INSTITUTE
OF
BIOORGANIC CHEMISTRY

POLISH ACADEMY OF SCIENCES

2008

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FOREWORD

The development of chemical and biological sciences together with the development of information technologies had tremendous impact on biology and medicine at the beginning of the 21st century. The statement saying that "chemistry is the language of molecular biology" involves essential implications facilitating the study of basic biological mechanisms and also a promise to use them for contemporary diagnostic and therapeutic methods, generally, for the improvement of civilization conditions.

The main objective of chemical and biological studies of the Institute of Bioorganic Chemistry PAS – nucleic acids – not only did not lose its importance in the years after sequencing of human genome and many fundamental plant and animal species, but it became more evident that the role of nucleic acids is not limited to only the stages of storing and transmitting genetic information. Nucleic acids, particularly ribonucleic acids, control and regulate all life processes to a greater degree than it has ever been assumed.

The Institute of Bioorganic Chemistry of the Polish Academy of Sciences is a research unit the role of which is to pursue basic research and advanced education at graduate and postgraduate level. The scientific aims of the Institute's researchers require interdisciplinary approach to the problems invoked by chemistry with fundamental biological problems at their molecular level.

At present, the Institute comprises 12 research laboratories and an affiliated Supercomputing and Networking Center, employing 353 people, including 29 professors, 50 Ph.D. scientists and 47 Ph.D. graduate students. The Ph.D. graduate program takes 4 years, combining the research work and a series of specialized lectures. Furthermore, the undergraduate students from Poznań schools of higher education studying biology, chemistry, biotechnology and pharmacy complete their M.Sc. degrees at the Institute's laboratories.

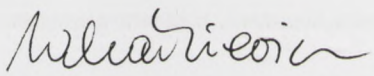
Major scientific interests of the Institute may be summarized as follows:

- chemical synthesis and stereochemistry of modified nucleosides, nucleotides and nucleic acids, including structural genes and model oligomers;
- chemistry and molecular biology of RNA;
- mechanisms of biomolecular recognition, including preparation of synthetic oligomer combinatorial libraries;
- conformation analysis of proteins, nucleic acids and their components by X-ray crystallography and high resolution NMR;
- molecular genetics of plant-microbe interaction, transformation of plant cells with foreign DNA and regeneration of transgenic plants expressing biomedical products;
- molecular biology of viruses and oncogenes;
- modelling and computation methods in molecular biology and chemistry;
- mass spectrometry of natural compounds esp. metabolomics and proteomics.

Although most scientific activities carried out in the Institute laboratories focus on basic research problems, some of them may become of practical importance. The newly organized Center of Excellence at the Institute aims at accelerating research, development and education by bringing together the most vital activities and resources from both the Institute and other academic and research institutions of widely understood "Scientific Poznań". One of the first goals of the Center is to form a platform for genomics and metabolomics devoted to medicine and biotechnology.

The Institute is a co-founder and member of the Center of Advanced Technologies and the Center of Advanced Informational Technologies.

Poland joined the European Union in 2004, and the coming years will have a profound impact on science that continues towards interdisciplinary integration and global cooperation.



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Research Support:	*Zofia Adamska, Krystyna Lembicz*
Ph.D. Students:	*Joanna Fogt, Zofia Jahnz-Wechmann*

Keywords: • *synthesis of nucleoside analogs* • *transglycosylation* • *glycosylic bond stability* • *antivirals* • *structure-activity relationship*

The research is concentrated upon the synthesis of modified nucleobases, nucleosides and nucleotides together with their structural features, chemical properties and biological activities. The main objects of interest are nucleoside analogs designed to mimic the natural substrates for: i) probing the structural and steric requirements of selected enzymes, ii) enhancing the specificity in enzyme inhibition, iii) developing new antiviral and anticancer agents. Studies in the field of synthesis and reactivity of nitrogen heterocycles also include the stability, solubility and transport characteristics important for the desired activity *in vivo* of the aforementioned analogs.

Current research activities

- search for new approaches towards synthesis of nucleobases and nucleosides (controlled degradation, rearrangements);
- selective alkylation and aralkylation of nucleosides and nucleotides;
- synthesis and characterization of nucleoside analogs that are potentially and actually biologically active, mainly ring analogs of pyrimidine and purine, acyclonucleosides, 2'-C-alkylribofuranosides, and 2',3'-dideoxynucleosides;
- attempts to define structure-activity relationships in base-modified acyclovir and ganciclovir analogs;
- synthesis and characterization of conformationally restricted nucleosides;
- study on the mechanism and applications of transglycosylation reactions in the nucleoside chemistry;
- determination of the structural factors influencing the stability of N-glycosylic bonds;

- reaction of partial acylation of the 2',3'-*cis*-diol system in the ribofuranosyl and 2'-C-alkylribofuranosyl nucleoside series;
- study on glycosylation reactions of naturally occurring poliphenolic compounds and biological activity of their sugar conjugates.

Major recent results

Tricyclic modification of the guanine moiety of acyclovir and ganciclovir

The linking of 1 and N^2 positions of the guanine moiety in two potent antivirals, acyclovir (ACV) and ganciclovir (GCV), with an etheno bridge to form the derivatives of the tricyclic 3,9-dihydro-9-oxo-5*H*-imidazo[1,2-*a*]purine system was found to modulate the biological and physical properties of these two compounds. The appended ring alone lowers the activity against herpes simplex virus type 1 and 2 (HSV-1 and HSV-2), varicella-zoster virus (VZV) and cytomegalovirus (CMV) by a factor of 10^2 or more. Further substitutions in that ring enhance the activity and make compounds more selective toward particular viruses. The substitution with phenyl group results in fluorescent analogs which, having biological activity similar to that of the parent compounds, seem to be promising candidates for noninvasive diagnosis of herpesvirus infections.

Reinvestigated mechanism of ribosylation reactions in the purine series

Acid-catalyzed synthesis of the N-glycosylic bond, one of the most important reaction in the nucleoside chemistry, proceeds in two steps: i) the formation of a kinetic glycosylation product, and ii) its rearrangement, called "transglycosylation", to a thermodynamically more stable nucleoside. It has been believed for years that glycosylation of all purine bases takes place in the sequence 3 → 9, as it has been demonstrated for adenine. However, as shown in our laboratory, 6-oxopurines (e.g. guanine, hypoxanthine) undergo glycosylation in a different way, and the site of initial substitution (N7 or N9) depends on the structure of substrates. More recently, we have shown that even in the case of adenine the site of initial ribosylation may vary depending on the reaction conditions and substitution of the substrate. Therefore, under strongly acidic conditions the kinetic product has got a structure of 3-ribosyladenine (isoadenosine), while the use of silyl method leads directly to 1- or 7-ribosylated adenine derivatives.

Application of 6-oxopurine nucleosides to synthesis of novel nucleosides via exchange methods

The reaction of 7-9 transglycosylation has found many applications in the nucleoside chemistry, especially for synthesis of acyclonucleosides (*e.g.* acyclovir, ganciclovir). Thus, a fully reversible 7-9 isomerization reaction of 6-oxopurine nucleosides (*e.g.* tetra-O-acetylguanosine) may serve either as a source of purine in the reaction with peracylated sugars and their analogs (transpurination approach), or as a source of sugar cations for ribosylation of other heterocyclic bases (transglycosylation method). A variety of aspects related to the mechanism of transglycosylation, *i.e.* regio- and stereochemistry, reversibility, effects of structural modifications and the role of catalysts are currently being studied in our laboratory.

Synthesis of novel acyclovir analogs via transpurination of guanosine

We have shown that a direct acid-catalyzed reaction of guanosine and 1,3-dioxolane leads to the formation of 9-(2-hydroxyethoxymethyl)guanine, *i.e.* acyclovir. Recently, the transpurination approach has been applied to the synthesis of novel analogs of acyclovir, substituted with an alkyl or aryl group at the positions 1', 4' and/or 5'. A series of new acyclonucleosides has been obtained in a direct one-pot coupling reaction with substituted derivatives of dioxolane. Because the 1,3-dioxolane system can be easily synthesized from a variety of 1,2-diols and aldehydes, the new approach offers a simple and inexpensive route towards guanine acyclonucleosides.

New derivatives of 2'-C-alkylnucleosides

2'-C-β-Methylribonucleosides have attracted much attention as potent inhibitors of viral RNA replication, including *Hepatitis* C virus RNA. A series of new and promising compounds of this type has been obtained in our laboratory. The new pyrimidine and purine 2'-C-alkyl-nucleosides are modified in either aglycon portion and/or in the sugar part. Their biological activity evaluation is currently in progress.

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LABORATORY OF STRUCTURAL CHEMISTRY OF NUCLEIC ACIDS

Head: ***Ryszard W. Adamiak***

Professors:	*Ryszard W. Adamiak*
Research Staff:	*Łukasz Bielecki, Mikołaj Olejniczak, Katarzyna Pachulska-Wieczorek, Mariusz Popenda*
Research Support:	*Grażyna Dominiak*
Ph.D. Students:	*Katarzyna J. Purzycka*

Keywords: • *retroviral RNA* • *regulatory RNA of pathogenic bacteria* • *RNA therapeutics* • *RNA chemistry* • *RNA structure and dynamics* • *RNA molecular biology* • *RNA hydration* • *RNA-metal, small ligand and protein interactions* • *^{1}H, ^{13}C, ^{19}F NMR spectroscopy* • *simulation of molecular dynamics* • *RNA structure prediction*

The Laboratory's current aim is to understand the structure, function and interactions of functionally important domains of RNA on atomic and molecular levels. The idea is to combine, within the group, the synthetic and structural lines of research in the RNA field. Particular areas of interest include retroviral RNAs and regulatory RNA of pathogenic bacteria and their interactions with metal ions, small inhibitory molecules and proteins, structure of RNA bulges, RNA hydration and mechanism of RNA helicity reversal. The RNA synthesis and chemistry are dedicated to structural studies and advancement of RNA therapeutics. The major methodologies are heteronuclear (^{1}H, ^{13}C, ^{31}P, ^{19}F) NMR, thermodynamics and optical spectroscopies, including time resolved spectrofluorimetry and FT-IR. Thanks to the access to in-house supercomputing and networking facilities (Poznań Supercomputing and Networking Center), simulation of molecular dynamics and RNA structure prediction are intensively conducted in our Laboratory.

Two groups exist within the Laboratory: Biomolecular NMR Group (Zofia Gdaniec) and Biomolecular Modelling Group (Tadeusz Kuliński). Also, after returning from his postdoctoral assistantship, Mikołaj Olejniczak started a new line of research on small bacterial RNAs.

Particular areas of interest include: (i) structure of retroviral RNAs (HIV-1, HIV-2), their interactions with metal ions and recombinant proteins (Tat-2, cyclin T1) and their accessibility in view of RNAi applications, (ii) structure and function of non-coding RNAs in pathogenic bacteria, (iii) NMR structure of non-helical RNA regions, 2'-O-MeRNA/LNA chimera duplexes, RNA triplet repeats, Z-RNA and (iv) RNA dynamics and

developing new MD approaches in a view of RNA structure and specificity of protein recognition and methods directed toward 3D RNA structure prediction.

Current research activities

- design and application of regioselectively modified oligoribonucleotides for ^{1}H, ^{13}C and ^{19}F NMR studies of RNA structure, dynamics and interactions with metal cations, small ligands and recombinant proteins;
- introduction of modified nucleosides, including photochemical probes, into oligoribonucleotides using chemistry and *in vitro* synthesis;
- RNA folding and its accessibility in the view of RNA interference-based therapeutics;
- studies on Z-RNA structure (NMR) and mechanism of "A→Z" transition;
- interactions at the RNA-protein interface (e.g. HIV-2 lider RNA, *Tat-2* and *NC-2* proteins);
- structure of non-coding regulatory RNAs of bacterial origin and their interactions with *Hfq* protein and the ribosome;
- developing new computational approaches for modeling of RNA structure and interactions (molecular dynamics thermocycler);
- developing tools and databases for the three-dimensional RNA structure prediction.

Major recent results

Z-RNA structure and mechanism of "A→ Z" RNA transition

Right-handed double helical DNA and RNA have in common one of the most intriguing structural features – the inversion of the helical screw sense, the phenomenon often called helicity reversal or helical transition. Following the discovery of the left-handed double helical Z-DNA form of $d(CGCGCG)_2$ in the crystal state (Rich *et al.*, 1978), a similar tendency of RNA double helices, i.e. poly[(CG)] (Tinoco *et al.*, 1984) and short duplexes like $(CGCGCG)_2$ (Adamiak *et al.*, 1985), to form left-handed Z-RNA was reported. The propensity of right-handed double strands to undergo the *A to Z* transition is most characteristic of RNA containing alternating CG base pairs. The left-handed RNA prevails at much higher salt concentration, e.g. 6 M $NaClO_4$ than that noted for the Z-form of $d(CG)_n$ duplexes (2.6 M NaCl).

In contrast to the thoroughly studied Z-DNA (due to excellent crystallisation properties of some Z-helix forming DNA duplexes; currently we have access to ca. 60 X-ray structures of Z-DNA), no Z-RNA structure of natural sequence has been known till very recently. This year, we reported the NMR structure of a half-turn left-handed RNA helix $(CGCGCG)_2$ determined in 6 M $NaClO_4$. This is the first nucleic acid motif determined at such high salt. Sequential assignments of non-exchangeable proton resonances of the Z-form were based on the hitherto unreported NOE connectivity path [$H6_{(n)}$-H5′/H5″$_{(n)}$-$H8_{(n+1)}$-H1′$_{(n+1)}$-$H6_{(n+2)}$] found for left-handed helices. Z-RNA structure shows several conformational features significantly different from Z-DNA. The intra-strand but not inter-strand base stacking was observed for both CpG and GpC steps. Helical twist angles for CpG steps have small positive (4-7°), whereas GpC steps have large negative (-61°) val-

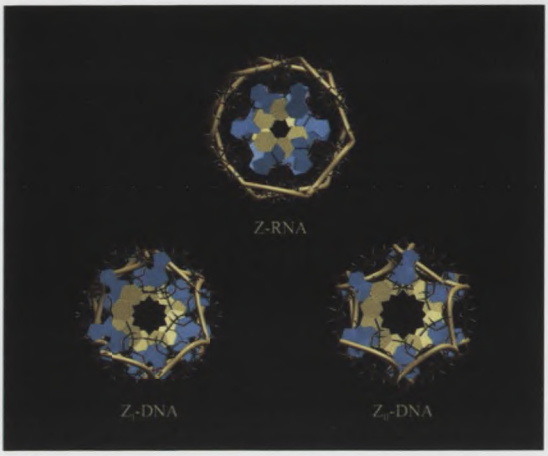

Figure 1. Full turn model of the Z-RNA helix compared to the models of Z_I- and Z_{II}-DNA. The Z-RNA model is significantly different from the Z_I- and especially Z_{II}-DNA. One of the spectacular differences is seen when viewing left-handed helices along their z-axes. In Z-DNA helices, CG base pairs are set away from the z-axis with their imidazole parts of guanine rings protruding onto the outer helix surface. Base pairs in the Z-RNA helix, due to their smaller x- and y-displacement parameters, are located much closer to the helix z-axis (Popenda et al., 2004).

ues. In the full turn model of Z-RNA (12.4 bp per turn), the base pairs are much closer to the helix axis than in Z-DNA, thus both the very deep, narrow minor groove with buried cytidine 2′-OH groups and the major groove are well-defined. The 2′-OH group of cytidines plays a crucial role in the Z-RNA structure and its formation; 2′-O-methylation of cytidine, but not of guanosine residues prohibits *A to Z* helicity reversal.

HIV-1 TAR RNA structure and interactions using ^{19}F NMR, metal ion-induced cleavages and Brownian dynamics

Currently, our interest focuses on the ^{19}F NMR study of interactions of these RNAs with metal ions and small ligands. The fluorine chemical shifts are extremely s ensitive to

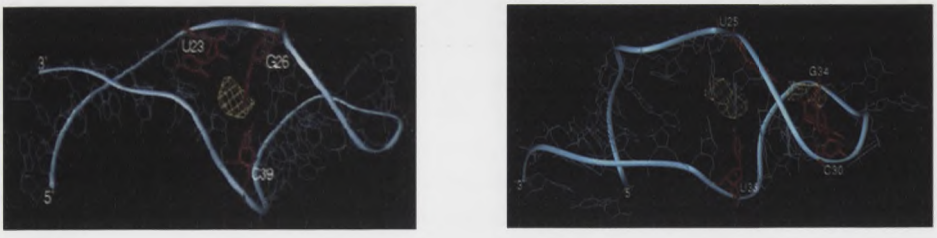

Figure 2. Visualization of the Brownian dynamics simulation showing magnesium cation (yellow sphere) interaction within the bulge site (left panel, Olejniczak et al., 2002) and apical loop of the HIV-1 TAR RNA (right panel, unpublished).

the nucleus environment, thus making fluorine an ideal NMR spin label in the study of nucleic acids interactions with proteins. In order to probe the interaction of TAR RNA with metal ions, apart from ^{19}F NMR, we applied several other approaches: specific metal ion cleavage, competitive inhibition experiments and Brownian dynamics simulations of cation diffusion. In case of HIV-1 TAR RNA, the bulge region of the TAR RNA is a metal ion-binding site. The combination of ^{19}F NMR and biochemical approaches with computational modeling offers a promising way to study RNA – metal ion in solution.

HIV-2 RNA leader domains

The genome of retroviruses, including the human immunodeficiency virus type 2 (HIV-2), consists of two identical RNA strands that are packaged as non-covalently linked dimers.

Currently, we are investigating magnesium dependent conformational equilibriums of TAR, TAR/-polyA DIS regions and their involvement in the HIV-2 leader RNA protein interaction and dimerisation *in vitro*.

The HIV-2 TAR RNA domain (TAR-2) plays a key role in the *trans*-activation of HIV-2 transcription as it is the target for the Tat-2 protein and several cell factors. We have shown that the TAR-2 domain exists *in vitro* in two global, alternative forms: a new, extended hairpin form with two conformers and the already proposed branched hairpins form. This points strongly to the structural polymorphism of the 5′ end of the HIV-2 leader RNA. The evidence comes from the non-denaturing PAGE mobility assay, 2D structure prediction, enzymatic and Pb^{2+}- or Mg^{2+}-induced RNA cleavages. Existence of the TAR-2 extended form was further proved by the examination of engineered TAR-2 mutants stabilized either in the branched or extended structure. The TAR-2 extended form predominates at the increased magnesium ion concentration.

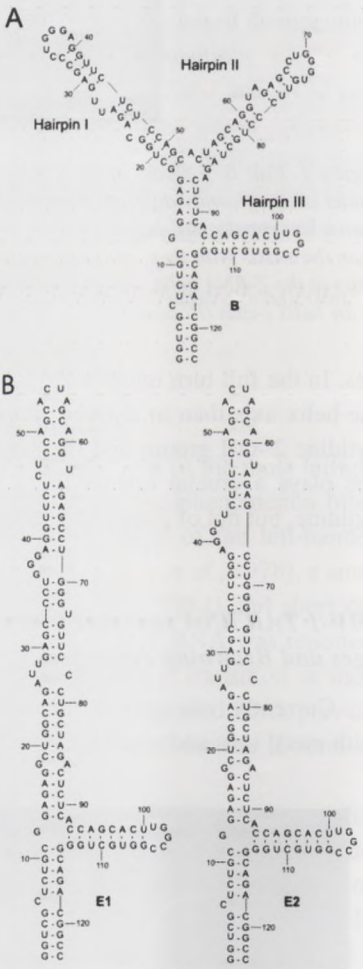

Figure 3.

