrenergic receptors prazosine and atenolol prevented the effect of carvedilol and adrenergic agonists on

nitrite accumulation and iNOS expression in lipopolysaccharide-stimulated cells. These results, together with the absence of scavenging properties of carvedilol against NO, imply that both carvedilol and adrenergic agonists suppress the lipopolysaccharide-evoked NO production by macrophages through the activation and modulation of signaling pathways connected with adrenergic receptors.

Effect of inorganic pollutants on bioluminescence of bacteria Photorhabdus temperata and Photorhabdus luminescens subsp. thracensis

Bioluminescence (BL) emitted by terrestrial bacteria Photorhabdus temperata and Photorhabdus luminescens subsp. thracensis was analysed to verify whether BL of Photorhabdus sp. can be the sensitive indicator of the presence of toxicants in water or water solutions. Exact concentrations of bright bacterial cells ($2.4*10^8$ cells) collected from the cultures in stationary phase were prepared. The salinity was adjusted using NaCl to 0.5, 1.0, 1.5 or 2.0 % and bacterial BL was measured continuously for the time period of 30 minutes. Longer time intervals were not tested because the decrease of BL after 15 or 30 minute co-cultivation of bacteria with metal ions is usually used for the calculation of EC50. BL of both Photorhabdus temperata and P. luminescens subsp. thracensis was quite stable and independent on the salinity. While no effect of salinity was observed, BL signal of bacteria cultivated in pH 6.2 was about 30% lower in comparison with bacteria cultivated in pH 8.2. It was the reason why we decided to use HBSS in further experiments. HBSS is the buffer without aldehyde (sometimes used as an external enhancer of BL) which does not interfere with BL emitted and ions and glucose present in the buffer ensure the viability of bacteria and their proper metabolism. In the following set of experiments, effect of $CdCl_2$, $CuSO_4$, $NiSO_4$, $HgCl_2$, and $K_2Cr_2O_7$ was tested. Bacteria P. temperata and P. luminescens subsp. thracensis were sensitive to metal ions and BL of these strains was decreased in the presence of metal ions similarly in both strains. The effect of $CdCl_2$ on BL of Photobacterium leiognathi (marine bacteria included in the commercial kit CheckLight ToxScreen On-Site Test) was quite comparable with effects reached using P. temperata and P. luminescens subsp. thracensis. It proves that BL of terrestrial bacteria could be potentially used for routine testing of water toxicity.

Oxidatively modified collagen lost its activating properties towards oxidative burst of phagocytes

Calf or rat skin type I collagens were isolated by a standard procedure involving acetic acid extraction, salting out with NaCl and using ion exchange chromatography on DEAE-cellulose. The collagen concentration was determined according to the modified Biuret method. Collagen samples were subjected to the oxidative modification by incubation of collagen solutions (1 mg/mL in 10 mM HCl, pH adjusted to 5.0-5.5) with different oxidants: hydrogen peroxide (100 mM or 300 mM), hydroxyl radical (100 mM $FeSO_4$/2 mM H_2O_2 or 50 mM $FeSO_4$/5 mM H_2O_2), peroxyl radical (obtained by the thermal decomposition of 200 mM ABAP), and sodium hypochlorite (5% NaOCl). After the oxidation treatment all samples were subjected to an extensive dialysis against 10 mM HCl. Collagen samples dissolved in 50 mM acetic acid and dialyzed against 10 mM HCl only were designated as non-modified (control) samples.

Thermal denaturation of the non-modified and modified collagen samples was performed on UV-VIS spectrophotometer, equipped with thermal gradient device. The heating rate used was 0.5^{o}C/min within the temperature range 22-50°C. The observed denaturation temperatures of rat skin collagen samples were as follows: Td1 = 35.06± 0.2^{o}C (non-modified collagen), Td1 = 30.58 ± 0.2^{o}C (hydroxyl radical modified collagen), Td1 = 31.78± 0.2^{o}C and Td2 = 35.76± 0.2^{o}C (hydrogen peroxide modified collagen), and Td1 = 31.30 ± 0.2^{o}C and Td2 = 35.36± 0.2^{o}C (peroxyl radical modified collagen). The denaturation profiles visible in spectrophotometer were also verified on the microcalorimeter. Similar results were obtained for calf skin collagen. It is obvious from the data that the used procedures led to the modification of collagen samples characterised by the decrease in denaturation temperature.

Two different methods were used to evaluate the capacity of collagen samples to scavenge peroxyl radicals – TRAP (total peroxyl radical-trapping antioxidative parameter) and ORAC (oxygen radical absorbance capacity). Both methods revealed that even non-modified (native) collagen has a very low capacity to scavenge peroxyl radicals. This capacity was further diminished in oxidatively modified collagen samples.

It is obvious from the results that native collagen itself has a capacity to induce a spontaneous oxidative burst of isolated neutrophils. Oxidatively modified collagen samples lost their capacity to induce the oxidative burst of neutrophils the changes being independent on the type of oxidative

treatment. Furthermore, the effect of both native and oxidatively modified collagens on the oxidative burst of neutrophils activated by opsonized zymosan particles, phorbol myristate acetate, calcium ionophore or formyl-Met-Leu-Phe was studied.

Plasma levels of myeloperoxidase are not elevated in patients with stable coronary artery

Plasma and serum levels of myeloperoxidase (MPO), a redox-active hemoprotein released by polymorphonuclear neutrophils upon activation, is now recognized as a powerful prognostic determinant of myocardial infarction in patients suffering acute coronary syndromes. However, there is limited information on whether systemic MPO levels are also elevated and of discriminating value in patients with stable coronary artery disease (CAD). Particularly, there are no significant clinical study representing different ethnic groups. Therefore, in cooperation with University of California at Davis, plasma levels of MPO and traditional CAD risk factors were quantified in African American and Caucasian patients (n=557) undergoing elective coronary angiography. Interestingly, MPO levels did not differ significantly between patients with or without CAD [421 pM (321, 533) vs. 412 pM (326, 500), $p>0.05$]. MPO levels were similar across ethnicity and gender, and correlated positively with CRP and fibrinogen levels ($r=0.132$, $p=0.002$ and $r=0.106$, $p=0.007$, respectively). Thus in summary, plasma MPO levels were not elevated in patients with stable CAD, suggesting that systemic release of MPO is not a characteristic feature of asymptomatic CAD.