Gel retardation assays reveal that both TAR-2 wt and its mutant, unable to form branched structure, bind Tat-2 protein with comparable, high affinity, while RNA hairpins I and II, derived from TAR-2 branched structure model, show much lesser protein binding. We propose that the internal loop region of the TAR-2 extended hairpin form is a potential Tat-2 binding site.

The dimerization signal called dimerization initiation site (DIS hairpin) is located in the downstream part of the untranslated leader of HIV-2 RNA. The HIV-1 and HIV-2

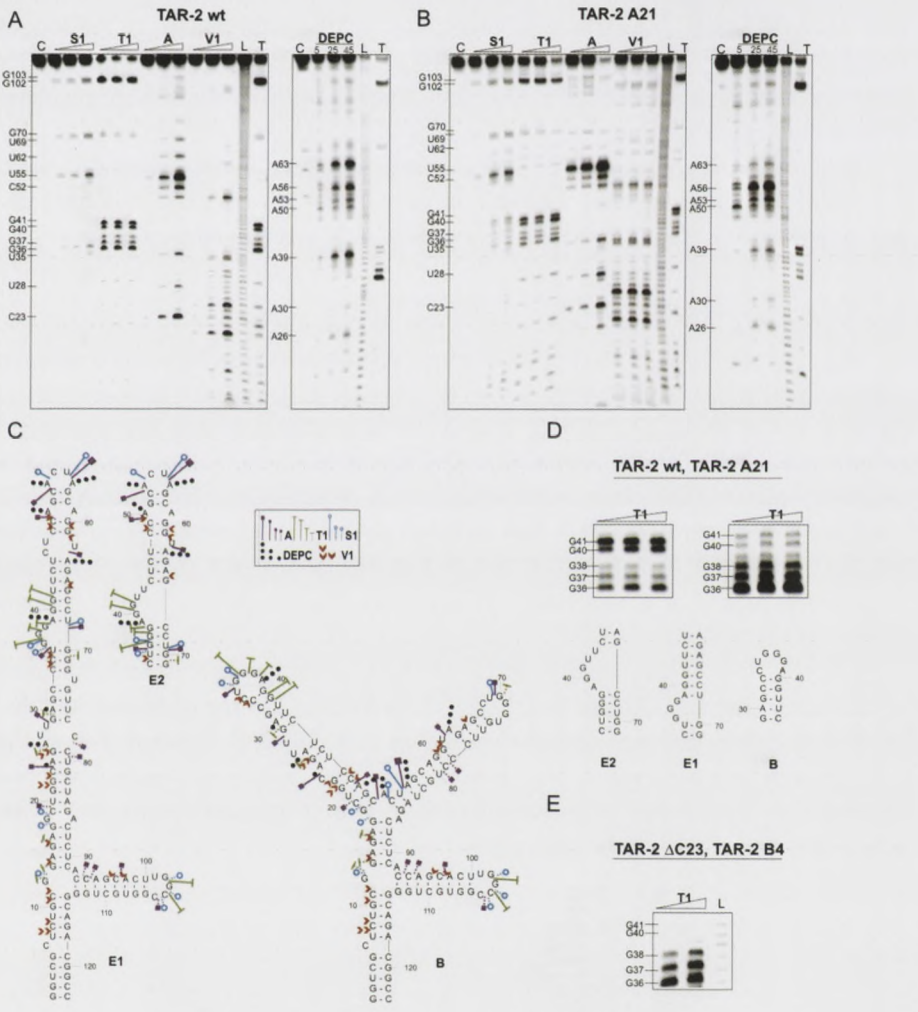

Figure 4. Secondary structure probing of HIV-2 TAR RNA. The RNA was treated with selected single-strand specific enzymes (S1, T1, A), diethypyrocarbonate (DEPC) and double-strand specific RNase V1. (A) Cleavage patterns obtained for the 5' end labelled TAR-2 wt transcript. Lanes C represent control sample with untreated RNA; lanes L, formamide ladder; lanes T, limited hydrolysis with RNase T1. (B) Cleavage patterns obtained for the 5' end labelled TAR-2 A21 mutant transcript. (C) A summary of the enzymatic cleavages and chemical modifications data for the TAR-2 wt RNA viewed on the secondary structure models (E1, E2 and B). For clarity, only the top part of the E2 conformer that differs from E1 is shown. Sites and intensities of cleavages with the respective reagents are indicated by symbols (see insert); the size of a symbol corresponds to the relative cleavage intensity. The weakest cleavages are not indicated. (D) Two different RNase T1 cleavage patterns observed for the particular G-rich region of both TAR-2 wt and A21 mutant along with respective secondary structure motifs. Both patterns point to the mixture of TAR-2 forms; the left pattern is consistent mostly with one of the extended (E1) and with the branched (B) structure models; the most often observed right pattern represents predominantly the second extended conformer E2. (E) RNase T1 cleavage pattern obtained for the TAR-2 B4 and ΔC23 mutants stabilized in the branched (B) form. (Pachulska-Wieczorek et al., 2006).

leader can adopt two alternative conformations: the branched multiple-hairpins (BMH) and, the most stable, long distance interaction (LDI) conformation. In the long-distance interaction structure, the DIS element is masked. In the BMH structure, the DIS hairpin is open for the "kissing-loop" dimerization. The LDI-BMH riboswitch regulates RNA dimerization *in vitro*.

Low resolution structure of DIS-2 is under study using diverse chemical, photochemical, enzymatic and calculation methods, in view of the RNA accessibility towards siRNA and the specificity of recombinant *NC-2* (nucleocapsid protein) interaction.

Regulatory RNAs of pathogenic bacteria

Many of the small, *trans*-encoded RNAs (sRNAs) in bacteria control translation and stability of mRNAs involved in the bacterial cell's response to environmental changes. They affect virulence of pathogenic strains, antibiotic resistance and host-pathogen interactions. Unlike eukaryotic microRNAs, bacterial sRNAs exert both positive and negative regulation of translation. The mRNA translation regulation by small RNAs involves incomplete pairing between sRNA and its target mRNA. The *Sm*-like *Hfq* protein facilitates this interaction and is required for the activity of most of sRNAs. This homohexameric, ring-shaped protein is a pleiotropic post-transcriptional regulator, which is abundant in many bacterial species.

The objective of the emerging group of M. Olejniczak is to understand the mechanism of interactions among sRNAs, their target mRNAs, *Hfq* and the ribosome, and the role of these interactions in the translation regulation of pathogenic bacteria. For this purpose, high-throughput assays using filter binding and fluorescence are being developed. Also, oligonucleotide inhibitors of crucial interactions will be designed, which may offer opportunities for new anti-bacterial strategies.

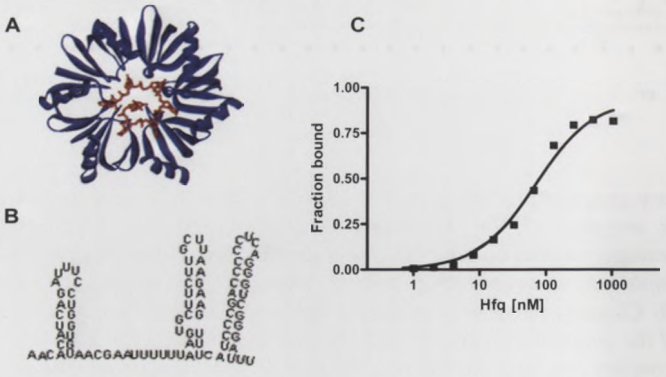

Figure 5. The interaction of E. coli Hfq protein with small RNA DsrA. A) crystal structure of S. aureus Hfq bound to 5'-AUUUUG; B) secondary structure of E.coli DsrA; C) equilibrium binding of E. coli Hfq to DsrA RNA.

The three-dimensional RNA structure prediction

The recently published RNA FRABASE is a web-accessible engine with a relational database, which allows for the automatic search of user-defined, three-dimensional RNA

fragments within a set of RNA structures. This is a new tool to search and analyse RNA structures, directed at the 3D structure modelling. The user needs to input either RNA sequence(s) and / or secondary structure(s) given in a 'dot-bracket' notation. The algorithm searching for the requested fragment(s) against all the 'dot-bracket' encoded RNA structures is very efficient. The database contains: (i) RNA sequences and secondary structures in the 'dot-bracket' notation, derived from over a thousand PDB-deposited RNA structures and their complexes, (ii) a collection of atom coordinates of unmodified and modified nucleotide residues occurring in RNA structures, (iii) calculated RNA torsion angles and sugar pucker parameters, and (iv) information about base pairs. Advanced query involves filters sensitive to: modified residue contents, experimental method used, and limits of conformational parameters. The output list of query-matching RNA fragments gives access to their coordinates in the PDB-format files, ready for direct download and visualization, conformational parameters and information about base pairs. The RNA FRABASE is automatically, monthly updated and is freely accessible at http://rnafrabase.ibch.poznan.pl (mirror at http://cerber.cs.put.poznan.pl/rnadb).

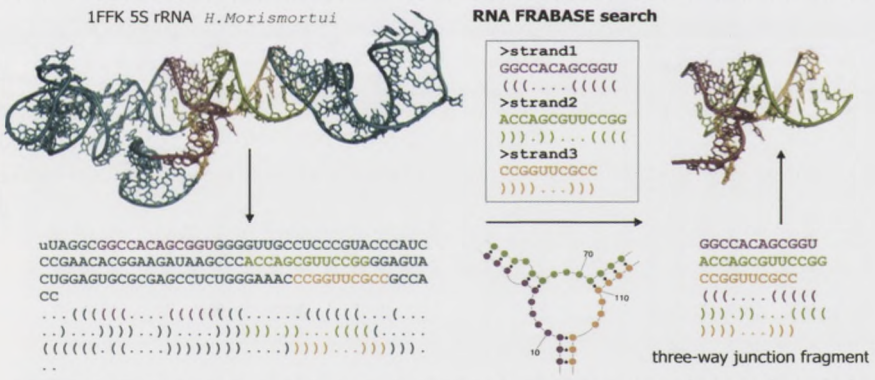

Figure 6. The encoding and searching concept of the RNA FRABASE presented for the H. Marismortui 5S rRNA structure /PDB code 1FFK/ (Popenda et al. 2008).

The RNA FRABASE is the central point in our 3D RNA structure prediction protocol using the structural templates approach.

RNA Molecular Dynamics

As a supporting method for our structural studies of regulatory RNA sequences, molecular dynamics simulations are performed using our own cumputational resources, as well as the supercomputers located in the Poznan Supercomputer and Networking Center affiliated to our Institute. The MD simulation method has been developed in our group for about a decade and, at the beginning it was used to study short DNA and RNA duplexes containing chemical modifications. Gradually, we have moved towards conformational research of longer RNAs involved into viral regulatory functions. Presently, the HIV-2 TAR and DIS sequences are the main object of our study. As an extension of our scope, we are developing novel simulation methods aim to get better access to the conformational space ("molecular dynamics thermocycler"), as well as evaluation methods for the analysis of our results.

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Keywords: • *NMR spectroscopy* • *RNA structure and dynamics* • *metal ion binding* • *RNA bulged duplexes* • *RNA structure calculation* • *virtual NMR laboratory*

In our group, high-resolution NMR spectroscopy is used to probe the structure and dynamics of ribonucleic acids in solution. Of our major interest are the structural studies of RNA fragments in order to understand the relationship between the structures of these molecules and their biological functions.

Current research activities

- conformational studies of bulged RNA duplexes;
- structural characterization of *LNA*-2-'O-MeRNA/RNA and 2-'O-MeRNA/RNA duplexes;
- conformational equilibria of RNA fragments containing CGG trinucleotide repeats;
- molecular modelling studies of RNA;
- application of NMR spectroscopy to study RNA-metal ions interactions;
- Virtual NMR Laboratory. A pilot project in collaboration with the Poznań Supercomputing and Networking Center.

Major recent results

Bulged RNA duplexes

Bulges are one of the frequently occurring secondary structural elements in nucleic acids. The bases that lack a pairing partner in the opposite strand of the double helix have been implicated to be functionally important for several RNA molecules. Distinct sequence features can influence conformation of the bulge: the type of the bulge, identity of the neighboring residues, sequence not adjacent to the bulge and length of the helix.

In order to gain insight into structural changes induced by the presence of adenosine bulge on RNA duplex, the solution structures of RNA duplex containing a single adenine

bulge and a reference duplex with all Watson-Crick base pairs have been determined by NMR spectroscopy. The reference duplex structure is a regular right-handed helix with all the attributes of an A-type helix. In the bulged duplex, single adenine bulge stacks into the helix and the bulge region forms a well-defined structure. The accommodation of the bulged adenine residue between the neighbouring base-pairs does not significantly disturb the regular A-RNA structure. Intrahelical position of bulged adenine A5 is stabilized by efficient stacking with 5' -neighboring residues G4.

a) 5'- $G_1C_2A_3G_4A_5G_6G_7A_8G_9C_{10}G_{11}$-3'
3'- $C_{21}G_{20}U_{19}C_{18}U_{17}C_{16}U_{15}C_{14}G_{13}C_{12}$-5'

b) 5'- $G_1C_2A_3G_4$ A_5 $A_6G_7A_8G_9C_{10}G_{11}$-3'
3'- $C_{21}G_{20}U_{19}C_{18}$ $U_{17}C_{16}U_{15}C_{14}G_{13}C_{12}$-5'

Figure 7. Superposition of ten lowest energy structures of reference (a) and bulged duplex (b) Bulge region: superposition of ten final structures. The view is into the major groove (c).

5'- $G_1U_2C_3G_4$ A_5 $G_6C_7U_8G_9$-3'
3'- $C_{17}A_{16}G_{15}C_{14}$ $C_{13}G_{12}A_{11}C_{10}$-5'

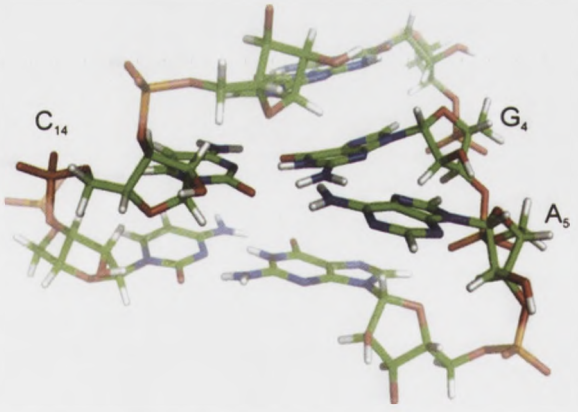


Figure 8. Portion of the single adenine bulged duplex structure showing the C14-G4-A5 base-triple platform and the adjacent C3-G15 and G6-C13 base-pairs.

Recently, we have determined an NMR structure of the duplex with bulged adenine residue flanked by two GC base pairs. NMR and molecular modelling data provide evidence for the presence of base-triple platform involving bulged adenine residue. A common feature of dinucleotide platforms is that they appear to mediate long-range RNA-RNA contacts. Although A-U-A platforms do not form stable structures in the absence of tertiary contacts, C14-G5-A5 platform forms a stable structural motif stabilized by stacking interactions and several noncanonical inter-base hydrogen bonds.

Structure of LNA-2-'O-MeRNA/RNA duplex

Oligonucleotides favoring N-type sugar conformation display an increased helical thermodynamic stability when hybridized with complementary DNA or RNA. LNA (*Locked Nucleic Acid*), a nucleoside analogue containing methylene bridge between C2' and C4' atoms of ribose ring, locks the ribose ring in C3'-*endo* conformation. When LNA is hybridized with RNA, it generates the most stable hybrids ever characterized.

2'-O-Alkyl modified ribose rings are also known to adopt preferentially C3'-*endo* sugar pucker. Although the structures of LNA-modified oligonucleotides have been recently obtained, there are only few high resolution structures of sugar modified oligonucleotides including 2'-O-methylated analogue. We have determined the solution structures of *LNA*-2'-O-MeRNA/RNA hybrid along with 2'-O-MeRNA/RNA reference hybrid.

5'- ^{M}A ^{M}C ^{M}G ^{M}U ^{M}G ^{M}C ^{M}A-3'	5'- ^{M}A ^{M}C ^{M}G ^{L}U ^{M}G ^{M}C ^{M}A-3'
3'- U G C A G G U-5'	3'- U G C A G G U-5'

Both duplexes retain features common for a right handed A-type helix conformation. LNA induces mostly local minor structural perturbations. The local geometric changes around the LNA residue result in a global structural effect. Analysis of helical parameters shows that the position of base-pairs relative to helix axis and their inclinations are significantly smaller in *LNA*-2'-O-MeRNA/RNA duplex.

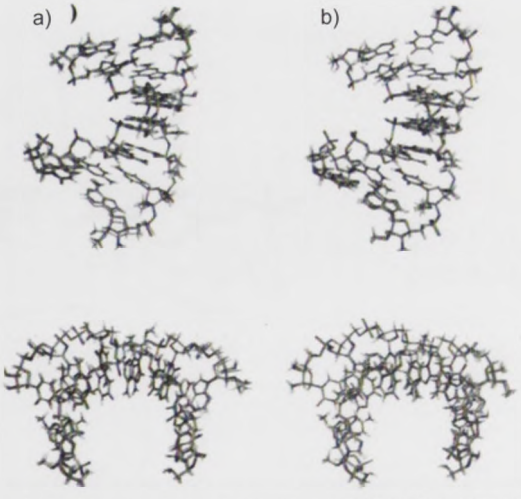
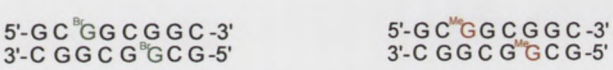


Figure 9. Superposition of ten lowest energy structures of 2'-O-MeRNA/RNA (a) and LNA-2'-O-MeRNA/RNA duplex (b).

Conformational equilibria of RNA fragments containing CGG trinucleotide repeats

NMR spectra of RNA duplexes composed of CGG trinucleotide repeats reveal a presence of multiple conformational equilibria associated with the presence of GG mismatches. There are four possible structures of GG mismatches that involve two hydrogen bonds. Three of them require one of the bases to be inverted either by switching the base from *anti* to *syn* or by reversing the direction of the strand. The presence of a bulky substituent, such as bromine atom or methyl group at the C8 position of guanosine, influences the rotation about the glycosidic bond, disfavoring the normal *anti* orientation of the base and promote the formation of ^{Br,Me}G(*syn*)-G(*anti*) base pair.