Modulation of retinoic acid induced neuronal differentiation of P19 mouse embryonal carcinoma cells

Retinoic acid (RA), the derivative of retinol, plays significant roles in the regulation of cell proliferation, differentiation, and apoptosis. In vitro, the pluripotent embryonal carcinoma (EC) and embryonic stem (ES) cells are induced by RA to differentiate into various lineages. It was documented previously that growth in serum-free media itself committed EC cells to neural differentiation and the addition of RA intensified this effect. Leukemia inhibitor factor (LIF) is a cytokine playing a key role in the regulation of self-renewal and maintenance of undifferentiated state in mouse ES cells. At the same time, LIF potentiates retinoic acid-induced neural differentiation of pluripotent mouse embryonal carcinoma P19 cells. The response of pluripotent cells to LIF is mediated mainly by the STAT3 and ERK signalling pathways. Interestingly, pro-neural effects of LIF and

partially also of retinoic acid are abolished by inhibition of the JAK2->STAT3 signalling pathway. In contrast, inhibition of the MEK1->ERK signalling pathway does not exhibit any effect. These results suggest that in neurogenic regions, cooperative action of LIF and other neuro-differentiation-inducing factors, such as retinoic acid, may be mediated by the STAT3 signalling pathway. Further, signaling pathways and transcriptional factors involved in embryonal cell differentiation are suggested to be sensitive to redox status of intra-cellular environment. Thus, it can be hypothesized that intracellularly produced reactive oxygen species (ROS) can modulate differentiation of EC/ES cells. Selected antioxidants (glutathione, N- acetyl cysteine, and ascorbic acid) together with inhibitors of intracellular ROS production (diphenylene iodonium chloride and apocynin) were tested to modulate RA-induced differentiation of P19 cells to neural-like cells. Interestingly, RA significantly increased intracellular production of reactive oxygen species which was inhibited by antioxidants. The RA-induced differentiation of P19 cells into neural-like cells was documented by downregulation of markers of undifferentiated stage of EC cells (E-cadherin and Oct-4) up-regulation of markers of neural cells (N-cadherin and IIIbeta-tubulin). To further characterize mechanism of antioxidant modulation of RA-induced EC cell differentiation an activity of RA-directed promotor the retinoic acid responsive element (RARE) was evaluated by luciferase reporter assay. Antioxidants significantly inhibited RA-induced activation of RARE. In summary, the obtained data suggest a role of reactive oxygen species in RA-induced embryonal pluripotent cell differentiation to neural lineages.

Aldose reductase inhibition abolishes glucose-induced endothelial dysfunction

Increased glucose utilization by aldose reductase, a rate-limiting enzyme of the polyol pathway, has been implicated in the pathogenesis of diabetic vascular complications. In this process, several biochemical mechanisms are involved, including depletion of reduced cofactors necessary for action of antioxidant enzymes or endothelial NO synthase. In this study, the effect of a novel aldose reductase inhibitor JMC2004 on hyperglycemia-induced endothelial dysfunction was studied. Bovine aortic endothelial cells (BAEC) were treated with glucose (30mM), JMC2004 (0,01mM), or glucose + JMC2004 for 24 h. Then the cells were stimulated with 0,001mg/ml of calcium ionophore A23187 and NO production was measured electrochemically using porphyrine-coated carbon NO electrode. After the measurement, cell supernatants were harvested and nitrite concentrations were evaluated using Griess reaction. Further, peroxyl and

hydroxyl radical-scavenging activity of JMC2004 was measured with luminol–enhanced chemiluminescence. Superoxide scavenging was measured colorimetrically using XTT. 24h incubation of the cells with 30mM glucose strongly diminished calcium ionophore-induced response. Concomitant treatment with JMC2004 restored NO production by 50%. This effect was probably antioxidant-independent, since JMC2004 did not exert any scavenging activity towards any of tested radicals. In conclusion, aldose reductase inhibition with JMC2004 was able to abolish hyperglycemia-induced endothelial dysfunction in bovine aortic endothelial cells.

Granted projects

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

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

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

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

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

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

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

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

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Structure and Dynamics of Nucleic Acids

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

We continued in our MD studies of Kink-turn (K-turn) motifs. K-turn motifs are asymmetrical internal loops, characterized by sharp bend of phosphodiester backbone. K-turn motifs can be considered as flexible molecular hinges which are able to allow biologically significant motions of ribosomal segments during individual stages of proteosynthesis. We complement our preceding studies by MD simulations of the system formed by K-turn 42 and GTPase-associated center (rGAC) (Fig. 1). The molecule consists of alternating rigid and flexible segments. The first flexible region (Hinge1) is formed by the highly anharmonic kink of K-turn 42. The second flexible region (Hinge2) is formed by the helix 42 – helix 43/44 junction of rGAC. The rigid segments are the two arms of helix 42 flanking the kink. The whole molecule ends up with compact helices 43/44 (Head). The direction of preferred motion at Hinge2 roughly coincides with the direction of the elbow-like motion of the K-turn 42 and both motions preferably shift

the rGAC towards or outwards the body of the ribosomal subunit. The two consecutive flexible elements create a highly versatile RNA limb characterized by a complex set of bending and twisting essential dynamical modes. In other words, MD simulations show that individual rRNA building blocks have contrasting intrinsic dynamical predispositions and consecutive rRNA segments can further create molecular structures with characteristic patterns of internal flexibilities.

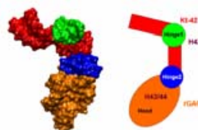

Fig. 1. Surface (left) and schematic (right) representations of the Kt-42 + rGAC rRNA system. Two flexible Hinges (green and blue) link three rigid segments – stems of Kt-42 (red) and Head (orange).

Extensive MD simulations were applied with different ion conditions and with two force fields (AMBER and CHARMM) to investigate the structure and dynamics of RNA tertiary motifs called kissing loop complexes from the Human Immunodeficiency Virus 1 (HIV-1) and the large ribosomal subunit of Haloarcula marismortui. This study represents extension of our previous investigations of kissing loop complexes from HIV-1 and Moloney Murine Leukemia Virus. We focused on characterization of base positions of unpaired flanking bases in multiple NMR and x-ray kissing loop structures. The standard MD simulations were further supplemented by locally enhanced sampling MD technique to enhance the sampling of the flanking bases. We found that an initial x- ray open conformation (Fig. 2) of bulged-out bases in HIV-1 complexes, affected by crystal packing, tends to convert to a closed conformation formed by consecutive stretch of four stacked purine bases. This is in agreement with those recent crystals where the packing is essentially avoided. We also observed variants of the closed conformation with three stacked bases, while nonnegligible populations of stacked geometries with bulged-in bases were detected, too. While the x-ray structures consistently show the flanking bases to be in bulged-out arrangement, the NMR experiments suggest their bulged-in orientation. The simulation results reconcile these differences observed in x-ray and NMR studies. Our results suggest that bulged-out geometries are somewhat more preferred, which is in accord with recent experiments showing that they may mediate tertiary contacts in biomolecular assemblies or allow binding

of aminoglycoside antibiotics.

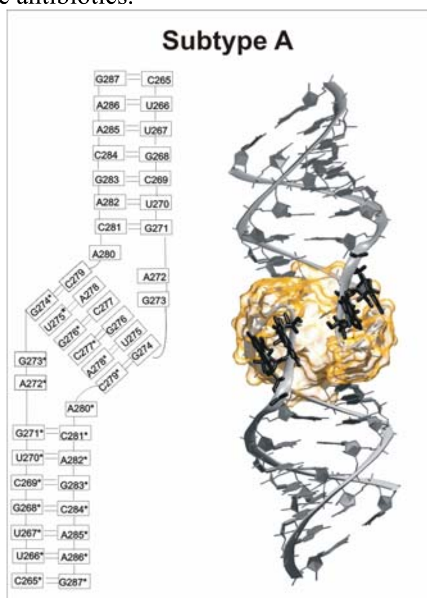

Fig. 2. Secondary (left) and tertiary (right) structure of HIV-1 DIS subtype A kissing loop complex (PDB code 1XPF). Flanking bases (A272, A272*, G273 and G273*) forming the open conformation (i.e., two separate two-base stacks) are highlighted in black. The cation binding pocket in the central part of the kissing loop complex is highlighted by transparent orange surface.

Helical junctions play an important role in biology. In DNA, their main significance is as central intermediates in homologous genetic recombination process which represents an important mechanism for the repair of damage to DNA that occurs in all cells. In RNA they are important architectural elements. We employed MD simulations to investigate the structural and dynamical properties of representative three-way junctions (3W-junctions) that are composed of three helices (P1, P2 and P3) (Fig. 3). Structured 3W-junctions typically include two coaxially stacked helices (P1 and P2) and can be categorized into three families: A, B and C. In our study, we focused on three representatives of the family C. These 3W-junctions are situated in functionally important sites of the large ribosomal subunit (like the Peptidyl Transferase Center, the GTPase-associated center and 5S rRNA). All three molecules exhibited two basic fluctuations on the nanosecond time scale: an anisotropic hinge-like motion between P2 and

P1/P3 helices and breathing-like motion of P1/P3 helices. We also found out that central regions of junctions are associated with strong ion binding sites which essentially contribute to stability of the structure. Our study is in a good agreement with x-ray and cryo-EM experiments and provides new information about structural dynamics of RNA junctions.

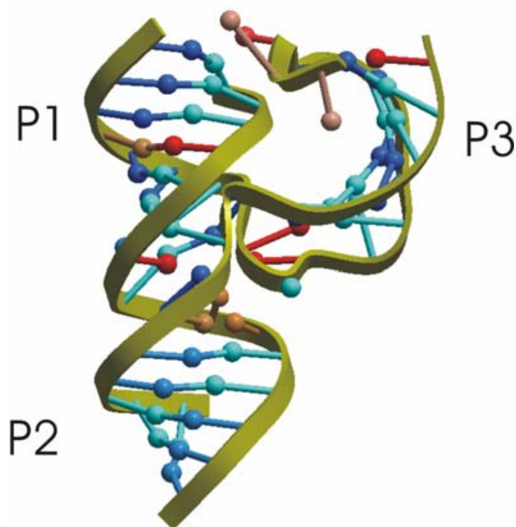

Fig. 3. Tertiary structure of 3W-junction from Peptidyl Transferase Center with depicted P1, P2, P3 helices.

Ribozymes represent highly structured noncoding RNA molecules with catalytic function. The activity of ribozymes often requires that specific regions have local structural flexibility and/or the ability to switch from one conformation to another. Understanding ribozyme function thus demands an accurate and comprehensive knowledge of the dynamics of the RNA's secondary and tertiary structure. An atomistic view of the structural dynamics as necessary for function of a ribozyme can only be obtained from few approaches, among them prominently MD simulations. We extended our previous work on Hepatitis Delta Virus (HDV) ribozyme (Fig. 4) to observe the conformational dynamics of G76 in atomistic detail and identify conformational substates of the catalytically essential trefoil turn that pivots around G76. In all available crystal structures, the conformation of G76 is

restricted by stacking with G76 of a neighboring molecule. However, in MD simulations G76 fluctuates between several conformations, including one wherein G76 establishes a perpendicular base quadruplet in the major groove of the adjacent P1 stem. Mutagenesis experiments suggest that the identity of the nucleotide in position 76 indeed contributes to the catalytic activity of a trans-acting HDV ribozyme. We also carried out MD simulations to study the role of dangling nucleotides at the 5' end of the genomic HDV ribozyme. This 5'-sequence contains a clinically conserved U-1 that is essential for fast cleavage. MD simulations demonstrate that a U-1 forms the most robust kink around the scissile phosphate, exposing the cleavage site to the catalytic C75 in a previously unnoticed U-turn motif found also, for example, in the hammerhead ribozyme and tRNAs. The results demonstrate that a common structural U-turn motif is used in distinct ways to accelerate catalysis in the HDV and hammerhead ribozymes.

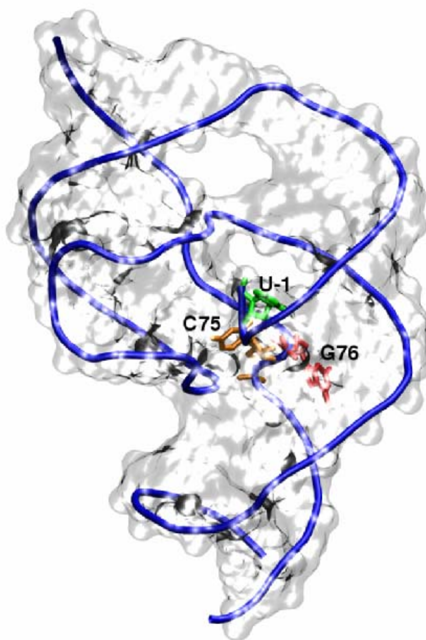

Fig. 4. Surface and ribbon representation of the precursor structure of Hepatitis Delta Virus ribozyme. Selected residues from the catalytic core are highlighted.