In order to understand the nature of the equilibria that are present in RNA fragments containing CGG trinucleotide repeats, we have studied oligoribonucleotides containing 8-bromoguanosine and 8-methylguanosine in place of guanosine:



Both molecules adopt alternate conformations in a concentration- and ionic strength-dependent manner. NMR spectroscopy and native polyacrylamide gel electrophoresis have been used to provide evidence that these molecules are involved in multiple equilibria between monomer, dimer and tetramer molecularities. At pH 6.8 and low ionic strength, molecules are in equilibrium between single strand and duplex. At higher salt concentration, double and four-stranded structures exist in solution. Higher strand concentration drives equilibrium toward quadruplex form.

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Research Support: *Bartłomiej Gramowski*

Keywords: • *RNA structure and dynamics* • *structure – function relationships* • *molecular recognition* • *RNA – RNA and RNA – protein interactions* • *oligonucleotide analogues* • *rational drug design* • *3D modeling of RNA and RNA – protein complexes* • *Molecular Dynamics simulations* • *MD forcefield development* • *free energy calculations* • *replica exchange MD*

Our research group concentrates on the modeling, simulation and visualization of RNA three-dimensional structures in order to understand their dynamics and biological functioning. To determine the rules that govern the processes of the RNA folding and molecular recognition, we use different computational approaches for identification of intra- and intermolecular interactions, non-standard interactions, water-mediated hydrogen bonding and their participation in the stabilization of RNA structural domains and RNA complexes under different ionic conditions. We are working on the optimization of different numerical methods for the prediction of binding affinities and stability changes of RNA, such as free energy perturbation or methods combining molecular mechanics and continuum models. We use them for the prediction of the properties of modified nucleosides in structure of RNA and new nucleoside and oligonucleotide analogues for the design of effective drugs.

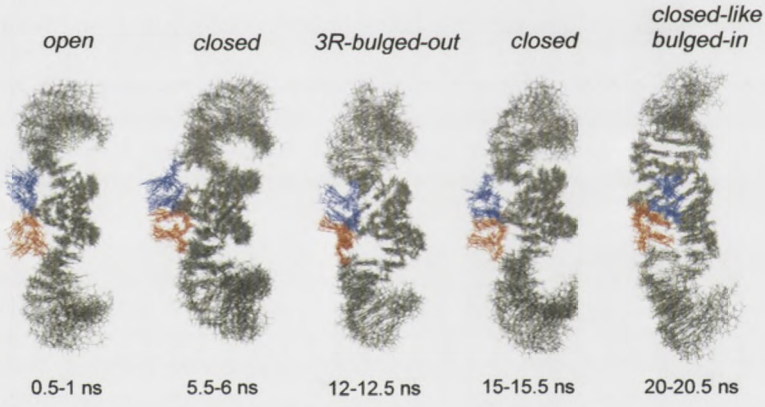
Current research activities and major results

Conformational transitions of flanking purines in HIV-1 RNA DIS kissing complexes

Dimerization of HIV-1 genomic RNA is initiated by kissing loop interactions at the Dimerization Initiation Site (DIS). Dynamics of purines that flank the 5' ends of the loop-loop helix in HIV-1 DIS kissing complex were explored using explicit solvent molecular dynamics (MD) simulations. Multiple MD simulations (200 ns in total) of X-ray structures for HIV-1 DIS subtype A, B and F revealed conformational variability of flanking purines. In particular, the flanking purines, which in the starting X-ray structures are bulged-out and stack in pairs, formed a consecutive stack of four bulged-out adenines at the beginning of several simulations (*closed* conformation). This conformation is observed in the crystal

structure of DIS subtype F with no interference from crystal packing. However, as the simulations progressed, the four continuously stacked adenines showed conformational transitions from the bulged-out into the bulged-in geometries, resembling the structure suggested by a recent NMR investigation.

Our simulations of HIV-1 RNA DIS kissing-loop complex were performed with the CHARMM force field and were compared with the simulations carried out with the AMBER force field by the J. Sponer group. Both CHARMM and AMBER simulations detected similar main substrates of flanking bases, including the *closed* conformations. Whereas in CHARMM simulations the *closed* conformation was always unstable and converted into the bulged-in geometries within 2-16 ns., the AMBER force field showed modest but persistent preference for the bulged-out *closed* geometry or its variants.



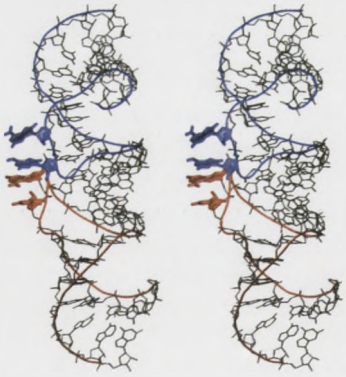


Figure 10. Conformational transition from the bulged-out open to the bulged-in closed-like conformation through the bulged-out closed conformation in HIV-1 DIS kissing complexes, observed in MD simulations. The flanking bases in both strands are shown in colors.

Validation of MD derived structural models* of *the apical loop of TAR by comparing the calculated and experimentally observed NMR data

Our recent molecular modeling and MD simulation studies revealed the coexistence of alternative conformations: one stabilized by the cross-loop base pair between C30 and G34 and the other stabilized by the interactions characteristic for U-turn motif.

To assess the reliability of MD-derived structural models of the dynamic multifunctional RNA hairpin, a comparison between back calculated from MD trajectories and ex-

perimentally observed proton chemical shifts has been carried out. Our results confirmed the coexistence of alternative conformations of the apical loop of HIV-1 TAR RNA hairpin in the solution. The comparison between the calculated and observed proton chemical shifts proved to be a good tool for validation and refinement of MD derived structures of dynamically inter-converting RNA conformational substrates.

Structural analysis of the 3D model of cyclin T1 and its complex with HIV-1 TAR RNA

The gene expression of the human immunodeficiency virus type 1 (HIV-1) during its life cycle is regulated by positive transcription elongation factor b (P-TEFb). It contains cellular cofactor cyclin T1 (CycT1), which forms a ternary complex with viral transcriptional transactivator (Tat) protein and transactivation response (TAR) RNA element thereby activating cyclin dependent kinase 9 (Cdk9), which stimulates transcription at the level of chain elongation. The lack of detailed structural information on cyclin T1 hinders an understanding of the molecular determinants underlying its interaction with target macromolecules, especially HIV-1 Tat – TAR nucleoprotein complexes. Apparently, CycT1 belongs to the group of proteins which are partially unstructured under physiological conditions and assume a defined three-dimensional structure upon binding to another protein, nucleic acid or macromolecular complex. Such examples are numerous among proteins involved in regulatory functions in eukaryotic cells. Many studies provided evidence that the TRM in CycT1 is disordered in the *apo* form and adopts a structure upon binding to Tat and TAR. Therefore, the analysis of structural basis for the cyclin T1 interaction with HIV-1 Tat and TAR require extensive structural analysis of this ternary complex. We have built several homology models of human CycT1 (1-272) based on the available structures of related cyclins, and studied them by extensive molecular dynamics (MD). Our MD simulations suggest that the proposed 3D models of the CycT1 have structurally stable modules in its cyclin box core. We have identified the dynamic modes of structural adaptation within the protein, which may have implications for the mechanism of RNA binding and the formation of the full functional complex. We have also analyzed possible RNA binding interface within the protein and generated models of the CycT1 – TAR RNA complexes, using structural motifs previously proposed by us for HIV-1 TAR RNA.

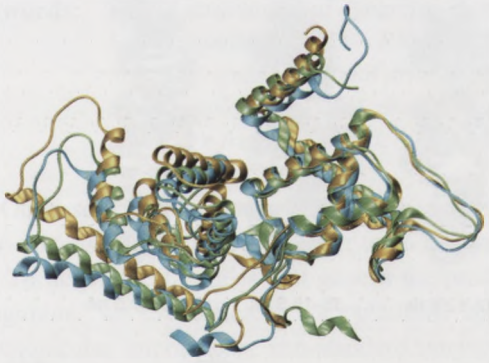


Figure 11.

Modeling of ErmC' methyltransferase - RNA interactions

ErmC' is an enzyme that confers bacterial resistance to the macrolide-lincosamide-streptogramin B group of antibiotics by catalyzing the N6-methylation of a specific adenine residue (A-2058 in *Escherichia coli*) in the peptidyl transferase loop of bacterial 23S rRNA. The smallest, 27 nt model RNA oligonucleotide that shows methyl-accepting activity should adopt a stem-loop structure, where the substrate adenosine is an unpaired base

located within the loop. The model for ErmC' interaction with the minimal RNA substrate has been previously proposed by J. Bujnicki's group, based on available structural and mutagenesis data. To elucidate the details of ErmC' – RNA interaction, we have undertaken molecular dynamics (MD) simulations studies of the model of the complex. Our MD simulations of the RNA substrate and ErmC' – RNA complex models have suggested that the existing 3D model of RNA is structurally unstable and requires improvement. Thus, we have generated new models of RNA substrate, using structural motifs identified with the aid of the RNA FRABASE server. Our new models were subjected to MD simulations in the explicit solvent. We have also performed MD simulations of the full-length ErmC' enzyme (i.e. extended to contain eight N-terminal residues that are intrinsically disordered, but may be involved in RNA binding). From the results of MD simulations, we have identified the dynamically cross-correlated residues within the enzyme, which have implications for the mechanism of RNA binding and catalysis of the methyltransfer reaction.

Figure 12.

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LABORATORY OF RNA CHEMISTRY

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Research Staff: *Ewa Biała, Elżbieta Kierzek*

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Keywords: • *modified nucleotides* • *locked nucleic acids and modified locked nucleic acids* • *oligonucleotides synthesis* • *thermodynamics of RNA* • *thermodynamics of modified oligonucleotides* • *antisense oligonucleotides* • *isoenergetic RNA microarrays* • *analysis of RNA structure and interactions*

The research in the group has focused on many problems of chemical synthesis, thermodynamic properties, as well as on structure and function of oligoribonucleotides and RNA. Apart from oligoribonucleotides and RNA containing natural nucleotides, we are interested in the studies of oligomers containing modified nucleotides. "RNA chemistry" in the group is not only a tool for the chemical synthesis of model oligoribonucleotides, but also an approach for understanding various behaviors and functions of RNA. Recently, we have been intensively working on chemical synthesis of locked nucleic acids (LNA), particularly, synthesis of LNA nucleotides containing natural and modified nucleobases such as 4-thiouracil and 2,6-diaminopurine. The major application of modified oligoribonucleotides are isoenergetic RNA microarrays which we are using to study the structure and interactions of RNA.

Another part of our research concerns thermodynamics of nucleic acids. We have been involved in the studies of thermodynamic properties of nucleic acids since 1984, when we started the collaboration with Professor Douglas H. Turner (University of Rochester, Rochester, USA) on this subject. Our recent studies have focused on thermodynamic stability of LNA-2'-O-methylRNA/RNA duplexes containing modified nucleotides in 2'-O-methylated strand. The modifications include LNA-cytidine, LNA-uridine, LNA-guanosine, LNA-adenosine, LNA-2,6-diaminopurine riboside, LNA-2-thiouridine, LNA-4-thiouridine and some of their 2'-O-methyl analogs. We determinated the influence of a single substitution of modified nucleotides on thermodynamic stability of 2'-O-methylRNA/RNA duplexes when modified nucleotides were placed at 5'- and 3'-side, as well as at the center of the duplexes. Based on thermodynamic stability of 2'-O-methylRNA/RNA and LNA-2'-O-methylRNA/RNA duplexes, the nearest neighbor parameters were calculated. Recently, we have also found that pyrene pseudonucleotide at 3'-side of LNA-2'-O-methylRNA/RNA duplexes uni-

versally enhances thermodynamic stability of those duplexes irrespective of the type of nucleotides placed in opposite strand.

The researches presented above were dedicated to determination of the thermodynamic rules necessary for preparation of isoenergetic RNA microarrays. Using phosphoramidite approach, the 853-isoenergetic pentanucleotides were synthesized (among 1024 of all possible pentanucleotides). Currently, the library of isoenergetic probes is used for printing of semi-universal microarrays and subsequently used for microarray mapping to study the structure and interaction of native RNA. Those studies concern various retrotransposon R2 5'RNAs from *Bombyx mori, Bombyx hercules, Bombyx prometheus, Bombyx cynthia and Bombyx pyri.* Using isoenergetic RNA microarrays, the secondary structure of those RNA was determined for the first time. Another objects of our interest are regulatory RNAs from *Escherisia coli* - DsrA RNA and OxyS RNA. Besides the secondary structure solving, we are also working on application of microarray mapping for determination of interactions of both regulatory RNAs with Hfq protein. Recently, we have expanded our research on the use of isoenergetic RNA microarrays as tool for searching single stranded fragments in RNA structure, which particularly strongly bind short modified oligonucleotides. In case of pathogenic RNA, strong binding of short oligonucleotide should inhibit process of pathogenesis. As a model for those investigations, we are using fragment X of 3'UTR genomic HCV.

We also are working on developing allelespecific digestion of pathogenic RNA based on antisense oligonucleotides strategy. In our approach, we are using gap-mer type strongly modified antisense oligonucleotides. The differences in thermodynamic stability of complementary and single mismatched LNA-DNA/RNA duplexes are used to achieve specific hydrolysis of mutated at single nucleotide polymorphism (SNP) position pathogenic RNA in the presence of ribonuclease H.

Current research activities

- chemical synthesis of nucleosides, nucleotides and oligoribonucleotides also containing modified nucleotides such as 2'-O-methylnucleotides, LNA nucleotides, and modified LNA nucleotides;
- thermodynamic stability of RNA and DNA duplexes containing natural and modified nucleotides (modified 2'-O-methylnucleotides, LNA nucleotides, and modified LNA nucleotides);
- construction of isoenergetic RNA microarrays based on short, modified oligonucleotide probes;
- application of isoenergetic RNA microarrays (microarray mapping) to determine the structure and interactions of RNA with other biomolecules;
- allelespecific hydrolysis of pathogenic RNA with modified antisense oligonucleotides.

Major recent results

Thermodynamics of LNA-2'-O-methylRNA/RNA duplexes containing modified nucleotides in 2'-O-methylated strand.

In collaboration with Douglas H. Turner, we studied the thermodynamic stability of 2'-O-methylRNA/RNA duplexes containing LNA-nucleotides and modified LNA-nucleotides. We found that the presence of LNA nucleotides enhances thermodynamic stability (free energy, $\Delta\Delta G^{o}_{37}$) of 2'-O-methylRNA/RNA duplexes in the range of 1.4-2.4, 0.3-0.6 and 0.1-1.4 kcal/mol when placed at central, 5'- and 3'-terminal position within the duplex, respectively (see below). Moreover, substitution of LNA-adenosine by LNA-2,6-diaminopurine riboside additionally increases thermodynamic stability by ca. 1 kcal/mol. Recently, we have also found that the placing of pyrene pseudonucleotide at 3'-side of 2'-O-methylRNA strand of 2'-O-methylRNA/RNA duplexes enhances thermodynamic stability evenly by ca. 2.3 kcal/mol, irrespective of the nature of nucleotide in opposite RNA strand. Furthermore, the influence of single mismatches on thermodynamic stability of 2'-O-methylRNA/RNA duplexes was also established and we found that the presence of a single mismatch diminishes thermodynamic stability of duplexes by 1.6-7.1 kcal/mol, depending on the type of mismatch. Based on collected data, the thermodynamic parameters of nearest-neighbor for 2'-O-methylRNA/RNA and LNA-2'-O-methylRNA/RNA duplexes were calculated.

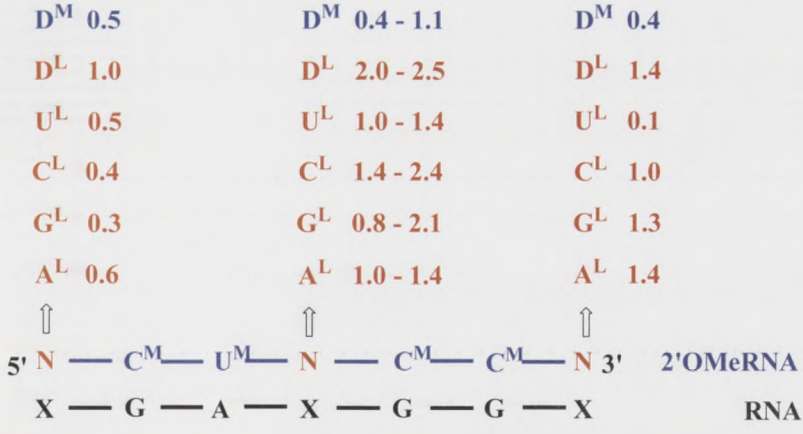

Figure 1. Schematic presentation of LNA-nucleotides' influence on enhancement of the thermodynamic stability ($\Delta\Delta G^{o}_{37}$) of 2'-O-methylRNA/RNA duplexes (nucleotide X in RNA strand is complementary to LNA-nucleotide).

Developing a new type of microarrays based on pentanucleotide isoenergetic probes (isoenergetic RNA microarrays).

Original type of microarays was introduced to develop a new method (microarray mapping) to study the structure and interactions of RNA. The microarrays are built on penta/hexanucleotide probes, which are modified with various LNA-modified nucleotides in a way that thermodynamic stabilities of the hybridization duplexes formed between oli-

gonucleotide probes on microarray and complementary fragments of target RNA are thermodynamically similar as much as possible (in consequence, the hybridization duplexes are isoenergetic), and, additionally, more favorable. Pentanucleotide fragment from 5'-side of a probe is responsible for recognition of complementary RNA fragment. For some of the probes, LNA-guanosine at 3'-side is added, which based on nonspecific (specific only for cytidine) interaction additionally enhances stability of hybridization duplex by ca. 1.5 kcal/mol. In consequence of isoenergetic character of hybridization duplexes, only the presence of single stranded fragment of target RNA determines hybridization results (in opposite to nonisoenergetic microarrays for which thermodynamic stability of hybridization duplexes is also key factor). At present moment, we have 853 penta/hexanucleotide probes, all able to form hybridization duplexes more favorably (free energy) than -6 kcal/mol.

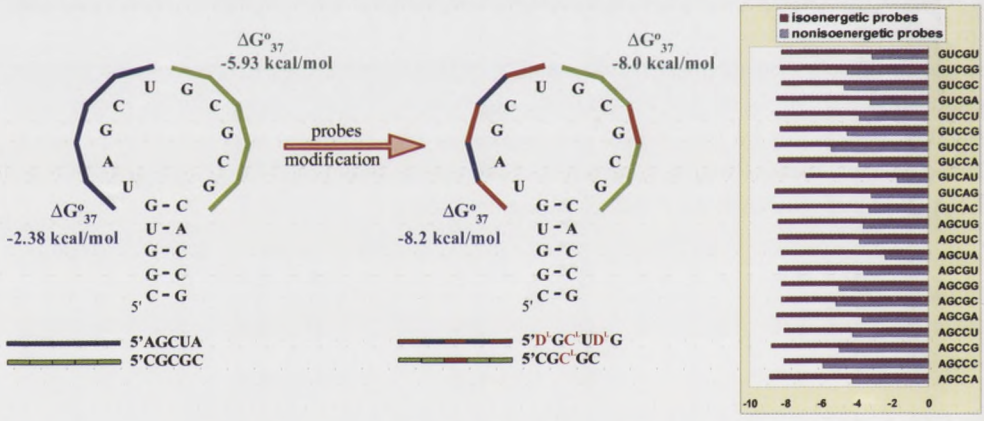

Figure 2. Schematic transformation of nonisoenegetic (unmodified) probes (left side) into isoenegetic (modified) probes (right side) with LNA-nucleotides and modified LNA-nucleotides. On the right side of the diagram, there is the comparison of free energies of the fragment of nonisoenegetic (blue bars) and isoenegetic (purple bars) pentanucleotide library.