Complex molecular shapes of ribosomal RNA molecules are stabilized by recurrent types of tertiary interactions involving highly specific and conserved non-Watson-Crick base pairs, triples and quartets. We analyzed the intrinsic structure and stability of the P-motif and the four basic types of A-minor interactions (types I, II, III and 0) which represent the most prominent RNA tertiary interaction patterns refined in the course of evolution. In the studied interactions the electron correlation component of the stabilization usually exceeds the Hartree-Fock (HF) term, leading to a strikingly different balance of forces compared to standard base pairing stabilized primarily by the HF term. In other words, the A-minor and P-interactions are considerably more influenced by the dispersion energy compared to canonical base pairs which makes them particularly suitable to zip the folded RNA structures which are substantially hydrated even in their interior. Continuum solvent COSMO calculations confirm that the stability of the canonical GC base pair is affected (reduced) by the continuum solvent screening considerably more than stability of the A-minor interaction. Among the studied systems the strong A-minor II and weak A-minor III interactions require water molecules to stabilize the experimental geometry. Gas-phase optimization of the canonical A-minor II A/CG triple without water results in geometry which is clearly inconsistent with the RNA structure. The gas-phase structure of the P-interaction and the most stable A-minor I interaction nicely agrees with the geometries occurring in the ribosome. A-minor I can also adopt an alternative water mediated substate rather often observed in x-ray and molecular dynamics studies. The A-minor I water bridge, however, does not appear to stabilize the tertiary contact and its role is to provide structural flexibility to this binding pattern within the context of the RNA structure. Interestingly, the insertion of polar water molecule in the A-minor I A/CG tertiary contact occurs into the A/C tertiary pair stabilized primarily by the HF (electrostatic) interaction energy while the dispersion-controlled A/G contact remains firmly bound. Thus, the intrinsic balance of forces as revealed by QM calculations nicely correlates with many behavioral aspects of the studied interactions inside RNA. Comparison of interaction energies computed using quantum chemistry and AMBER force field reveals that common molecular mechanics calculations perform rather well, except that the strength of the P-interaction is modestly overestimated.

Granted projects

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

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

GA CR 203/05/0388, Conformational dynamics of nucleic acids. Principal investigator: V. Sychrovský, co-investigator: J. Šponer, 2005 - 2007

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

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

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

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PhD thesis defended in 2007

Ing. Filip Rázga, PhD, Structure and dynamics of rRNA: A computational study

ANALYSIS OF CHROMOSOMAL PROTEINS

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The ends of eukaryotic chromosomes are fixed due to their large size, resulting in a topological problem for cells to overcome when the genetic information in the double helix DNA has to be accessed. The superhelical tension and other topological consequences resulting from the separation of the two DNA strands must be resolved by topoisomerases in order to complete DNA replication, transcription and recombination. Two types of topoisomerases, both of which catalyze cleavage and religation of the DNA, are depicted in Fig. 1.

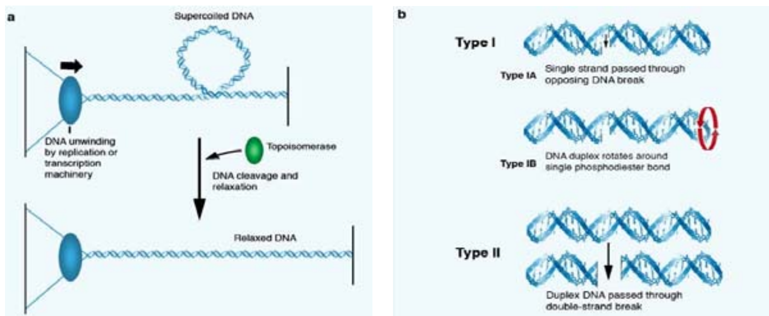

Fig. 1. DNA topology and topoisomerases. (a) DNA unwinding generates positive supercoiling which is resolved by topoisomerases. (b) Types of topoisomerases.

Human cells contain six DNA topoisomerases: Topo I (type IB), mtTopo I (mitochondrial, type IB), Topo IIα/β (type II) and Topo IIIα/β (type IA). Whereas the knowledge of mtTopo I and Topo IIIα/β is still fragmented, human topo I and topo II are intensively studied due to the fact that the enzymes are target of a large number of clinically effective anticancer drugs used in treatment of human malignancies.

DNA topo IIα is an essential nuclear enzyme and its unique decatenation activity has been implicated in many aspects of chromosome dynamics such as chromosome replication and segregation during mitosis. We have demonstrated that chromatin-associated protein HMGB1 (a member of the large family of HMG-box proteins with possible functions in DNA replication, transcription, recombination and DNA repair, reviewed by Štros et al.: Cell Mol. Life Sci., 64, 2007, 2590-2606) promotes topo IIα-mediated catenation of circular DNA (Fig. 2), relaxation of negatively supercoiled DNA and decatenation of kinetoplast DNA.

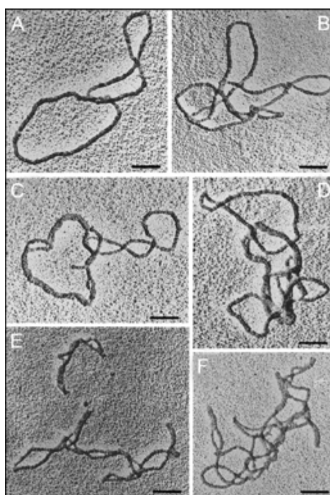

Fig. 2. The complex multimers formed by topoisomerase IIα and HMGB1 consist of fully catenated DNA molecules. Multimers were formed with plasmid DNA of 9.1 kb (panels A–D) or 2.8 kb (panels E–F), HMGB1 and human topoisomerase IIα. The samples were deproteinized and coated with RecA protein as single-stranded (panels A–D) or double-stranded DNA (panels E–F) before visualization by electron microscopy. All negatives were shot at 50 000 magnification. The bars represent 0.1 μm.

HMGB1 could interact with topo IIα and this interaction, like the stimulation of the catalytic activity of the enzyme, required both HMG-box domains of HMGB1. A mutant of HMGB1, which cannot change DNA topology stimulated DNA decatenation by topo IIα indistinguishably from the wild-type protein. Although HMGB1 stimulated ATP hydrolysis by topo IIα, the DNA cleavage was much more enhanced. The observed abilities of HMGB1 to interact with topo IIα and promote topo IIα binding to DNA suggested a mechanism by which HMGB1 stimulates the catalytic activity of the enzyme via enhancement of DNA cleavage (Fig. 3, see also Štros et al.: Nucleic Acids Res., 35, 2007, 5001-5013). Our recent data also suggested that over-expression of HMGB1 could cell-specifically transactivate the human topo IIα gene promoter and up-regulate cellular expression of the enzyme (Štros et al., submitted).

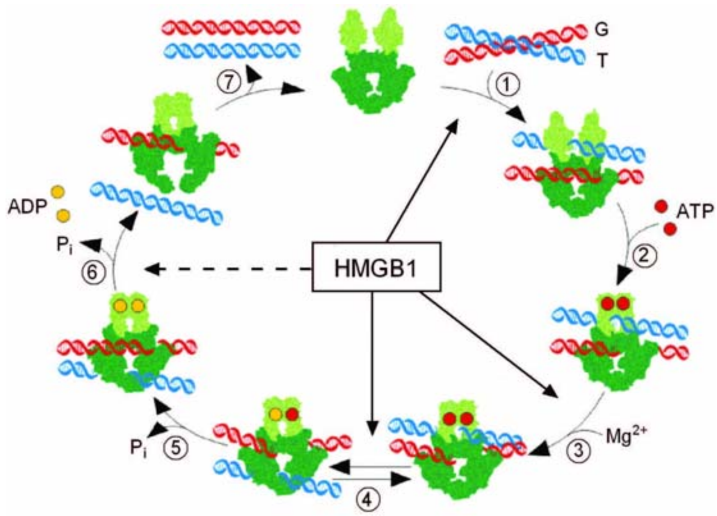

Fig. 3. HMGB1 and catalytic cycle of DNA topoisomerase IIα. Individual steps of the topo II catalytic cycle which are stimulated the most by HMGB1 are indicated by arrows: Step 1 (DNA binding), Steps 3 and 4 (DNA cleavage). The dashed arrow indicates the HMGB1-mediated partial relief of topo IIα inhibition by catalytic inhibitor ICRF-193.

The observed differences in HMGB1-mediated transactivation of the human topo IIα gene promoter in Saos-2, H1299 or MCF-7 cells suggested that the status of tumor suppressors (Rb-null Saos-2 versus Rb-plus H1299 or MCF-7 cells) might be essential for the effect of HMGB1.

We and others have previously shown that HMGB1 and HMGB2 (at >100:1 molar ratio of HMGB-to-DNA) could introduce into closed circular DNA negative supercoils in the presence of topoisomerase I (Sheflin and Spaulding: Biochemistry, 32, 1993, 3238-3248; Štros et al.: Nucleic Acids Res., 22, 1994,1044-1051). Our recent data also indicated that HMGB1 could stimulate activity of isolated topoisomerase I at 1:1 molar ratio of HMGB-to-topo I, suggesting a possibility of a direct physical interaction. In addition, HMGB1 seems to be involved in regulation of cellular expression of human topoisomerase I as revealed by our transfection experiments using siRNA specifically targeting HMGB1. As HMGB1/2 proteins are significantly up-regulated in most cancer cells (reviewed by Štros et al.: Cell Mol. Life Sci., 64, 2007, 2590-2606), HMGB1 and/or other HMGB-type proteins could have a significant impact on the formation/stability of topo I/II-DNA cleavable complexes, and correspondingly on the efficacy of anticancer drugs specifically targeting the latter enzymes. Thus, HMGB-type proteins could be important players in response of human cancer cells to topo I and II inhibitors, most likely in “concert” with other proteins, such as tumor suppressor proteins.

Granted projects

GA AS CR IAA400040702, Hemicatenated DNA loops: their occurence in human genome and recognition by tumor suppressor protein p53. Principal investigator: M. Štros, 2007 - 2009

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

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CD SPECTROSCOPY OF NUCLEIC ACIDS

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Intramolecular and intermolecular guanine quadruplexes of DNA in aqueous salt and ethanol solutions

DNA guanine quadruplexes are all based on stacks of guanine tetrads, but they can be of many types differing by mutual strand orientation, topology, position and structure of loops, and the number of DNA molecules constituting their structure. We have studied a series of nine DNA fragments (G3Xn)3G3, where X = A, C or T, and n = 1, 2 or 3, to find how the particular bases and their numbers enable folding of the molecule into quadruplex and what type of quadruplex is formed. We show that any single base between G3 blocks gives rise to only four-molecular parallel-stranded quadruplexes in water solutions. In contrast to previous models, even two Ts in potential loops lead to tetramolecular parallel quadruplexes and only three consecutive Ts lead to an intramolecular quadruplex, which is antiparallel. Adenines make the DNA less prone to quadruplex formation. (G3A2)3G3 folds into an intramolecular antiparallel quadruplex. The same is true with (G3A3)3G3 but only in KCl. In NaCl or LiCl, (G3A3)3G3 prefers to generate homoduplexes. Cytosine still more interferes with the quadruplex, which only is formed by (G3C)3G3, whereas (G3C2)3G3 and (G3C3)3G3 generate hairpins and/or homoduplexes. Ethanol, which is a more potent DNA guanine quadruplex inducer than are ions in water solutions, promotes intramolecular folding and parallel orientation of quadruplex strands, which rather corresponds to quadruplex structures observed in crystals.