Microarrays mapping as new method to study structure of native RNA as well as theirs interactions with another biomolecules

Based on application of isoenergetic RNA microarrays a new method to studies of RNA structure was introduced (microarray mapping). The isoenergetic microarrays method provides information on single stranded fragments in target RNA. The results from microarrays mapping are used as constraints in Mfold or RNAstructure programs to predict secondary structure of RNA based on experimental results. The structure of several RNAs were studied including retrotransposon R2 5'RNAs from *Bombyx mori, Bombyx hercules, Bombyx prometheus, Bombyx cynthia* and *Bombyx pyri.* Microarray mapping was applied to study also regulatory RNA from *Escherisia coli* – DsrA RNA and OxyS RNA. Beside studies of the secondary structure we are also working using isoenergetic

microarray method on interactions of both regulatory RNAs with Hfq protein and fragments of regulated RNA.

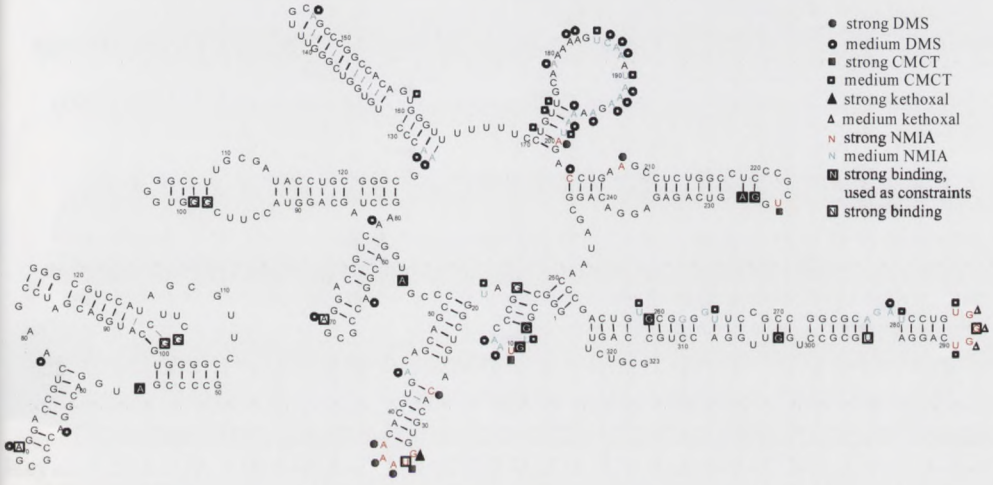

Figure 3. Prediction of secondary structure of R2 Bombyx mori 5' RNA from RNAstructure 4.4 using chemical mapping and microarray hybridization constraints or hybridization constraints alone at room temperature in buffer 200 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.0. On the left,there is fragment R2 5'RNA between 50 and 124 nucleotides which form pseudoknot structure (not predictable by RNAstructure program).

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LABORATORY OF RNA BIOCHEMISTRY

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Keywords: • *RNA structure and function* • *ribozymes* • *in vitro selection method* • *nucleic acids-based technologies* • *non-coding RNA regions* • *IRES-dependent translation*

The major efforts of our laboratory have been focused on elucidation of the role of metal ions in the structure formation and functioning of ribozymes derived from hepatitis *delta* virus and on searching for novel RNA motifs capable of specific binding of selected divalent metal ions.

More recently, we have commenced the studies aimed at structural and functional characterization of the conserved, non-coding regions of hepatitis C viral RNA as well as the IRES elements found in some cellular mRNA molecules. The goal of the ongoing studies is also to establish the rules which could facilitate designing nucleic acids-based molecular tools for targeting of such highly structured RNA regions.

Major recent results

Antigenomic delta ribozyme variants obtained by the in vitro selection method

In the genomic RNA strand of the hepatitis *delta* virus (HDV), as well as in its antigenomic counterpart generated during virus replication *via* the double rolling circle mechanism, there are two sequences with ribozyme activities, called the *delta* ribozymes. Despite large progress in elucidation of the structure and mechanism of catalysis of the *delta* ribozymes, the existing data do not, however, reveal how their catalytic centers might have evolved. It is interesting to know whether a similar or better ribozyme performance could be achieved by catalytic centers that are composed of nucleotides other than the wild-type residues. High sequence conservation of ribozyme regions of viral RNAs precludes answering this question. Simultaneous testing of a very large number of ribozyme variants with multiple mutations is, however, possible with the use of the *in vitro* selection methodology.

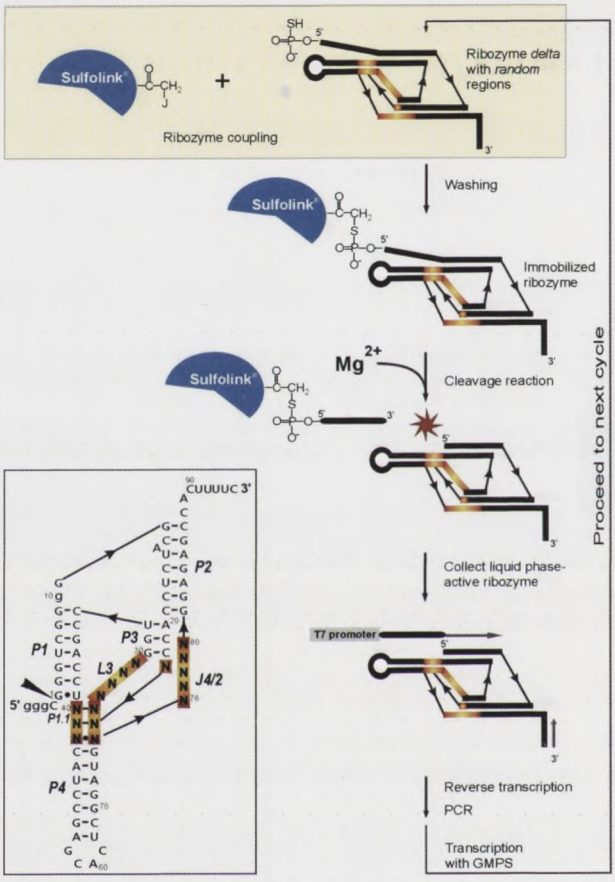

Figure 1. Combinatorial library of cis-acting antigenomic delta ribozyme (inset) and a scheme of the selection-amplification procedure used to search for its catalytically active variants. The randomized regions are boxed.

We used the *in vitro* selection method to search for catalytically active variants of the antigenomic delta ribozyme with mutations in the regions that constitute the ribozyme active site: L3, J1/4 and J4/2. In the initial combinatorial library, sixteen nucleotide positions were randomized and the library contained a full representation of all possible sequences. Following ten cycles of selection-amplification, several catalytically active ribozyme variants were identified. It turned out that one-third of the variants contained only single mutation G80U and their activity was similar to that of the wild-type ribozyme. Unexpectedly, in the next one-third of the variants, the C76 residue, which was proposed to play a crucial role in the ribozyme cleavage mechanism, was mutated. In these variants, however, a cytosine residue was present in a neighboring position to the polynucleotide chain. It shows that the ribozyme catalytic core possesses substantial 'structural plasticity' and the capacity of functional adaptation. Four selected ribozyme variants were subjected to more detailed analysis. It turned out that the variants differed in their relative preferences towards Mg^{2+}, Ca^{2+} and Mn^{2+} ions. The differences in their catalytic activity seem to be a consequence of the different abilities of various metal ions both to perform a chemical reaction as well as to aid the formation of ribozyme structural core.

Trans-acting delta ribozymes as molecular tools in the strategy of directed degradation of RNA targets

The *delta* ribozymes acting *in trans* are potentially attractive tools in biochemical and biomedical applications. We have recently proposed the use of antigenomic *delta* ribozyme as molecular scissors for the purpose of correct processing of the 3' ends of RNA transcripts obtained *in vitro* with T7 RNA polymerase. The transcripts were extended with the sequence of only seven nucleotides complementary to the ribozyme's recognition site. Subsequently, these extra nucleotides were cleaved off with *trans*-acting *delta* ribozyme.

Among all known ribozymes, *delta* ribozymes have some unique characteristics, which make them very attractive *trans*-acting molecular tools in the regulation of gene expression. However, earlier reports have suggested that some other factors besides RNA accessibility may limit the efficiency of RNA targeting by ribozymes.

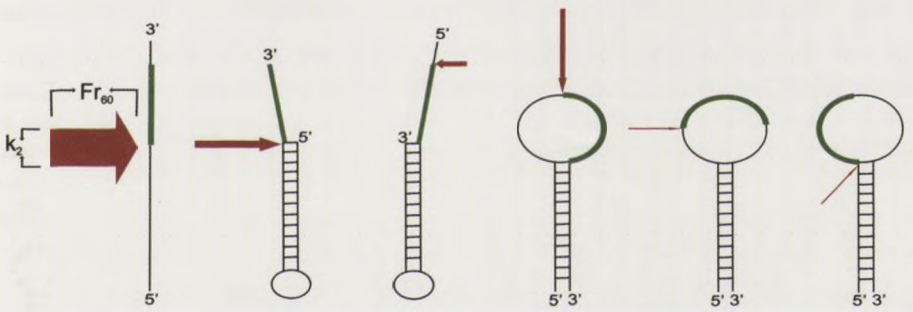

Figure 2. Schematic representation of the differences in cleavage rates (k_2) and cleavage extents after 60 minutes (Fr_{60}) determined for six model RNA substrates, in which the ribozyme recognition sequence (marked with green boxes) was embedded into a different structural context.

The aim of our recent work was to shed some more light on factors influencing the effectiveness of *delta* ribozyme cleavage of structured RNA molecules. An oligoribonucleotide that corresponds to the 3'-terminal region X(+) of HCV RNA as well as yeast $tRNA^{Phe}$ were used as representative RNA targets. Only a few sites susceptible to ribozyme cleavage were identified in these targets using a combinatorial library of ribozyme variants, in which the region responsible for ribozyme-target interaction was randomized. On the other hand, the targets were fairly accessible for binding of complementary oligonucleotides, as was shown by 6-mers DNA libraries and RNase H approach. Moreover, the specifically acting ribozymes cleaved the targets precisely, but with unexpectedly modest efficacy. To explain these observations, six model RNA molecules were designed, in which the same 7-nucleotide-long sequence recognized by the *delta* ribozyme was always single-stranded, but embedded into different RNA structural context. These molecules were cleaved with differentiated rates, and the corresponding k_2 values differed almost 50-fold. This clearly shows that the cleavage of structured RNAs might occur to be much slower than the cleavage of a short unstructured oligoribonucleotide, despite full accessibility of the targeted regions for hybridization. Restricted possibilities of conformational transitions, which are necessary to occur on the cleavage reaction trajectory, seem to be responsible for these differences. Their magnitude, which was evaluated in this work, should be taken into account while considering the use of *delta* ribozymes for practical applications.

Folding of the conserved non-coding sequences of HCV RNA genome and the complementary sequences of the replicative viral strand

Hepatitis C virus contains a single-stranded, positive-sense RNA molecule, ca. 9600 nucleotide-long, which includes a single large open reading frame and two untranslated regions at the 5' and 3' ends of the genome. Both untranslated regions are highly conserved and they play important roles at the key steps of the viral life cycle: translation and replication. Better knowledge on spatial folding of these regions of viral RNA is important for understanding the HCV functioning in the cell. This will also be helpful in designing more effective nucleic acids-based antiviral agents.

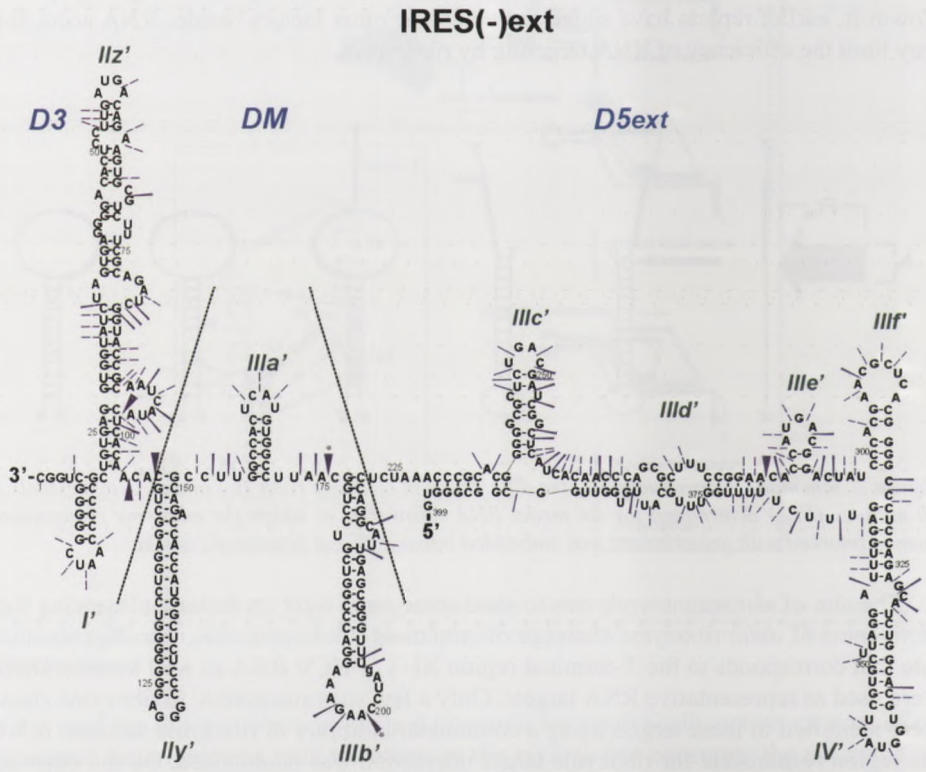

Figure 3. Probing of the secondary structure of RNA IRES(-)ext by Pb^{2+}-induced cleavage method. The sequence corresponds to the 3'-terminal region of the hepatitis C viral replicative strand.

We have investigated the folding of two oligoribonucleotides, X(+) RNA, which corresponds to the 3' terminal region of genomic HCV RNA, and X(-) RNA – the 5' terminal region of complementary RNA strand generated during virus replication. We proposed a new secondary structure model of X(+) RNA consisting of four hairpin motifs. In this model, two short hairpins might form a hypothetical pseudoknot by changing their base-pairing systems. Such structural dynamics might be important for the biological function of that region, i.e. for switching between its involvement in replication or translation. On the other hand, the analysis of X(-) RNA folding supported a three-stem-loop secondary structure model for this RNA.

For the 3' terminal region of HCV replicative strand, the IRES(-) region, two secondary structure models have been proposed in the literature. The aim of our recent studies was to better characterize folding of that region, especially within its domain which was controversial in the previously proposed models, and to investigate the ability of IRES(-) to interact with distant regions located either upstream or downstream in the replicative RNA strand. For structural analysis, oligoribonucleotides representing selected domains of the earlier proposed models were also synthesized, their structures were analyzed in solution and the results were compared to those obtained with the full-length molecule. Such 'structural fingerprinting' gave better insight into the IRES(-) structure. We showed that in the case of IRES(-) RNA consisting of 3' terminal 374 nucleotides three domains: *D3*, *DM* and *D5* fold independently on one another. However, when the RNA molecule is extended by additional 25 nucleotides domains *D3* and *DM* fold autonomously, but a part of domain *D5* interacts with the upstream sequence stretch of the viral replicative strand.

Structural basis for recognition of Co^{2+} by in vitro selected RNA aptamers

The studies on RNA motifs capable of binding divalent metal ions have largely been focused on Mg^{2+}-specific motifs and the information concerning the interactions of other ions with RNA are still very limited. The application of the *in vitro* selection method has allowed us to isolate two RNA aptamers that bind specifically Co^{2+} ions. Subsequently, these aptamers were used as research models to study the structural basis of the molecular mechanism emphasizing binding of Co^{2+} to RNA molecules.

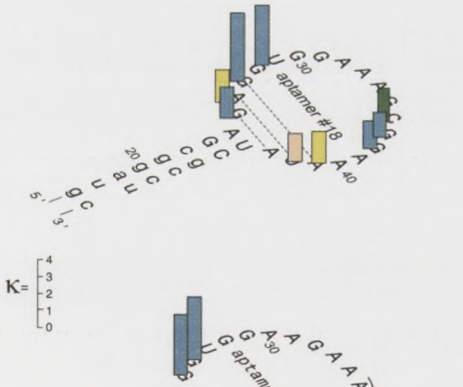

Figure 4. Summary of the interference effects within the Co^{2+}-binding aptamer no. 18 and no. 20 defined by NAIM. The histogram represents the secondary structure of the aptamer loop regions. The bars are correlated with the determined magnitude of interference (κ values). Dotted lines in aptamer no. 18 mark the loop E-like base pairing, and the solid line in aptamer no. 20 indicates the nucleotides involved in dimer complex.

We showed the importance of the N7 atoms of guanine residues in the arrangement of Co^{2+}-binding sites using phosphorothioate 7-deazapurine analogs and the NAIM approach. In both aptamers, several tandems of guanines involved in Co^{2+} binding or development of aptamer structures were identified. Additionally, other elements of their secondary structures, such as the E-like loop and kissing complex motifs, seemed to be important in the formation of the architecture of metal ion-binding sites. In the case of aptamer no. 20 that contains the self-complementary 35-ACGCGG-40 sequence, we confirmed the appearance of a kissing loop complex motif in the presence of Co^{2+} or Mg^{2+} ions. This observation was in line with NAIM results showing that N7 atoms of G39 and G40 interfere with Co^{2+} binding. The importance of the purine-rich stretches involved in stacking interactions for the binding of Co^{2+} also has to be pointed out. The regions 19-AGGCGAGAGG-28 in aptamer no.18 and 19-AGGCGAGG-26 in aptamer no. 20 contain a tandem of guanine residues with the highest $c^7G\alpha S$ interference values thus indicating direct metal ion coordination. Moreover, the binding of Co^{2+} induces RNA conformational changes that result in the stabilization of the aptamer structures, which was confirmed by two independent methods, hybridization of semi-random library and RNase H digestion, and temperature dependent UV melting.

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LABORATORY OF BIOCONJUGATE CHEMISTRY

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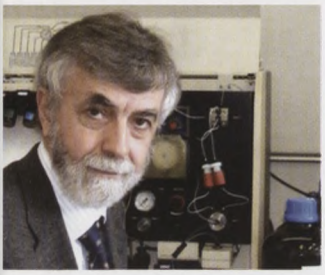

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Keywords: • *RNA/DNA chemical synthesis* • *protecting groups* • *thermolabile protecting groups* • *synthetic oligonucleotide combinatorial libraries (SOCL)* • *DNA sequencing* • *epigenetic DNA methylation* • *isothermic library*

Chemical synthesis of nucleic acids' fragments and their analogues is the main objective and a tool used in the research performed in the Laboratory of Bioconjugate Chemistry. What Francis Crick once said *DNA is such an important molecule that it is almost impossible to learn too much about it* (1979) continues to be contemporary. Thus, all efforts in the Laboratory aim at synthesis and study of nucleic acids' fragments and at applying the accumulated information in practice as well.