Conformations of DNA strands containing GAGT, GACA, or GAGC tetranucleotide repeats

Alternating guanine-adenine strands of DNA are known to self-associate into a parallel-stranded homoduplex at neutral pH, fold into an ordered single-stranded structure at acid pH, and adopt yet another ordered single-stranded conformer in aqueous ethanol. The unusual conformers melt cooperatively and exhibit distinct circular dichroism spectra suggestive of a substantial conformational order but their molecular structures are not known yet. To find more about the primary structure specificity of the unusual conformational variability of the (GAGA) DNA strands, we have performed parallel comparative studies of related tetranucleotide repeats $(GAGC)_5$, $(GAGT)_5$, and $(GACA)_5$. The general conclusion following from these comparative studies is that the primary structure specificity is fairly high, indicating that not only guanines but also adenines play a significant role in the stabilization of these unusual structures. $(GAGC)_5$ is a hairpin or a duplex depending on DNA and salt concentration. Neither acid pH nor ionic strength or the presence of ethanol changed the secondary structure of $(GAGC)_5$ in a significant way. $(GACA)_5$ forms a weakly stable hairpin in neutral aqueous solutions but forms a duplex at acid pH where cytosine is protonated. $(GAGT)_5$ behaves most similar to $(GAGA)_5$. Salt induces its hairpin to duplex transition at neutral pH and an isomerization into another, probably parallel stranded, duplex takes place at acid pH. $(GAGT)_5$ is the only of the three present 20mers that responds to ethanol like $(GAGA)_5$.

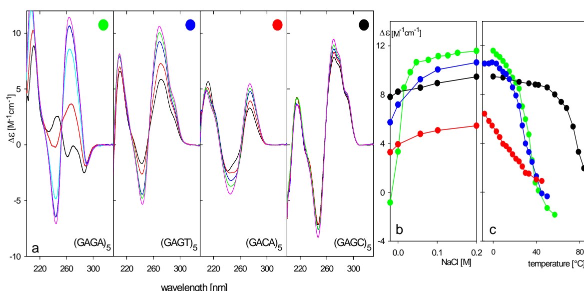

Fig. 1. (a) Changes in CD spectra induced by increasing concentration of NaCl (from black to violet spectra); (b) NaCl dependences and (c) temperature dependences in 0.2 M NaCl monitored by $\Delta\varepsilon$ values of the positive long-wavelength maximum.

Oligo(dT) is not a correct native PAGE marker for single-stranded DNA

Polyacrylamide gel electrophoresis is a widely used method to study short DNA fragments in solution. It is, however, a relative method requiring length markers to assess mobility, shape, flexibility, and molecularity of the DNA structures of interest. In recent literature we have encountered the use of oligo(dT) fragments as the native PAGE length markers. We have shown that this practice is inadequate because oligo(dT) migration is strongly retarded in native polyacrylamide gels. This conclusion is qualitatively true irrespective of the conditions of electrophoresis, oligo(dT) length, and gel concentration. Depending on their length, oligo(dT) fragments migrate 2-4 times slower than that would correspond to their nucleotide number. This leads to erroneous conclusions, e.g., determination of the number of associated molecules in guanine quadruplexes or other DNA complexes.

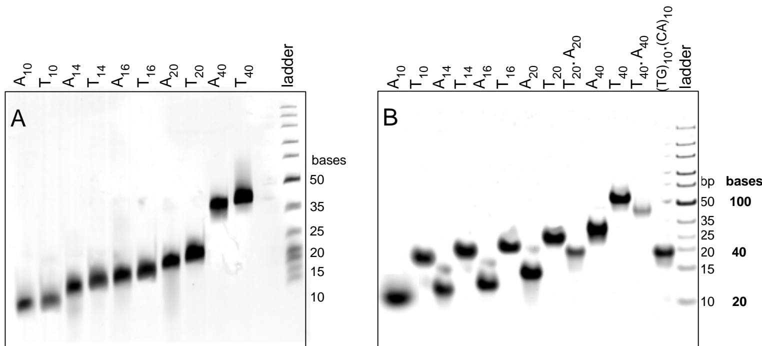

Fig. 2. Polyacrylamide gel electrophoreses: (A) denaturing in TBE + 7 M urea running at 52°C for 50 minutes; (B) non-denaturing in Britton-Robinson buffer and 0.15 M NaCl, pH 7.4 running at 2°C for 18 hours.

G-quadruplex structure in the c-myc promoter region

Aberrant c-myc expression is a key step in the initiation and progression of many cancers. Initiation of c-myc transcription has been hypothesized to depend on the formation of a G-quadruplex DNA structure within the c-myc promoter. The presumable region of its formation has a sequence AGGGGCGCTTATGGGGAGGGTGGGGAGGGTGGGGAAGGTGGGG AGGAGAC. We divided this region into seven fragments, each with at least four blocks of repeated guanines and evaluated each for the ability to

form quadruplex. All the fragments formed parallel quadruplexes as shown by CD. The middle fragment, AG3TG4AAG3TG4 (designated Pu18), which was suggested in literature to form an intramolecular quadruplex, generated a four molecular quadruplex. The fragments containing more than four G blocks formed more types of quadruplexes. In accord with other studies, we found that the most probable location of quadruplex formation within the c-myc promoter was the sequence TG4AG3TG4AG3TG4AAGG, designated Pu27 (in bold above). We propose that this sequence adopts a parallel intramolecular quadruplex that is distinct from previously proposed antiparallel arrangements of the quadruplex of this sequence.

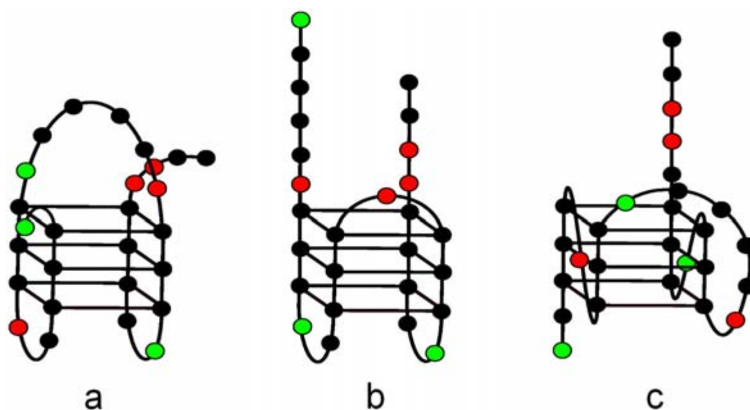

Fig. 3. Proposed models of quadruplex of the Pu27 c-myc oncogene fragment: (a) antiparallel quadruplex of a basket type proposed by Simonsson et al., (b) antiparallel quadruplex of a chair type proposed by Siddiqui-Jain et al., and (c) our proposed parallel quadruplex. The black balls indicate guanines, green indicate thymines, and red indicate adenines.