The main interests focus on search and studies of the properties of oligonucleotides with the desired properties derived through the use of approaches offered by *combinatorial chemistry.*

The main activities fall into several groups:

- chemical synthesis of oligonucleotides and their polyamine analogues;
- analysis of properties of oligonucleotides and their analogues using methods of combinatorial chemistry;
- studies on thermolabile protecting groups;
- synthesis and analysis of properties of nucleic acids components – nucleobases and nucleoside analogs – as compounds potentially interfering with epigenetic DNA methylation;
- analysis of combinatorial problems related to DNA sequencing.

Chemical synthesis of oligonucleotides requires using appropriate protecting groups and coupling methods. The approach most widely applied in our practice is the phosphoramidite one. When synthesis of modified oligonucleotides is considered, although the unmodified oligonucleotides can be obtained by this approach repetitively in high yields, in preliminary studies it requires establishing compatible protecting groups to be used for

these modified units. Thus, a search for new protecting groups remains one of the objectives of our synthetic work.

Combinatorial chemistry in the studies of properties of nucleic acids

The above outlined chemical and biological interests together form the main research objective that is the development of a new technology: *Synthetic Oligonucleotide Combinatorial Libraries* (SOCL). The *SOCL* is a representation of oligonucleotides of a given composition and length permanently bound to a solid support (Fig. 1). The SOCL technology is a non-biological system of molecules selection (*chemoselection*), allowing a search for *library elements* strongly interacting with the chosen acceptor molecules. The SOCLs of a dispersed format are studied (one bead of support – one oligonucleotide sequence). The SOCL technology of an integrated format is under development as well.

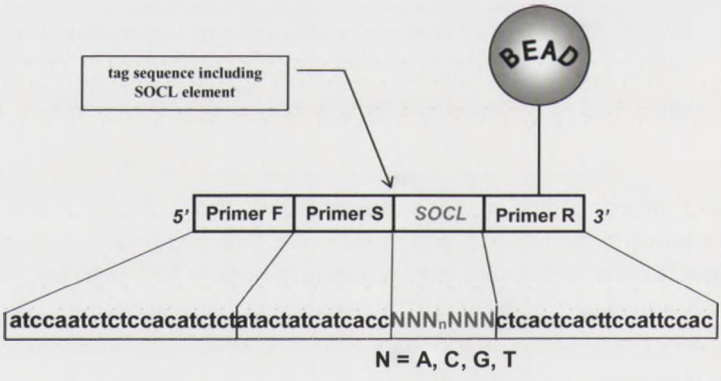


Figure 1. Structure of tag on single bead of SOCL.

In order to include nucleoside analogs into SOCLs, an alternative approach to library element structure elucidation has to be developed. This is due to possible effect of modification on action of DNA/RNA polymerase either inhibitory or causing a loss of information concerning base modifications. Thus, we decided to check whether mass spectroscopy can be a possible alternative method for direct sequencing of the elements of modified SOCLs. The prerequisite condition is the use of an appropriate linker between a solid support of SOCL and oligonucleotides that would be stable during libraries' synthesis and selection and cleavable prior to mass spectroscopy. Photolabile and chemically cleavable linkers are studied.

Synthesis of polyaminonucleosides, polyaminooligonucleotides, their analogues and studies of their properties studies by SOCL approach

The ability of natural polyamines to stabilize nucleic acids' tertiary structures and their complexes is well known. In recent years, oligonucleotide derivatives bearing various polyamine residues attached at different positions in nucleic acids have been synthesized, and their properties have been investigated. A stabilizing effect of polyamine modification in

the complexes of nucleic acids has been observed. We study polyaminooligonucleotides modified at base moieties.

The synthesis of 3'-phosphoramidites of polyamino-2'-deoxynucleosides was carried out. The method developed in our laboratory allows introducing symmetric polyamines (*e.g.* putrescine, spermine, *etc.*) into heterocyclic bases of four major deoxynucleosides. Spermine is the most promising polyamine in relation to DNA. Thus, spermine residue was attached to appropriately modified 5'-*O*-dimethoxytritylated 2'-deoxynucleosides at *N*-6 position of dA, *N*-4 position of dC, *N*-2 position of dG, and *C*-5 position of T *via* a methylene group. The amino functions of spermine moieties are protected with trifluoroacetyl groups to eliminate side reactions during a coupling step of automated DNA synthesis.

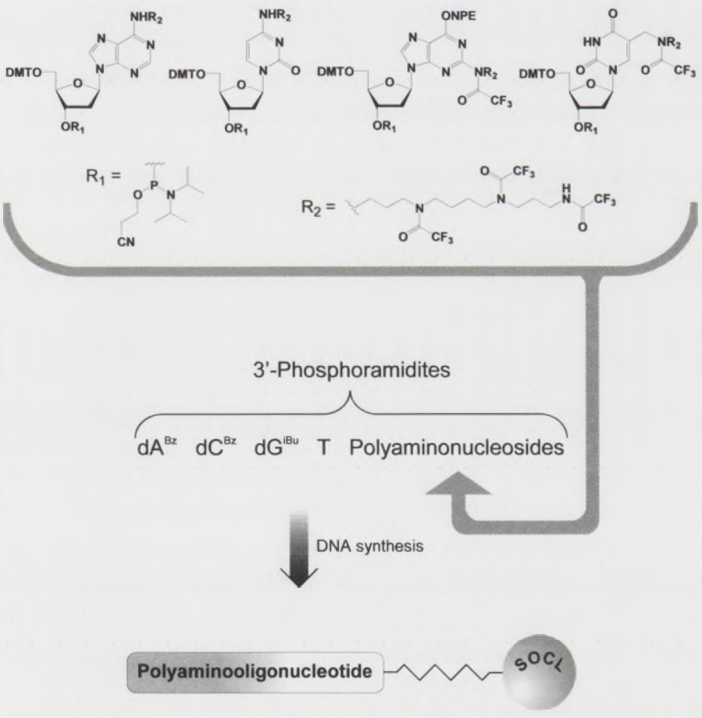


Figure 2. Polyaminooligonucleotides are synthesized on the modified polystyrene support using standard 2'-deoxynucleoside 3'-phosphoramidites as well as 3'-phosphoramidites of N-trifluoroacetyl protected polyamino-2'-deoxynucleosides.

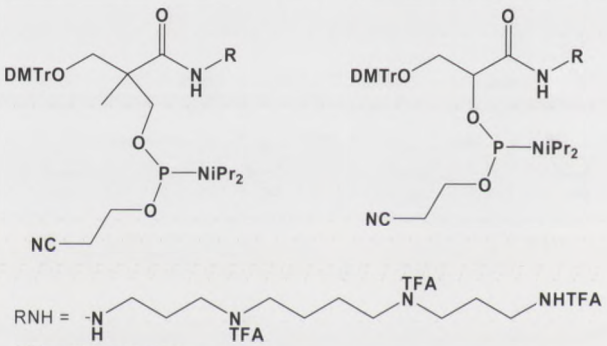
Finally, polyamino-2'-deoxynucleosides as 3'-phosphoramidites (Fig. 2) are used to obtain different synthetic polyaminooligonucleotides as well as their combinatorial libraries.

Polyaminonucleosides derived in a 2'-O-methylribonucleosides series were obtained as well. Thus, the derivatives of adenosine and cytidine were obtained carrying spermine moiety at 6- and 4-positions, respectively, as well as their 5'-O-dimethoxytrityl-3-phosphoramidites. These building blocks were successfully used in oligonucleotide synthesis.

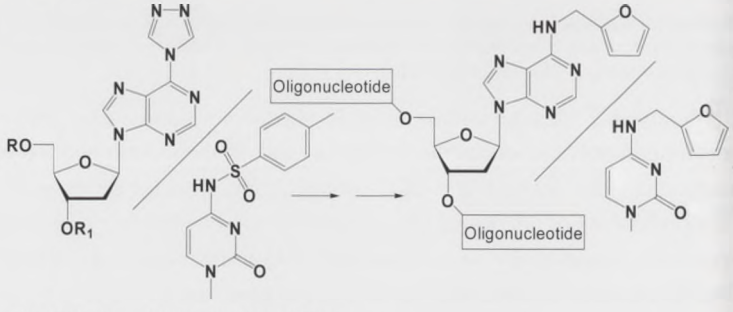
Recently, we have started structural studies of polyaminooligonucleotides using NMR spectroscopy. Thus, a double stranded octamer d-CAGC^{Sp}CGAC-3'/5'-GTCGGCTG was

obtained and analyzed with 600 MHz Bruker NMR spectrometer. The preliminary results suggest that spermine moiety is not involved in specific interactions within the duplex.

Studies on synthesis and properties of oligonucleotides containing polyamine moieties attached to non-nucleoside units were initiated in order to gain better insight into mechanisms of stabilization of nucleic acids' complexes with polyamines as well as to check whether a higher conformational flexibility of an internucleotide unit carrying a polyamine function can be beneficial both for complex stability and synthetic availability of these oligonucleotide analogs. The structure of two exemplary polyaminonucleoside analogs as phosphoramidites derivatives are shown in the scheme below.



We continued our studies on a possible biological function of some nucleoside modifications. Thus, we used 6-(1,2,4-triazol-4-yl)-purine 2'-deoxyribonucleoside and 4-N-p-toluenesulphonyl-2'-deoxycytidine, precursors for obtaining polyaminonucleosides, as precursors of 6-N-furfuryl-2'-deoxyadenosine and 4-N-furfuryl-2'-deoxycytidine, respectively. The dimethoxytritylated precursors were transformed into 3'-(2-cyanoethyl)-N,N-diisopropylphosphoramidites and used in oligonucleotide synthesis. The deprotection procedure involved the reaction with furfurylamine and led to the synthesis of oligonucleotides carrying 6-N-furfuryl-2'-deoxyadenosine (2'-deoxyriboside of kinetin) and 4-N-furfuryl-2'-deoxycytidine units, respectively. Their template properties during enzymatic DNA synthesis with DNA polymerases led to interesting conclusions concerning possible biological function of these nucleoside modifications (scheme below).



R = DMT, R_2 = $P(OCH_2CH_2CN)N(i\text{-}C_3H_7)_2$

Recently, we have started the studies aimed at finding the compounds potentially interfering with epigenetic DNA methylation. Thus, we synthesize and analyze properties of nucleic acids' components – nucleobases and nucleoside analogs – as potential drug candidates with anticancer and antiviral properties.

Development of new thermolabile protecting groups

Thermolabile protecting groups have been recently reported for the protection of phosphate/thiophosphate function (M. K. Chmielewski *et al.*, J. Org. Chem. 68, 10003, 2004). A deprotection under neutral aqueous conditions is a very attractive feature of these protecting groups. In our laboratory, we continue to study this type of groups and, especially, new type of thermolabile groups containing a 2-substituted pirydyl moiety.

The N-(2-pyridyl)aminoethanol derivatives (2-PAE), are among the promising candidates for efficient precursors of thermolytic protecting groups. The crystallographic analysis and physical data of N-(2-PAE) alcohols showed that the N-benzyl,N-(2-pyridyl)-aminoethanol has an optimal structure to build good protection groups (Fig. 3).

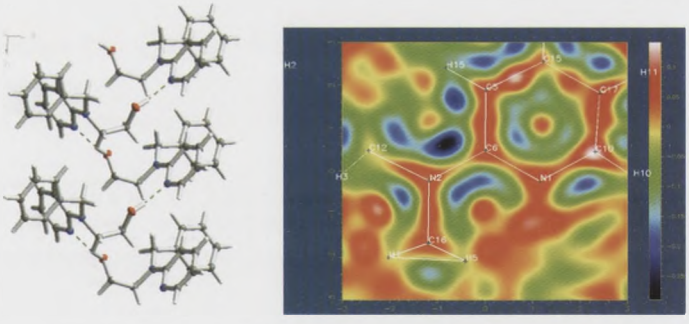


Figure. 3. The structure of supramolecular spiral chains and difference Fourier maps (Fo-Fc) of N-benzyl-N-(2-pyridyl)aminoethanol.

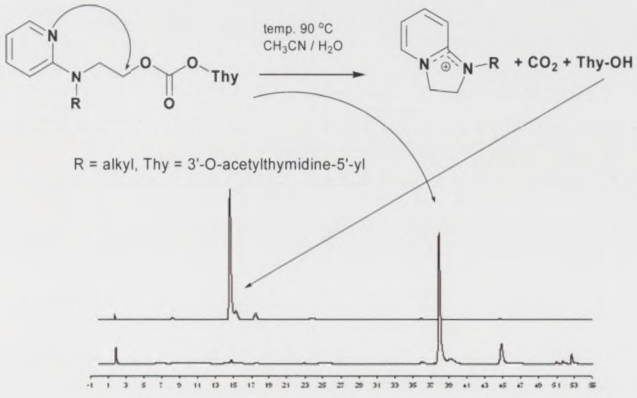


Figure 4. Scheme and HPLC analysis of removal reaction of N-benzyl-N-(2-pyridyl)aminoethyl TPG.

This observation will be confirmed by studying the properties as 3'-O-acetyl-5'-O-carbonates of thymidine. The thermolytic protection of hydroxyl function is most successfully

done through the use of carbonates. In such cases, the de-protection involves release of carbon dioxide (Fig. 4).

Modifications of aptamers by a post-selection approach

Most active aptamers that are available through SELEX and similar approaches are nucleic acids sequences of relatively large size. We believe that this length can be diminished without loss of activity using an aptamer sequence as a starting one. Thus, we propose using secondary structure analysis *in silico* based on thermodynamic data to plan and perform structure modifications of starting aptamer sequence as an effective approach to shorten aptamers. Then, new oligonucleotides can be further modified by internucleotide element exchange (*e.g.* nucleobase, sugar residue, internucleotide bonds modifications) to enhance aptamers' activity similarly as in post-SELEX approach.

Recently, we applied successfully this approach to the aptamer (RT36HIV1) with inhibitory properties against reverse transcriptase of HIV-1.

Current research activities

- synthesis of SOCLs, their application in chemoselection and sequencing of nucleic acids; application in studies of triplex DNA;
- synthesis of polyaminooligonucleotides and their analogues and studies of their properties by SOCL approach;
- development of new algorithms useful in DNA sequencing by hybridization (SBH);
- development of new thermolabile protecting groups;
- structural analysis of polyaminooligonucleotides using NMR spectroscopy.

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LABORATORY OF PLANT MOLECULAR BIOLOGY

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Keywords: • *plant genes* • *plant proteins* • *organ-specific gene expression* • *RNA silencing* • *microRNA* • *symbiotic nitrogen fixation* • *edible vaccines* • *ABC transporters* • *ferritins* • *plant transformatio* • *DNA microarrays*

The studies conducted in the Laboratory of Plant Molecular Biology concentrate on three major problems: (i) plant genes, their structure and functioning; (ii) structure and function of the products of plant gene expression, RNA and proteins; (iii) RNA as an important factor controlling plant gene expression. The major object of our studies is *Medicago truncatula*, a model legume plant. Sometimes, however, in well-founded situations our experiments involve also other plants. For example, we use *Arabidopsis thaliana* – to investigate selected problems concerning RNAi phenomenon; *Lupinus luteus* and *Lupinus angustifolius* – to investigate specific aspects of symbiotic interactions between legumes and rhizobium species or *Nicotiana benthamiana* and *Hordeum sativa* – to study plant-virus interactions.

In order to broaden our knowledge about the role of individual genes, proteins and RNAs in plant development or plant – microbe interactions, appropriate biomolecules are isolated and subjected to a detailed analysis using various molecular and cytological methods. Recently, the latter have been significantly extended by a wide spectrum of techniques used in modern functional genomics and proteomics (e.g.: DNA microarrays, 2D protein electrophoresis, RNAi-based technologies). In addition, plant transformation systems available in the laboratory provide an important way of testing the *in vivo* expression of recombinant DNA. These systems were also adapted for engineering transgenic plants producing medicinally important proteins.

Current research activities

- identification and characteristics of genes and proteins involved in RNAi phenomenon in legumes;
- identification and characteristics of small regulatory RNAs – elucidation of their biogenesis and function in plant cells;

- application of RNAi and microarray technology in functional genomics of cultivable plants;
- identification of ATP-binding cassette transporter genes in legumes;
- elucidation of the physiological functions of ABC-transporters in legumes;
- structure determination and expression analysis of plant symbiotic genes using differential screening and DNA microarray technology;
- iron metabolism, the role of oxidative stress in legume symbiosis and molecular links between symbiotic and pathogenic interactions;
- phylogenetic and computation analyses of plant genes and bacterial nodulation genes, elucidation of the co-evolutionary correlations of symbiotic systems;
- developing plant systems for the expression of pharmaceutically important proteins, including recombinant vaccines.

Major recent results

Ferritins and iron management in legume plant development and symbiosis

Iron-containing proteins play a crucial role in many important processes such as respiration, photosynthesis and DNA or hormone syntheses. Although cellular iron is mostly bound in proteins and their cofactors, it may be released to the cytoplasm in various stress situations. The physico-chemical properties of iron ions (small ionic radius, high reactivity, and especially the capacity for catalyzing the formation of hydroxyl radical, the most potent oxidizing agent known in nature) make them responsible for the damage to nucleic acids, proteins, and lipids. Sequestration seems to be the most effective way to limit iron noxiousness. All organisms from bacteria to animals possess a special "protein cage" in which iron may be kept harmless, but available for all vital processes, and this is ferritin. Ferritins are 24-subunit, spherical protein complexes, capable of the reversible transfer of iron between liquid and solid phases. Iron is then concentrated inside the protein cavity (up to 4500 iron atoms) as a hydrated ferric oxide. Not only iron sequestration, but also removal of dioxygen from the cytoplasm (mineral core formation) make ferritin an effective part of the antioxidant system.

An ability to form symbiotic associations with rhizobia and to utilize atmospheric nitrogen makes legumes ecologically successful. Symbiotic nitrogen fixation, however, is a phenomenon particularly dependent on iron-containing proteins. Free-living bacteria turning into microsymbionts (bacteroids) triple their cytochrome accumulation and synthesize nitrogenase. The latter may contain more than 30 atoms of iron and can constitute more than 10% of total bacterial proteins. To protect this enzyme from oxygen-induced inactivation and to supply symbionts with enough oxygen for their respiration, a plant fills the bacteroid cytoplasm with another iron-containing protein, leghaemoglobin (Lb).

Lb may account for up to 30% of all soluble proteins of the infected cell, which makes a nodule one of the most iron-loaded organs of a plant. The nodules are better equipped with all kinds of antioxidant systems (i.e. ascorbate-glutathione pathway or superoxide dismutases) than the parent root. This ensures effective detoxification of the superoxide radical and excess H_2O_2. However, during senescence, a rise in proteases activities, a decline in the antioxidant pool, and increased ROS accumulation promote nodule decay. Protein

degradation and membrane disruption lead to the significant increase of free iron concentration. Such "unattended" iron accelerates the spread of nodule tissue disruption and nitrogen fixation termination. Both the rapidity of senescence and the extent of stress tolerance of symbiotic interactions may be, to a great extent, defined by the structure and developmental pattern of the nodules (indeterminate- versus determinate-type of nodule). Our first model for research on iron management in symbiotic nitrogen fixation is yellow lupine. Possession of long-lasting meristems as well as clearly divided developmental zones makes the general anatomy of lupinoid nodules similar to those of an indeterminate type. In addition, because of the special evolutionary position of lupines with those of the earliest branching lineages within the Papilionoideae subfamily, this plant system appears very attractive to study.

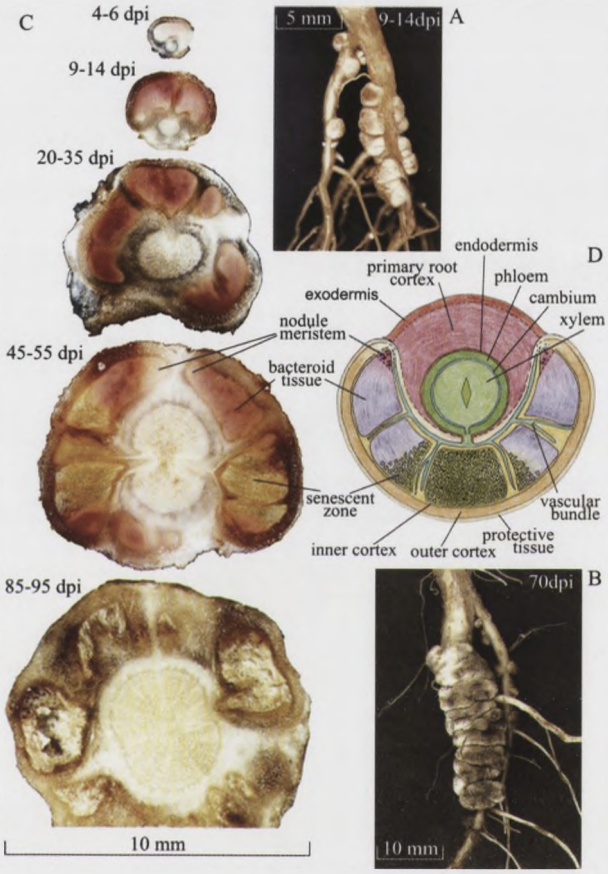

Figure 1. Lupinus luteus root nodules. (A) Young nodules: because of very early division of laterally-positioned nodule meristem and its growth in all directions, the lupin nodule escapes from its cylindrical shape. (B) As a result of the activity of lateral meristems, the initially spherical nodules grow laterally encircling the root. (C) Cross-sections through nodules at different stages of development. (D) Schematic diagram of the mature nodule.

Three ferritin genes were identified in lupine. They were differently regulated in response to iron and ABA, similarly to ferritin genes in maize and *Arabidopsis*. Analyses of both RNA and protein samples, as well as tissue fragments originating from the same plants allowed us to correlate the changes in ferritin gene expression, during development and senescence of a nodule, with specific tissues of this organ. It is characteristic that during indeterminate-type lupine nodule formation, ferritin synthesis is associated mainly with differentiating bacteroid tissue, and correlates with the expression of leghaemoglobin genes. This may indicate a more complex function for ferritins than just acting as an iron-storage complex (changes in ion status, changes in oxidative states, etc.). We have also found that the senescence processes initiated in the oldest lupine nodule zones reactivate ferritin synthesis in differentiated bacteroid tissue of the adjoining, younger, and still active zones. The latter indicates that in indeterminate-type nodules, ferritin may be an element of the senescence delay mechanism. Such a delay could prolong

the time of nitrogen fixation and allow for more effective remobilization of iron to the floral parts of the plant. This may also partially explain the high iron content of legume seeds making nutritional advantage of this group of plants.

ABC transporters in legumes

ATP binding cassette (ABC) proteins (Fig. 2) form the largest known protein family, members of which operate in all living cells from bacteria to man. A major function fulfilled by these enzymes is membrane translocation of a wide range of unrelated molecules. Importers constitute mainly the prokaryotic members of the ABC family and are part of uptake systems for nutrients like amino acids, vitamins, sugars or metal ions. Exporters are found in both prokaryotes and eukaryotes and are involved in extrusion of signalling molecules, toxic substances as well as targeting of membrane components.

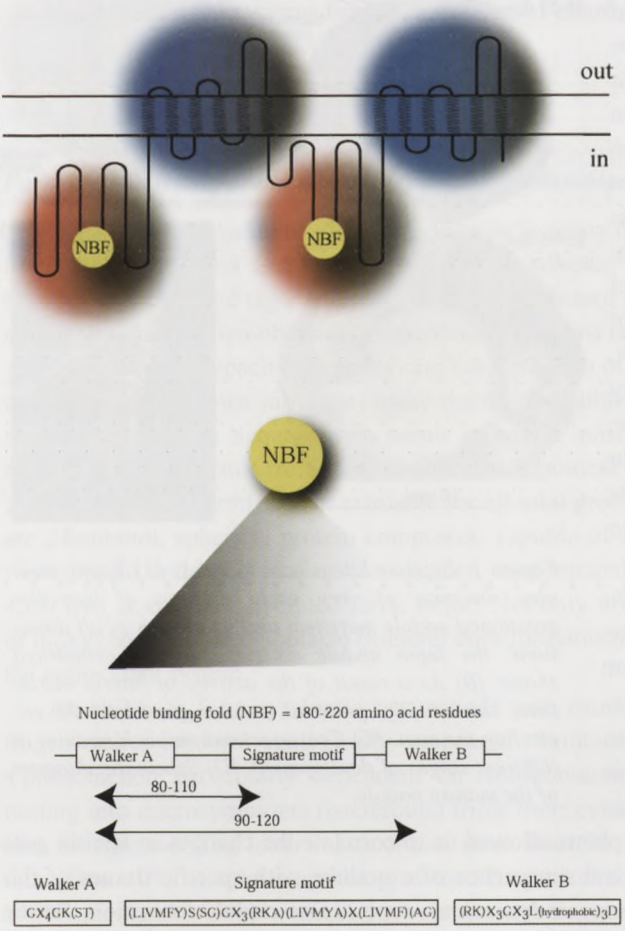

Figure 2. Schematic diagram showing ABC transporter. The two transmembrane domains (shaded blue), each generally containing six membrane-spanning regions (zigzag), and the cytosolic domains (shaded red), each with a characteristic region (nucleotide-binding fold, NBF) involved in ATP binding, are indicated. The bottom section shows the general organisation of an NBF, and the order of the Walker A and B boxes and the ABC signature.

One of the remarkable observations from the analysis of plant genomes inventories e.g. *Arabidopsis thaliana*, *Oryza sativa* and recently *Lotus japonicus* was identification of a visibly larger number (>120) of putative ABC transporter coding genes comparing to other multicelullar organisms (~50). Within described plant ABC transporters, there are enzymes participating in the cross talk between plants and other organisms. A particular type of plant-microbe interaction is symbiosis of the legumes exemplified by *Medicago truncatula* and endosymbiotic prokaryotes known as Rhizobia. There is a number

of data showing that their interplay depends on the exchange of specific signaling molecules (Nod factor, flavonoids) and leads to the formation of a new root organ (nodule). To date, nothing is known about ABC transporters in *Medicago*. This family of proteins seems to play an important role in the symbiosis process for the Rhizobia where transport of regulatory molecules and nutritive elements is crucial for successful colonization and fulfilled by an ABC transport system.

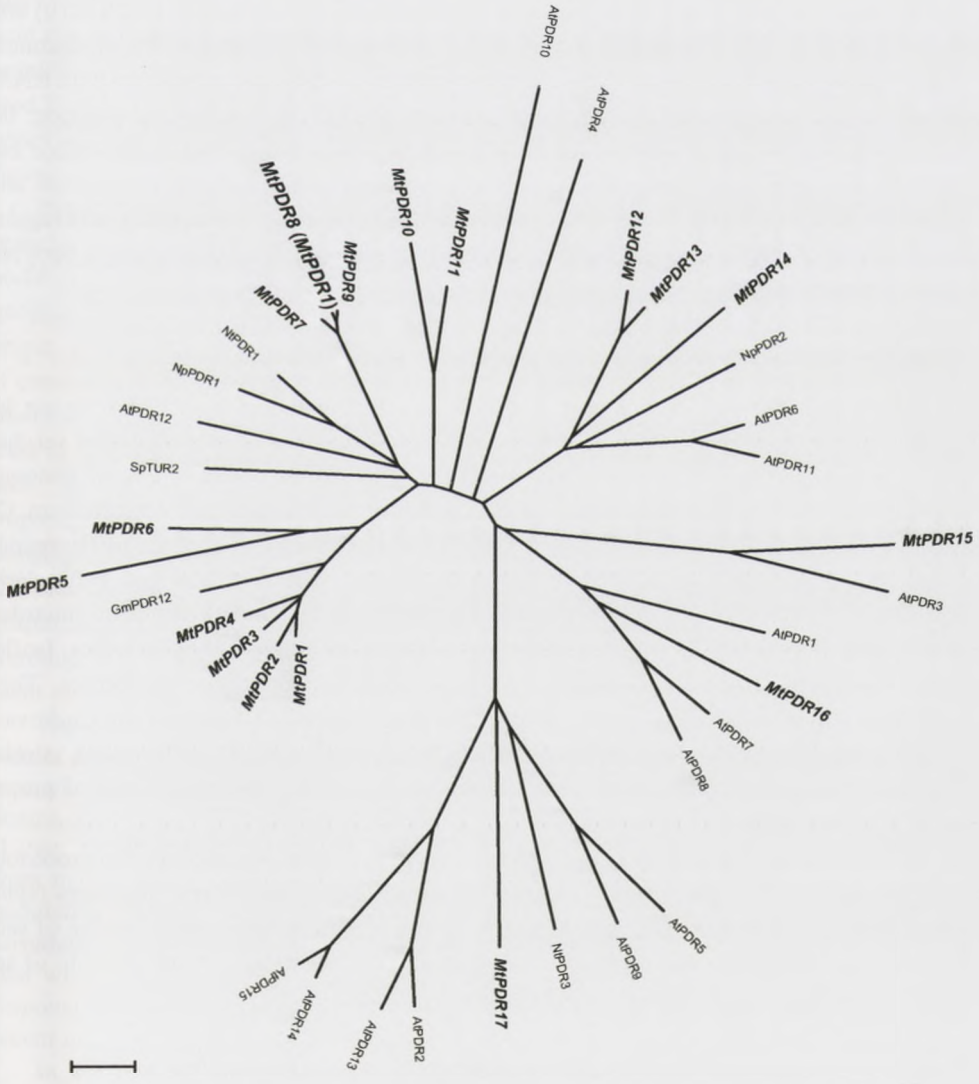

Figure 3. PDR like ABC transporters from Medicago (Mt), Arabidopsis(At), tobacco (Np) Glycine (Gm). SpTUR2 PDR from Spirodela polyrhiza.

In order to expand our knowledge about ABC transporters in legumes, we performed a systematic search for their genes in *Medicago truncatula*. At current state of art, 19 genes coding PDR like ABC transporters have been identified (Fig. 3). Within the identified genes

there are certain ones with broad expression pattern in all plant organs and others which transcriptional activity is strictly restricted, for example to flowers. Majority of them are expressed in root tissues. PDR like transporters seem to be involved in a secretion of compounds that play a defensive role against pathogens. In roots of *Medicago* seedlings, we observed (northern blot, qPCR) that the expression of certain PDRs is induced after infection with soil borne pathogenic fungi *Fusarium culmorum*. The expression of these genes was also up-regulated in the leaves after treatment with spores of fungus *Phoma medicaginis*.

We have obtained a cDNA sequence of the selected PDR transporter (MtPDR10) and analyzed in more detail its spatial and temporal expression in *Medicago*. The biochemical studies (subcellular localization of the protein, promoter GUS assays) combined with RNAi and phenotype analysis are ongoing to elucidate the MtPDR10 physiological function. Interestingly, we also observed that Rhizobia producing fully active nod factor induce expression of certain ABC transporters similarly to pathogens like *Fusarium culmorum* and Rhizobial strains deficient in nod factor production. A fine-tuning in perception and regulation of symbiotic infection and defence is probably required for discrimination between symbionts and pathogens.

Secondary metabolites production and distribution in legumes

Medicago truncatula is not only a rapidly developing model plant for studying legumes' biology (easily transformable, has a short life cycle and an abundant seed set, has simple, relatively small diploid genome, is self fertile and is the object of intense genomic studies), but also an excellent species for the studies on unique secondary metabolism. Of particular interests are isoflavonoids – a subclass of flavonoids. In contrast to flavonoids found throughout the plant kingdom, isoflavonoids are more restricted and particularly prevalent in *Leguminosae*. Apart of natural role of isoflavonoids in plant microbe-interactions, for humans they represent compounds with health promoting activities. Isoflavonoids exhibit estrogenic, antiangiogenic, antioxidant and anticancer properties. They were shown to be implicated in prevention of the postmenopausal disorders and cardiovascular disease. The biosynthetic pathway leading to the production of isoflavonoids is relatively well understood with several characterized enzymes, but the mechanisms of proper distribution are under investigation.

We are conducting the HPLC, LC/MS analysis of secondary metabolites from root/root exudates and leaves of *Medicago*. The analyses are performed at different life stages (symbiosis, pathogen infection). We would like to gain more information about profile of secondary metabolites, especially phenolic compounds in order to understand the role of secretion systems like ABC transporters in their distribution.

Biogeographic approaches in the phylogenetic studies of the root-nodule bacteria

In our research, we have focused on evolution and dispersal of symbiotic genes in the genus *Bradyrhizobium*. We put particular emphasis on phylogenetic analyses of nodulation genes. The latter are involved in the biosynthesis of nodulation factor – a rhizobium-produced molecule responsible for legume recognition and induction of root nodule meristem. The nodulation genes belong to the most specific component of rhizobium genome, being usually located on symbiosis plasmid or chromosome-located symbiosis island.

Unlike most non-symbiotic genes used in phylogenetic reconstructions, nodulation genes evolve much more rapidly, which is a characteristic of primary importance in biogeographic studies. In the case of a typical conservative housekeeping gene, the biogeographic structure is usually "erased" by more dynamic migration and recombination processes. We have developed protocols enabling PCR amplification of several nodulation genes, despite the high sequence divergence that characterizes nodulation genes. Our primary *nod* phylogenetic gene marker has become nodulation *nodA* gene encoding a fatty acid transferase. Apart from the *nodA* gene marker, we have developed protocols enabling amplification of other nodulation genes, first of all, the genes involved in modifications of Nod factor's reducing end which appear to be critical in the legume-host recognition. These genes are responsible for fucosylation (*nodZ*), sulfation (*nodH*), fucose acetylation (*nolL*), fucose sulfation (*noeE*), and fucose methylation (*noeI*).

Phylogenetic analyses of *nodA* gene have revealed the presence of seven major branches, known as clades I-VII, within the monophyletic *Bradyrhizobium* branch. Some *nodA* clades – clades I, II and IV and VII – comprise strains in a pattern reflecting their geographical origin. Thus clades I and IV encompass strains of a predominantly Australian origin, suggesting that they have diverged and differentiated in Australia. Likewise, clade II consists of strains isolated mainly in Europe from Genisteae and serradella legumes. It has been suggested that this clade which dominates in European soils, may have differentiated in the Mediterranean in response to the differentiation of Genisteae legumes in this area. The large and highly differentiated clade III represents strains isolated mainly in subtropical and tropical regions. Some of its inner groups (sub-clades) show geographical relationships, suggesting that they have evolved in a geographical separation with respect to the other groups. Thus phylogenetic studies of nodulation genes allow deducing a geographical origin of a given strain; moreover, they enable to differentiate between native and introduced strains. The latter fact is of economical importance, especially in the management of strains used for artificial inoculation.

We applied "the biogeographic" approach to elucidate the origin of lupine and serradella *Bradyrhizobium* strains isolated in Western Australia and the Western Cape province of South Africa. Thanks to the unique position of European lupine and serradella strains on the *nodA* gene tree – all strains belonged to a distinct branch referred to as clade II - we assumed that we could differentiate between the strains nodulating native legumes from those that have been introduced with seeds from Europe. Our study based on both nodulation and housekeeping gene trees has revealed that all strains isolated from lupine or serradella nodule have European origin, which supports an idea of an accidental introduction with soil-contaminated lupine and serradella seeds. The same mechanism has been responsible for dissemination of Australian native *Bradyrhizobium* strains following the recent introduction of Australian *Acacia* spp in southern Europe, Brazil and South Africa.