Granted projects

GA AS CR A100040701, Biophysical Properties of Biologically and Medically Important Regions of Human DNA. Principal investigator: M. Vorlíčková, 2007 - 2011

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

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PLANT DEVELOPMENTAL GENETICS

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DIPLOMA STUDENTS

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

In complex plant genomes, containing widespread repetitive sequences, it is important to establish genomic resources that enable us to focus on particular part of the genome. We have constructed a number of different genomic libraries (chromosome specific cDNA library, BAC library), which are used to study evolution of sex chromosomes in the model dioecious plant Silene latifolia (white campion). We employ chromosome microdissection as a tool to study structure and evolution of separated chromosomes and sub-chromosomal parts. This technique enabled us to reveal a role of chromosomal rearrangements in the evolution of S. latifolia sex chromosomes. Moreover, we deciphered a role of different repetitive elements in degeneration of non-recombining Y chromosome. This research

is realized in a close collaboration with Prof. Alex Widmer (ETH Zurich) and Dr. Ray Ming (U of Urbana-Champaign).

In comparison to mammalian sex chromosomes that evolved 300 mya, sex chromosomes of S. latifolia appeared approximately 20 mya. Here, we combine results from physical mapping of sex-linked genes using polymerase chain reaction on microdissected arms of the S. latifolia X chromosome, and fluorescence in situ hybridization analysis of a new cytogenetic marker, Silene tandem repeat accumulated on the Y chromosome. The data are interpreted in the light of current genetic linkage maps of the X chromosome and a physical map of the Y chromosome. Our results identify the position of the centromere relative to the mapped genes on the X chromosome. We suggest that the evolution of the S. latifolia Y chromosome has been accompanied by at least one paracentric and one pericentric inversion (Fig. 1). These results indicate that large chromosomal rearrangements have played an important role in Y chromosome evolution in S. latifolia and that chromosomal rearrangements are an integral part of sex chromosome evolution.

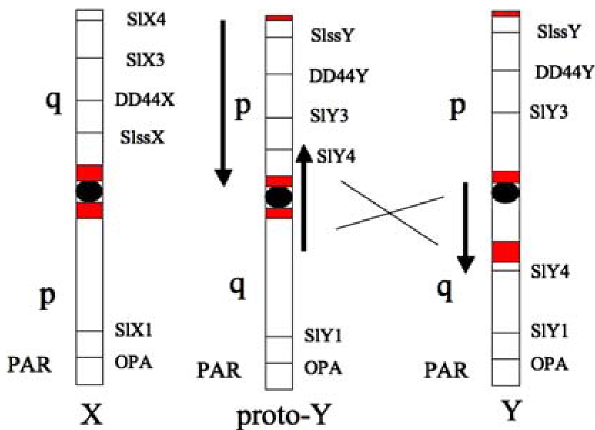

Fig. 1. A model indicating the evolutionary steps that led to the formation of sex chromosomes in S. latifolia. Arrows indicate regions that underwent chromosomal inversions. Red rectangles indicate the position of the STAR-Y repetitive DNA. The order of genes on the X chromosome corresponds most likely to the original arrangement of genes on ancestral autosomes from which the sex chromosomes evolved. The proto-Y chromosome shows the arrangement of genes and the STAR-Y repeats on the evolving Y chromosome after the Wrst paracentric inversion. The second pericentric inversion formed the current Y chromosome. The position of the STAR-Y repeats within the q arm of the Y chromosome may be a result of not only the inversion event but also the expansion of other Y chromosome centromeric repeats (not to scale).

A recent progress in plant molecular biology has led to enormous available data of DNA sequences, including complete nuclear genomes of Arabidopsis, rice, and poplar. On the other hand, in plant species with more complex genomes, containing widespread repetitive sequences, it is important to establish genomic resources that help us to focus on particular part of genomes. Laser technology enables to handle with specific subcellular structures or even individual chromosomes. Here we present a comprehensive protocol to isolate and characterize DNA sequences derived from the sex chromosomes of white campion (Silene latifolia). This dioecious plant has become the most favorite model to study the structure, function, and evolution of plant sex chromosomes due to a large and distinguishable size of both the X and Y chromosomes. The protocol includes a versatile technique to prepare metaphase chromosomes from either germinating seeds or in vitro cultured hairy roots (Figure 2). Such slides can be used for laser chromosome microdissection, fluorescence in situ-hybridization mapping, and immunostaining. Here we also demonstrate some applications of the laser-dissected chromosome template, especially a modified FAST-FISH technique to paint individual chromosomes, and construction and screening of chromosome-specific DNA libraries.

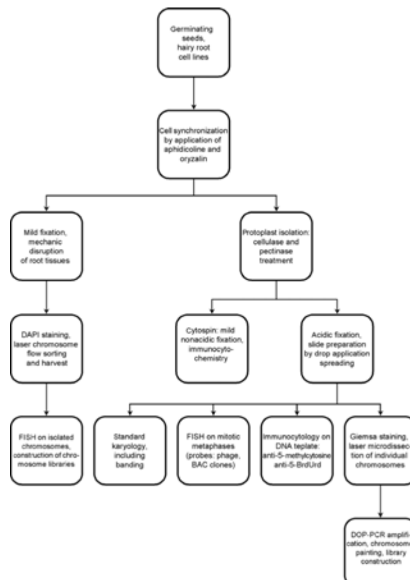

Fig. 2. Flow diagram of protoplast technologies applied to prepare mitotic chromosomes of Silene.

Patterns of gene expression are studied in order to map developmental processes leading to the suppression of male stamen organ formation in females and carpel formation in male campion plants. Genetic analysis using various kinds of molecular markers and various approaches should reveal not only positions of genes involved in development of sexual organs but they should also bring the data on mechanisms connected with the basic processes taking part in the chromosome making. All the analyses of function are interpreted in evolutionary context. This research is done in collaboration with Prof. Ioan Negrutiu (ENS de Lyon) and Dr. Gabriel Marais (CNRS Lyon).

Understanding the origin and evolution of sex chromosomes requires studying recently evolved X–Y chromosome systems such as those in some flowering plants. We describe Y chromosome deletion mutants of Silene latifolia, a dioecious plant with heteromorphic sex chromosomes. The combination of results from new and previously described deletions with histological descriptions of their stamen development defects indicates the presence of two distinct Y regions containing loci with indispensable roles in male reproduction. We determined their positions relative to the two main sex determination functions (female suppressing and the other male promoting). A region proximal to the centromere on the Y p arm containing the putative stamen promoting sex determination locus includes additional early stamen developmental factors. A medial region of the Y q arm carries late pollen fertility factors. Cytological analysis of meiotic X–Y pairing in one of the male-sterile mutants indicates that the Y carries sequences or functions specifically affecting sex chromosome pairing.