In these, as well as in subsequent studies, we have shown that clade II dominates in Europe. On the other hand, only a minority of *Bradyrhizobium* strains isolated from native American lupines grouped in this clade. We have hypothesized that clade II bradyrhizobia have evolved in Europe, most likely in the Mediterranean. Some of these strains later moved

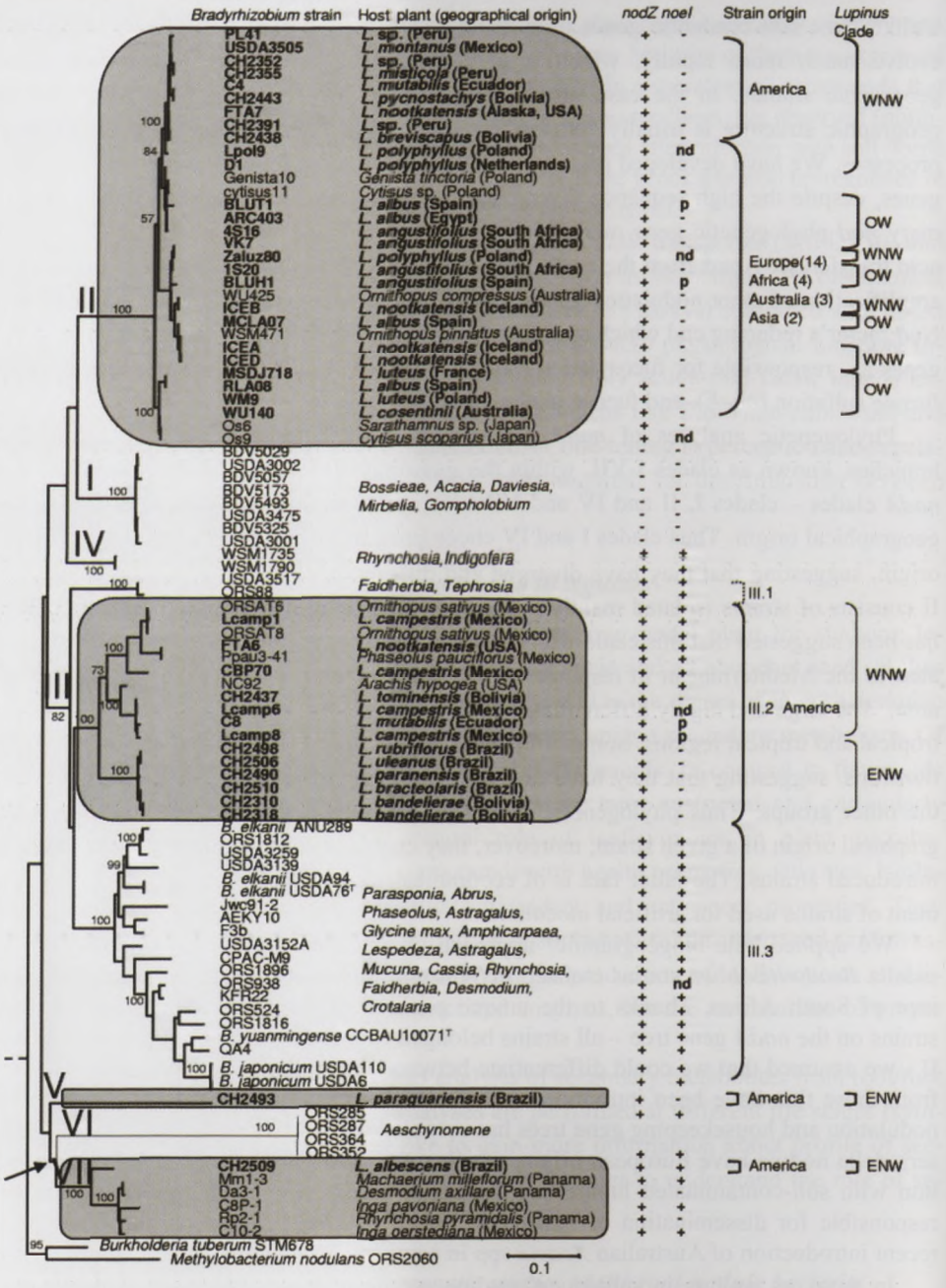

Figure 4. Maximum likelihood (ML) phylogenetic tree of Bradyrhizobium nodA genes. Lupinus strains are indicated in bold; clades to which they belong are in shaded blocks. Scale bar indicates the number of substitutions per site. Host plant species are indicated on the right side of the tree together with geographical origins in nodA clades containing Lupinus strains. Succeeding columns show results of nodZ and noeI PCR amplifications on test strains, geographical origin of lupine strains, and finally Lupinus clades corresponding to the phylogeny that divides lupines among three major groups, WNW: Western New World, ENW: Eastern New World, OW: Old World (Europe and Africa).

to the Americas. The American lupins were predominantly nodulated by strains, those *nodA* sequences group in clade III. This well differentiated clade groups strains isolated mainly from tropical and subtropical legumes. However, all lupine strains belonged to a sub-clade III.2 a group of strains originating exclusively from the Americas (although not only from the lupines). This suggests that sub-clade III.2 has differentiated in the Americas although its differentiation was, most likely, not connected to the evolution of lupines in the Americas.

ENOD40 – structural and functional studies

Plant development is a process requiring precise and multilevel regulation. The recent discoveries point to the important role of non-protein coding genes in many processes occurring in plant cell, e.g. developmental processes, growth coordination, or exact adjustment of gene expression in the course of organogenesis. *ENOD40* is one of the first genes activated in the course of legume plant symbiosis with nitrogen fixing bacteria. It possesses intriguing structural features such as lack of long conserved ORF, presence of two small conserved ORFs and conserved transcript structure. The ENOD40 gene seems to be of main biological function as its homologues have been identified in *Poaceae* and *Solanaceae* and the transcripts comprising ENOD40 like structures have been found in many angiosperms families. The experimental evidence points to both: small ORFs and non-coding RNA domains as important for *ENOD40* function.

We have demonstrated that legume plant *L. luteus* has two ENOD40 genes, one of them – ENOD40-1 encodes the shortest known legume ENDO40 transcript. The secondary structure of the *L. luteus* ENDO40-1 mRNA was elucidated using biochemical and computational methods. The differences observed between the models postulated for *ENOD40-1* from *L. luteus* and *G. max* suggest that the transcript can adopt two alternative structures depending on the conditions.

The translational activity of *L. luteus* ENDO40-1 ORF A was tested *in vivo*. The undertaken experiments have proved that the starting codon is surrounded by sequences promoting efficient translation. Also, the phylogenetic analysis provides evidence that ORF A is of translational activity.

The phylogenetic analysis, based on cDNA sequence and the features of peptides coded by the short conserved ORFs clusters *L. luteus* ENOD40 gene with plants of determinate nodules, whereas meristem of *L. lupinus* nodules is active during the whole nodule ontogeny. Clustering *L. luteus ENOD40* genes with *Lotus* and *Glycine* genes is probably linked to the specificity of indeterminate nodule development within genus *Lupinus*. Combining the phylogentic data with RNA structure allows to propose that ENOD40 domain 4 is important for symbiosis function/development in legume plant of group II (as *Medicago*).

Development of effective method of gene silencing in legumes

To elaborate an effective method of gene silencing in legumes, we are testing three currently available techniques inducing the RNAi phenomenon in plants. The first technique is based on the agroinfiltration of leaves/roots with the suspension of *Agrobacterium tumefaciens/rhizogenes* transformed with binary vectors into which a fragment of the targeted gene is inserted. The second technique consists in virus-induced gene silencing-

VIGS. For this purpose, we use BBMV (Broad Bean Mottle Virus), which infects a wide spectrum of legume species and BMV (Brome Mosaic Virus), which infects *Nicotiana benthamiana*. The third possibility to induce gene silencing in plants is the application of the biolistic method. The main object of our studies is *Medicago truncatula*. However, some specific aspects of plant-microbe interactions are also tested on three lupine species: *Lupinus angustifolius, Lupinus luteus,* and *Lupinus albus*.

The experiments involving transgenic *N. benthamiana* plants (constitutively expressing GFP) suggested that two methods, agroinfiltration and VIGS, should be most efficient in legumes. Currently, virus- and Ti plasmid-based vectors targeting *M. truncatula* endogenous loci coding for ABC transporters are being tested.

Transgenic plants as bioreactors of medicinally important proteins

The production of recombinant proteins in plants has many potential advantages for generating biopharmaceuticals relevant to diagnostics and clinical medicine. For the past few decades, the researchers have developed various plant transformation techniques enabling the integration of a gene of interest with the host plant genome. The transformed cells are positively selected and regenerated into transgenic plants in which the foreign protein is synthesized. Among the proteins of great practical interest that can be expressed in plants, there are subunit vaccines, inducing a protective immune response in human and animals. Plant-expressed antigens have been shown to induce immune response when administered parenterally or orally.

Since 1996, we have been working on the expression of viral and parasitic antigens with potential immunogenic function in plants cells. Our experiments have been concentrated mainly on: *hepatitis B* surface antigen – HBsAg, Classical Swine Fever Virus E2 protein – CSFV E2 and *Fasciola hepatica* serine protease. The following specific objectives are involved in the research: (i) plant transformation with gene encoding viral/parasitic antigen to obtain its constitutive or organ-specific expression. Specific protocols have already been elaborated to produce transgenic plant through the combination of generative reproduction and vegetative propagation; (ii) preparation of a prototype of the edible vaccine This step includes the liophylization of transgenic plant material and the formulation of tablets with a standardized antigen content; (iii) oral immunization of laboratory or target animals with the prototype vaccine. In the case of *hepatitis B*, we have demonstrated that the antigen expressed in plants administered orally can induce a production of specific anti Hbs-Ag antibodies in mice, as well as in human volunteers. It was the first demonstration that the oral route of plant derived antigen delivery is effective in inducing a specific immune response.

MOLECULAR VIROLOGY GROUP

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Keywords: • *RNA viruses* • *retroviruses* • *genetic polymorphism of RNA viruses and retroviruses* • *genetic RNA recombination* • *error prone replication* • *viral replicase* • *reverse transctiptase* • *RNAi phenomenon* • *viral vectors inducing RNAi* • *antiviral drugs*

Despite extraordinary progress that has recently been made in biological sciences, viral infections still remain one of the most serious problems in medicine and agriculture. In addition, unique features of viruses make them very interesting objects of basic studies and useful tools in modern biotechnology.

The research conducted by the Molecular Virology Group is especially focused on RNA viruses and retroviruses. They are the only known living species that use RNA to store their genetic information. Another characteristic, common to both RNA viruses and retroviruses, is their enormous genetic variability. There are several lines of evidence that an individual RNA virus or retrovirus does not form a genetically homogenous population, but rather a set of different viral variants. In consequence, RNA-based viruses can easily adapt to environmental changes, escape from immunological response or produce drug-resistant mutants.

Currently, we are using three systems to investigate various processes responsible for the generation of genetic diversity in RNA viruses and retroviruses: one *in vivo* system, developed with brome mosaic virus (BMV – a model (+) RNA virus of plants), and two *in vitro* systems, used for the studies of genetic diversity in human immunodeficiency virus (HIV-retrovirus) and in hepatitis C virus (HCV – (+) RNA virus).

In our studies, we also attempt to take advantage of the fact that RNA viruses infect, spread and express genetic information in higher organisms without altering their genomes. As a result, there is a possibility that specially prepared RNA virus-based vectors can be applied to avoid genetic manipulations at the DNA level. Currently, we are testing two series of viral vectors: one is used for silencing of selected host genes by inducing RNAi phenomenon, and the other for expressing specific genes in plant cells.

Current research activities

- involvement of BMV replicase proteins in genetic RNA recombination;
- role of RNA structure in non-homologous recombination between genomic molecules of BMV;
- homologous RNA recombination as a factor stabilizing the viral genome;
- determination of structural requirements for site-specific non-homologous RNA recombination;
- cloning and expression of HIV reverse transcriptase, its components and drug resistant mutants;
- reverse transcriptase as a key factor responsible for the genetic variability of HIV;
- cloning and expression of *hepatitis C* virus (HCV) polymerase and its mutants;
- determination of mechanisms involved in the development of chronic hepatitis C;
- BMV- and BBMV-based vectors inducing RNAi phenomenon in plant cells;

Major recent results

Frequent homologous crossovers between the same segments of RNA virus genome

Enormous genetic variability is one of the unusual features of RNA viruses. Numerous experiments reveal two main sources of genetic polymorphism that contribute to the rapid evolution of RNA viruses: error-prone replication and RNA recombination. The former introduces into the viral RNA genome a wide spectrum of point mutations at the rate of 10-4 to 10-5 per nucleotide per replication cycle. The latter is a widespread phenomenon described in many groups of RNA and in retroviruses. In spite of intensive studies, the mechanism of RNA recombination is not well understood. The copy choice mechanism, which is the most widely accepted, assumes that RNA recombinants result from template switching by viral RNA polymerase (RdRp) during RNA replication. Depending on the primary structure of the recombining molecules and on the location of junction sites, two types of recombination events have been recognized: homologous and nonhomologous, with the former being 10-fold higher than the latter in the case of brome mosaic bromovirus (BMV).

There is little information about homologous recombination in natural virus populations, because recombination products do not differ from parental RNAs. The crossovers in poliovirus RNA tended to occur within potential inter- and intramolecular heteroduplex regions, whereas in mouse hepatitis coronavirus the crossovers were found at apparent hot spots. However, the hot spots in mouse hepatitis virus appeared to result from selection pressure rather than from molecular constraints.

Previously, we demonstrated frequent homologous crossovers among molecules of the RNA3 segment in the tripartite brome BMV RNA genome. To further our knowledge about mechanisms of viral RNA genome variability, we have studied homologous recombination in BMV RNA1 and RNA2 components during infection. We have found that basal RNA-RNA crossovers could occur within coding regions of both RNAs, although recombination frequencies slightly varied at different RNA sections. In all cases, the frequencies were much lower than the rate observed for the intercistronic recombination hot spot in BMV RNA3. Probability calculations accounted for at least one homologous crossover per RNA molecule per replication cycle. In addition, we have demonstrated an efficient repair of mutations within the conserved 3' and 5' noncoding regions, most likely due to error-prone BMV RNA replication. Overall, our data verify that homologous crossovers are common events a during virus life cycle, and we discuss their importance for viral RNA genetics.

A novel subgenomic RNA in Brome Mosaic Virus

Single-stranded positive-sense RNA viruses utilize various strategies for expression of their RNA genomes, most notably via subgenomic RNAs (sgRNAs). The *Coronaviridae* and *Arteriviridae* families of the order *Nidovirales* express 6 to 7 proteins via sgRNAs, while the *Closteroviridae* generate between 6 and 11 sgRNAs. In some viruses, e.g., in Brome mosaic bromovirus (BMV), sgRNAs arise via internal initiation by the viral RNA polymerase (RdRp) on genomic minus-sense RNAs. It is also likely that other viruses copy their sgRNAs from prematurely terminated minus strands. In *Nidovirales*, the noncontiguous RNA leaders are joined to variously located sequences during minus-strand synthesis, followed by sgRNA transcription, whereas toroviruses combine discontinuous and nondiscontinuous processes to produce their sgRNAs. The formation of sgRNAs can also result from premature termination of positive-strand synthesis. In closteroviruses, a highly structured sequence region produces 5'-terminal sgRNAs by pretermination and additionally serves as a promoter for synthesis of another downstream sgRNA, with possible overlapping of termination and initiation signals.

The previously described 3' sgRNA4 of BMV is transcribed from an intergenic 100-nucleotide (nt) promoter (*sgp*) that consists of the core domain, a transcription enhancer, and the poly(A) tract. The more-upstream 150-nt sequence functions in *cis* as an internal replication enhancer (IRE) for positive-strand RNA3 amplification and carries a conserved B-box motif. In addition, we have recently reported that the poly(A) tract of the sgp can function as an efficient replicase detachment/reattachment site.

Recently, we have discovered a novel 5' sgRNA of BMV (sgRNA3a) that arises by premature internal termination and that encapsidates in BMV virions. Cloning and sequencing revealed that, unlike any other BMV RNA segment, sgRNA3a carries a 3' oligo(A) tail, in which respect it resembles cellular mRNAs. Indeed, both the accumulation of sgRNA3a in polysomes and the synthesis of movement protein 3a in *in vitro* systems suggest active functions of sgRNA3a during protein synthesis. Moreover, when copied in the BMV replicase *in vitro* reaction, the minus-strand RNA3 template generated the sgRNA3a product, likely by premature termination at the minus-strand oligo(U) tract. Deletion of the oligo(A) tract in BMV RNA3 inhibited synthesis of sgRNA3a during infection. We propose a model in which the synthesis of RNA3 is terminated prematurely near the sg promoter. The discovery of 5' sgRNA3a sheds new light on the strategies that viruses can use to separate replication from the translation functions of their genomic RNAs.

A universal BMV-based RNA recombination system

RNA recombination is a very common phenomenon. It has been observed in all types of viruses using RNA as a carrier of genetic information. Moreover, it has been shown that RNA recombination enables the exchange of genetic material not only between the same or similar viruses, but also between distinctly different viruses. Sometimes it also permits crossovers between viral and host RNA. Most of the collected data suggest that RNA recombinants are formed according to a copy choice model. A viral replication complex starts nascent RNA strand synthesis on one template, called RNA donor and then switches to another template, called RNA acceptor. Accordingly, two main factors are thought to affect RNA recombination: the structure of recombining molecules and the ability of the viral replicase to switch

templates. To gain more knowledge of the mechanism of RNA recombination, several model experimental systems have been created. They provided us with some specific data describing homologous and/or non-homologous recombination in particular viruses. As a result, a wide spectrum of the RNA motifs supporting recombination has been identified. In general, the collected data suggest that there exist two major types of RNA structural elements that induce recombination events: (i) universal ones mediating template switching by different viral replicases and (ii) virus-specific ones. Unfortunately, up till now there has been no *in vivo* recombination system that could be used to test the recombination activity of any given RNA sequence and, consequently, to verify the above hypothesis and find some general laws governing the studied process.

In our earlier studies on genetic RNA recombination, we used the well-characterized heteromolecular (a recombinationally active sequences (RAS) were placed in two different segments of the viral genome) *in vivo* system developed in BMV. Unfortunately, it has one serious limitation. It was designed in such a manner that viable RNA3 recombinants could easily form only if a sequence derived from the 3'-portion of RNA1 or RNA2 was used as a RAS. To solve this problem, we have recently created a new BMV-based recombination system. It has been constructed in such a way that both tested RASes are placed in the same segment of the BMV genome (in the modified RNA3 molecule); therefore, we have called this system homomolecular. To prove the usefulness of the homomolecular system, we have employed it to examine the recombination activity of sequences derived from the hepatitis C virus (HCV) genome. The examined sequences have been inserted into RNA3 as direct or inverted repeats. This has demonstrated that the 101 nt hypervariable region of HCV efficiently supports both homologous and non-homologous crossovers, while the most conservative 98 nt portion of HCV's 3'-UTR induces only non-homologous recombination events. Moreover, a direct comparison of the hetero- and homomolecular systems revealed crucial differences between the mechanisms of homologous and non-homologous recombination. The former involves preferentially two different segments of the BMV genome and the latter occurs more easily between the same genomic RNAs.

Searching for general rules in RNA recombination

Retroviruses and bromoviruses belong to different systematic classes. In spite of obvious differences, the mechanisms of their replication share a few similarities, and it was suggested that they might evolve from common ancestor. Recently, we have shown that in BMV, like in retroviruses, recombination is indispensable for the effective virus replication. Consequently, we have attempted to determine if the same or different RNA structural motifs are capable of inducing recombination in bromoviruses and retroviruses. To this end, we have compared the recombination activity of HIV-1 reverse transcriptase (HIV-1 RT) and BMV RNA-dependent RNA polymerase (BMV RdRp) towards two viral sequences considered as homologous recombination hot-spots, namely HIV-1 region R and BMV region R. We have noticed that annealing and precise base-pairing between the nascent and acceptor strands are crucial for recombinant formation, and that the longer double strand structure is formed, the higher is the frequency of crossovers. Secondary structures present in the recombining molecules also influence the efficiency of crossovers. Relatively weak structures cause the replicase pausing and induce crossover, whereas more stable

ones although efficiently stop polymerase do not mediate crossovers because they prevent the annealing of the nascent and acceptor strands. Surprissingly, we have also found that sequence R coming from BMV more effectively induces template switching by HIV-1 RT than HIV-1-derived region R.

Two types of viral populations identified in children suffering from chronic hepatitis C

Hepatitis C virus (HCV) infections are considered a very serious world health problem: about 170 million people worldwide are infected. The first, acute stage of the disease is usually asymptomatic. However, just 15-30% of the infected eliminate the virus, while in the remaining patients chronic hepatitis C (CHC) is developed. After 10-30 years of CHC, cirrhosis occurs in 20-30% of patients, 5-10% of this group eventually suffering from hepatocellular carcinoma. Unfortunately, up till now no effective methods protecting against HCV or allowing for efficient CHC treatment have been elaborated. The basic cause of this situation is the lack of knowledge of the mechanism of CHC emergence and the factors affecting anti-HCV therapy. So far, no obvious correlations between the clinical parameters describing patients and the therapeutic outcome have been found.