We have also established a new auxiliary phylogenetic approach based on genomic in situ hybridization technique (GISH). We used an interspecific hybrid Silene latifolia x Silene viscosa to compare two different genomes simultaneously on one slide. By using GISH with genomic DNA from another closely related species as a probe, we directly compared the level of relatedness between the genomes of the studied species and parental species. This experimental design enabled us to approximately estimate evolutionary relationships between the genome of tested plant species and genomes of both parental species of the hybrid by using the ratio of intensities of fluorescence signals. We tested this technique in various Silene species and the results were in accordance with the topology of the phylogenetic tree we constructed based on rDNA sequences. The results were also well correlated with phylogenetic distances between species that we estimated from an rDNA-based phylogenetic tree. Our experimental approach could help to improve tree topology and serve as a useful

complementary tool in molecular phylogenetic studies in related species.

The main role of telomeres is to maintain stability and integrity of the nuclear genome. If telomeres are too short or aberrant, as in, e.g., telomerase deficient plants, repair processes are induced which can lead to non-standard structural changes of chromosomes. In plants, the naked chromosome termini are subjected to chromosome fusions employing the non-homologous end joining pathway (NHEJ). Central role in repair processes plays ATM, protein kinase responsible for DNA-damage sensing, processing the telomere deprived chromosome ends and the control of the cell cycle. We have shown that ATM, besides of known functions, acts as potent inhibitor of stochastic telomere shortening by the mechanism known as telomere rapid deletion (TRD). The length of telomeres also affects the expression of nearby genes. In order to study the possible telomeric chromatin modifications which can be involved in the modulation of gene expression we are using number of chromatin mutants of the model Arabidopsis thaliana plants. This work was realized in collaboration with Dr. Karel Riha (GMI Vienna) and Prof. Dorothy Shippen (TAMU Texas).

Granted projects

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

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

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

GA CR 521/05/2076, 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 522/06/0380, The study of genome instabilities in Arabidopsis mutants deficient for DNA repair and checkpoint proteins. Principal investigator: J. Široký, 2006 - 2008

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

Publications

Hobza, R., Vyskot, B.: *Laser microdissection-based analysis of plant sex chromosomes*. Methods in Cell Biology, 82, 2007, 433-453.

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PhD thesis defended in 2007

Mgr. Petr Mokroš, PhD., Structure and stability of nuclear genome in Arabidopsis thaliana

Mgr. Zdeněk Kubát, PhD., The roles of repetitive DNA sequences in evolution of plant sex chromosomes

Prestigious International Projects

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

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

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

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

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

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

Prestigious National Projects

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

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

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

ME, LC06004, Center of Basic Research, Integrated research of the plan genome. Principal investigator: B. Vyskot, 2006-2010

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

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

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

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

In the 2007 there was added a new part of reservation system – a web based tool for booking rooms in the IBP lodging house:

(http://www.ibp.cz/local/rezervace_pok.php).

Synchrotron Facilities for the Development of Science and Technology in Central and Eastern Europe

The conference was organized by our Institute in cooperation with European Commission in Brno, hotel International, from 20 to 21 November 2007. The main goal of the conference was the elucidation of the needs for synchrotron facilities and their beamlines in the new member states of the EU and their possible role in the development of these countries. The following questions were addressed:

- are synchrotron facilities needed, how do they contribute to the development of the region (science and technology, education, industry);
- will the construction of a synchrotron facility in the eligible region (using structural funds) contribute to the policy of cohesion and convergence;
- what are the optimum strategies of the management of a synchrotron facility, how can industry be attracted;
- what beamlines and techniques will provide new possibilities for the EU users?

Recognized european scientists (about 30 experts) including directors or members of high management of well known synchrotron facilities (ESRF, ELETTRA, SOLEIL, ANKA, ALBA) presented their opinions on the above mentioned questions. The numer of participants at the conference was approximately 200. Support for the establishment of the synchrotron facility in Brno was expressed also by the Mayor of the town, Mr. Roman Onderka. In the panel discussion, organized by Rober Jan Smits, director of DG Research, the following conclusion was achieved: ***Owing to their pronounced influence on the development of science, technology, education and other aspects of the human society, synchrotron facilities are needed for Central and Eastern Europe***.

Analytical Cytometry IV

The Institute of Biophysics AS CR, Department of Cytokinetics, has been a principal organizer of an international conference „Analytical Cytometry IV“ – the official biannual meeting of the Czech Society for Analytical Cytology, which took part from June 23 till June 26, 2007 in Brno, Czech Republic. The conference was held under auspices of Prof. Václav Pačes, the President of the Academy of the Sciences of the Czech Republic. The list of more than 170 participants included a number of international experts, such as the President of the International Society for Analytical Cytology – Prof. Paul Robinson (USA), Prof. David W. Galbraith (USA), Prof. János Szöllösi (Hungary), Dr. Gero Brochoff (Germany), Prof. Jacek Witkowski (Poland) and others.

The meeting has aimed to serve to those participants from both academia and applied research, who are interested in progressive methods in analytical cytometry, including multidisciplinary approaches covering flow cytometry, flow sorting, high throughput microscopy and related optical techniques, including image analysis, application of DNA microarray techniques, data management and data evaluation. A special attention has been paid to development and testing of novel drugs and stem cell techniques.

Structure, recognition, and processing of DNA damage by antitumour metal-based compounds

The Institute of Biophysics, AS CR, Department of Biophysics and Pharmacology, has organized an international workshop "Structure, recognition, and processing of DNA damage by antitumour metal-based compounds", which took part on November 29, 2007, in the Grand Hotel, Corso di Porta Nuova, 37122 Verona, Italy.

The introductory talk given by Viktor Brabec was followed by oral presentations in which the participants presented overviews of the activities and major achievements of their laboratories in last two years. The final part of the conference was devoted to closing remarks from each laboratory which claimed unanimous view on the high standard of the research and that collaborations established among participants of this workshop played an important role in a progress in the improvement of theoretical background needed for the development and design of new metallodrugs.

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