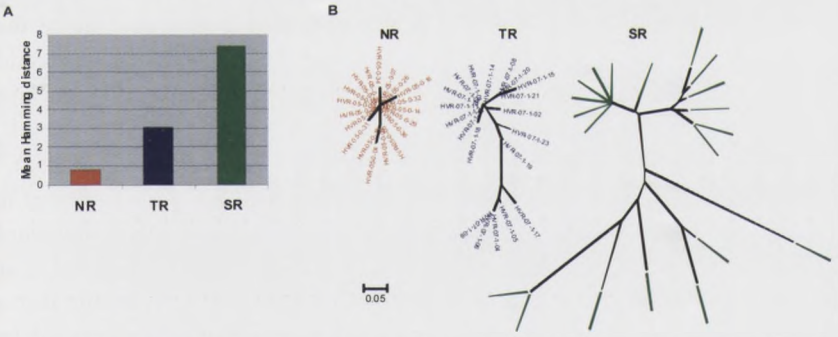

Figure 5. Two parameters mean Hamming distance (panel A) and phylogenetic tree (panel B) were proven to best reflect differences between the HCV populations isolated from patients with different response to antiviral treatment. Representative results obtained for nonresponding patients (red), patients with transient (blue) and sustain (green) response.

Recently, it has become increasingly clear that some specific features of the virus, especially its high genetic variability, might be responsible for the observed therapeutic problems. To verify the above hypothesis, we analyzed the changes occurring in the HCV populations isolated from children with CHC, all infected with HCV-1a and subjected to anti-HCV therapy. The structures of the viral quasispecies were established based on a 132 amino acid sequence derived from E2 protein – a portion including hypervariable region 1 (HVR1). As a result, two types of HCV populations were identified. The first type, found in patients who did not respond to therapy, contained a small number of closely related variants. The second type, characteristic for sustained responders, was composed of a large number of distantly associated equal rank variants. Phylogenetic analysis of nearly 300 HVR1 sequences showed that closely related variants are present in different nonresponding patients, suggesting that certain, still unidentified properties of the pathogen may be key factors determining the course of HCV infection and the result of treatment.

Identification of new HIV reverse transcriptase inhibitors

Despite the three decades of the extensive studies no effective methods of anti-HIV therapy have been developed. It was established that HIV needs only a few weeks or months to escape from immune response or to produce drug-resistant mutants. Assuming that detail biochemical studies of HIV-1 RT would help in searching of new inhibitors, we elaborated an effective system enabling production of recombinant HIV-1.

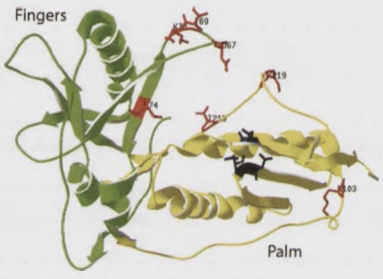

Figure 6. HIV-1 RT crystal structure. The location of the typical mutation observed in drug resistant variants is shown in red.

Based on this system, we produced a series of recombinant HIV-1 RTs resistant to commonly used anti-HIV drugs. Currently, they are used to determine anti-HIV activity of newly generated inhibitors. One of the tested options are DNA/RNA oligonucleotides. To find new, more effective oligonucleotide inhibitors of HIV RT, we looked for structural DNA motifs that are critical for HIV RT binding and affect at least one of its activities (polymerase, RNase and recombinase). To this end, the previously identified DNA aptamers influencing HIV RT were subjected to bioinformatic analysis. Based on the collected data, we designed new DNA oligonucleotides carrying the identified motifs. These potential HIV RT inhibitors were tested against polymerase activity (both on DNA and RNA templates), RNase H activity and recombinase activity. As a result, we identified several DNA aptamers which selectively inhibited RT polymerase activity without affecting its RNase activity. Additional experiments revealed that selected aptamers function both as competitors (they compete with other templates for RT binding) and as allosteric inhibitors. In general, two new DNA aptamers which efficiently inhibited HIV RT polymerase activity (over 95%) were found.

Single stranded DNA/RNA assisted template switching by HIV-1 RT

One of the main reasons for the ineffectiveness of anti-HIV therapy is the unusual genetic variability of RNA viruses and retroviruses such as HIV. Systematic analyses of HIV allowed researchers to identify two basic processes underlying such enormous genetic plasticity of this virus: error prone replication and RNA recombination. The enzyme that mediates both processes is a virion-associated protein, reverse transcriptase (HIV RT). Moreover, it has been demonstrated that recombination occurring via template switching by HIV RT is an essential part of a retroviral life cycle. Two obligatory, site-specific template switching events are required to produce proviral dsDNA, but additional crossovers can also occur along the whole genome. Thus, searching for driving forces of recombination seems to be of great importance for a better understanding of the molecular basis of HIV replication and production of new often drug resistant variants.

Earlier, we found that site-specific template switching by HIV RT frequently occurs when a local double-stranded structure formed by recombining molecules (an RNA donor and acceptor) is accompanied by specifically positioned short homologous sequences. A lack of the homologous regions in the donor and acceptor templates resulted in total inhibition of site-specific crossovers, even though the donor and acceptor templates were in close contact due to their hybridization. Our recent studies of universal motifs supporting template switching by HIV-1 RT (recombination hot spots) resulted in an elaboration of a new recombination system in which crossovers are supported by short 'helper' DNA or RNA, which is not a template for HIV RT, but selects proper donor and acceptor molecules and ensures their proximity. We have found that such a 'helper' can mediate precise transfer of HIV RT from the donor to the acceptor if both templates share short regions of homology. In our new system, one can produce DNA recombinants using RNA/RNA, RNA/DNA or DNA/DNA templates. In addition, one can assemble long DNA molecules from DNA/RNA fragments provided that the latter possess overlapping ends.

Virus-induced gene silencing

Viruses are obligatory parasites. It means that they cannot function without a host organism. To establish an infection, a virus has to overcome physical barriers, enter a plant cell and start to multiply genomic molecules. In RNA viruses, the latter process is catalyzed by an RNA-dependent RNA polymerase (the enzyme synthesizes RNA using RNA as a template). Consequently, the dsRNA replication intermediate, which triggers the RNA silencing pathway, is generated in the virus-infected cells.

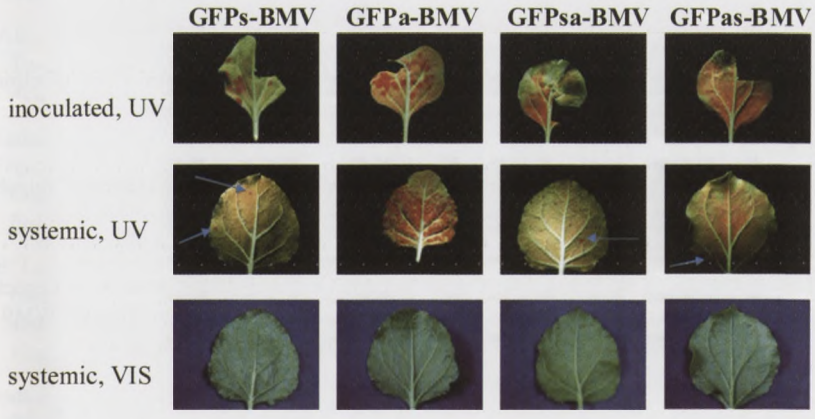

Figure 7. Application of BMV for gfp silencing in transgenic N. benthamiana. Transgenic N. benthamiana plants were inoculated with: GFPs-BMV (first column; gfp-derived fragment inserted in sense orientation), GFPa-BMV (second column; gfp-derived fragment inserted in antisense orientation), GFPsa-BMV (third column; gfp-derived sequence inserted in sense and antisense orientation) and GFPas-BMV (forth column; gfp-derived sequence inserted in antisense and sense orientation). First row: inoculated leaves – pictures taken under UV light. Second row: upper leaves (systemic infection) – pictures taken under UV light. Third row: upper leaves (the same as shown in the second row) – pictures taken under visible (VIS) light.

Most of the collected data indicate that the specific degradation of a virus genome occurs according to a similar scenario as RNAi or PTGS in animals and plants, respectively. The viral replication intermediate becomes a substrate for the RNase III-like enzyme (plant analog of Dicer), which digests dsRNA into small interfering RNAs (siRNA). Then, siRNA becomes part of an RNA-induced silencing complex (RISC) and targets it against viral genomic molecules (either against (-) or (+) RNA strands). This way, a plant can restrict or even eliminate viral infection.

Studies involving genetically modified viruses carrying host-derived exon sequences in their genomes (either the entire coding region or its fragment) revealed that RNA-based defense is capable of targeting both viral genomic molecules and host mRNA. If such a modified virus enters a plant cell, post-transcriptional silencing of a host gene is induced. Plant gene expression was also inhibited during infection with a virus bearing a promoter sequence in its genome. More detailed studies have demonstrated that, in this case, the host gene is silenced at the transcriptional level due to its promoter's methylation (RNA-directed DNA methylation). In general, these observations showed that modified viruses (RNA silencing-inducing viral vectors) could be used as effective tools to suppress host gene expression. Thus, a new technique called virus-induced gene silencing (VIGS) was developed.

Two series of BMV-based VIGS vectors are currently being tested in our laboratory. The first one was obtained by the duplication of the BMV RNA3 sub-genomic promoter just behind the coat protein-coding region. The second series was constructed on the basis of the BMV vector that we use to study the RNA recombination process.

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LABORATORY OF NUCLEOTIDE AND OLIGONUCLEOTIDE ANALOGUES

Head: ***Adam Kraszewski***

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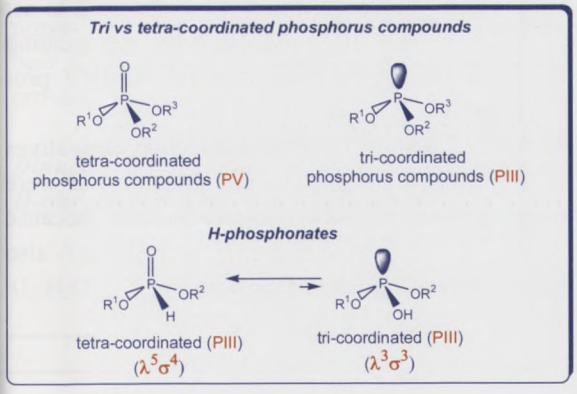
Research Support: *Marzena Szymczak*

Ph.D. Students: *Joanna Lutomska-Romanowska, Małgorzata Pietkiewicz*

Keywords: • *synthesis* • *H-phosphonate chemistry* • *nucleotides* • *oligonucleotides* • *analogues* • *antiviral* • *antisense oligonucleotides analogues* • *stereochemistry*

H-Phosphonates represent a class of phosphorus compounds with unique structure and chemical properties. Due to the presence of a phosphoryl group (P=O) and a tetrahedral structure, *H*-phosphonates bear strong resemble to phosphates (P^{V}), but the oxidation state (+3) clearly relates them to phosphites (P^{III}). Considering mechanisms of chemical transformations of *H*-phosphonates, in many cases these compounds behave similarly to phosphates, but at the same time they preserve characteristic features of phosphites. The above makes *H*-phosphonates exceptionally useful compounds in synthesis of phosphorus derivatives of natural compounds *e.g.* carbohydrates, nucleosides or oligonucleotides that are of high interest for chemical and biological studies. By investigating basic *H*-phosphonate chemistry, we were able to develop many efficient synthetic methods for the preparation of nucleotide analogues with P-N, P-S, P-Se and P-C modifications at the phosphorus centre. Biological activity of these compounds and some new analogues of nucleoside 2',3'-cyclic phosphates (*e.g.* phosphorothioate and phosphoroselenoate derivatives), have been evaluated in various *in vitro* systems containing thymidylate synthase or RNase A. Also, synthetic and enzymatic studies on new nucleotide analogues, dinucleoside phosphonate-phosphates have been initiated in our Laboratory. These compounds, bearing antiviral nucleoside moieties, can be considered as a novel type of potential antiviral and anticancer pro-drugs with tuneable pharmacokinetic and pharmacodynamic properties.

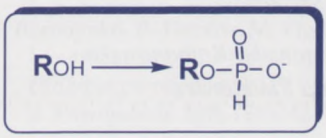


Current research activities:

- synthetic and mechanistic (including stereochemical) studies on nucleotide and oligonucleotide and their analogues.
- development of a new antiviral pro-drugs, antisense agents, *etc.*, based on modified nucleotides and oligonucleotides.

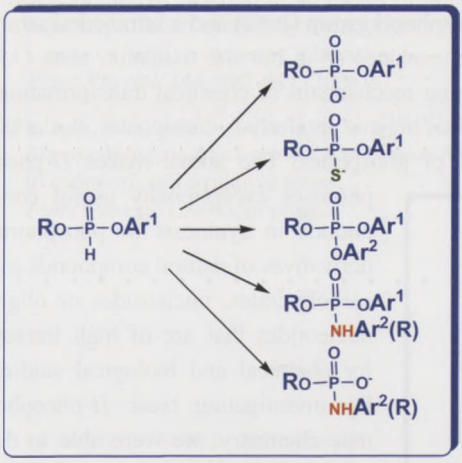
Major recent results:

Nucleoside H-phosphonates from nucleosides



We have developed a simple, efficient, and general method for synthesis of nucleoside *H*-phosphonates based on phosphonylation of suitably protected nucleoside by means of diphenyl *H*-phosphonate. Mild reaction conditions enable synthesis of nucleoside *H*-phosphonates both in deoxyribo and ribo series without the recourse to protecting groups in nucleobase moieties.

Anti-HIV pronucleotides based on aryl nucleoside H-phosphonates

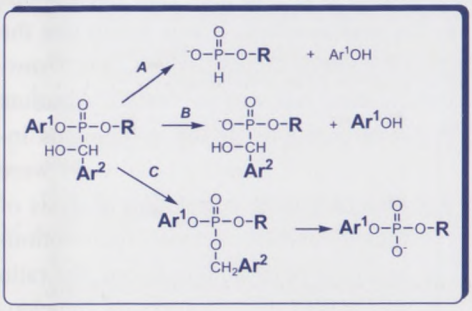


We have introduced to nucleic acids chemistry aryl nucleoside *H*-phosphonates which reactivity and other chemical properties were controlled by the use of variety of aryl group. These compounds could be converted into several types of nucleotides and their analogues, which were designed to act as an anti-HIV pronucleotides, representing of the second generation of anti-HIV pro-drug.

Several types of nucleotide derivatives disclosed high anti-HIV potency and were proved to act as pronucleotides because 2', 3'-dideoxyuridine derivatives also showed high anti-HIV activity.

Investigated pronucleotides were/are derivatives of:	Type of phosphate derivative investigated
2', 3'-dideoxyadenosine (ddA)	aryl phosphodiester
2', 3'-dideoxyinosine (ddI)	aryl phosphorothioate
2', 3'-dideoxyuridine (ddU)	di-aryl phosphotriester
3'-deoxy-3'-azidothymidine (AZT)	aryl phosphoramidate
	phosphoramidate

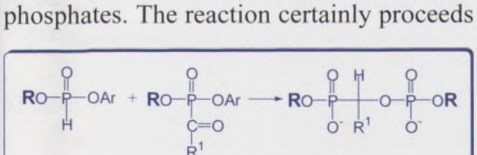
Aryl nucleoside α-hydroxyphosphonates a new type of anti-HIV pro-drug.



Aryl nucleoside α-hydroxyphosphonates are the products of addition of aromatic aldehydes to respective aryl nucleoside *H*-phosphonates. These compounds can decompose in several ways in dependence on phosphoester aryls and even in higher dependence on aryls of phosphonate hydroksymethylene part of molecule. In each case, they generate compound(s) which can be considered as a potential anti-HIV pro-drug.

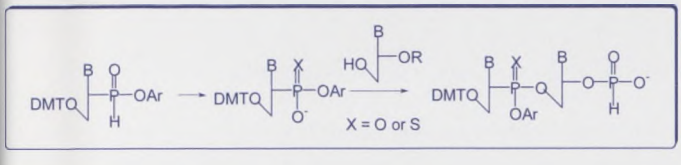
Dinucleoside phosphonates-phosphates – potential antiviral pro-drugs of second generation

Aryl nucleoside *H*-phosphonates and their P-acylated derivatives in the presence of tertiary bases undergo spontaneous condensation towards dinucleoside phosphonate-phosphates. The reaction certainly proceeds *via* respective *gem*-diphosphonate which in the case of aryl derivatives readily rearranges to stable, fully protected dinucleoside phosphonate-phosphates. After removal of phosphate and nucleoside protective groups, dinucleoside phosphonate-phosphates can be obtained. A possibility of introduction of nucleoside moieties with established antiviral activity (*e. g.* AZT and others) can make these compounds potential antiviral pro-drugs of second generation (pro-nucleotides) with tunable pharmacokinetic and pharmacodynamic properties as compared to parent nucleosides. Since dinucleoside phosphonates-phosphates bear phosphorylated nucleosides, they seem to be particularly good candidates as antiviral pro-drugs that may by-pass thymidylate kinase in TK^- cells.



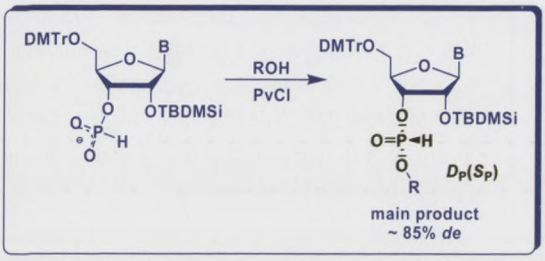
Dinucleoside phosphate and phosphorothioate building blocks from aryl nucleoside H-phosphonates.

Aryl nucleoside *H*-phosphonates are easily converted into aryl nucleoside phosphates (I_2/H_2O) or phosphorothioates (S_8) which after coupling with appropriate nucleoside unit and phosphonylation give respective dinucleoside 3'-5' phosphate or phosphorothioate 3'-*H*-phosphonate building blocks. These easily accessible synthetic intermediates permit, *inter alia*, preparation of the so-called chimeric oligonucleotide analogues in which selected modifications are placed only in pre-defined positions of an oligonucleotidic chain. At present, this is the main focus of our synthetic investigations aiming towards development of a new chemical method for a multigram scale preparation of oligonucleotide analogues for the purpose of an antisense therapy.



Stereoselective condensations of nucleoside H-phosphonates

We have identified the sources of stereoselectivity observed during the condensations of ribonucleoside 3'-*H*-phosphonates with nucleosides and alcohols. It was found that the investigated reactions owe their stereochemistry to a *Dynamic Kinetic Asymmetric Transformation* and the role of base, acid, and nucleophilic catalysis was evaluated. Absolute configurations of the compounds involved in the reaction pathways were assigned using correlation analysis of their ^{31}P NMR spectra. Upon optimization of reaction conditions, the ratio of $D_P(S_P)$:$L_P(R_P)$ isomers of dinucleoside *H*-phosphonates was increased from the initial 3:1 to ca. 9:1.



Concurrently, a comprehensive stereochemical D_P/L_P notation was devised as a convenient alternative to the R_P/S_P notation for stereochemical analysis of physical, chemical, and biological properties of nucleotide and oligonucleotide analogues bearing any type of tri- or tetra-coordinated phosphorus moiety.

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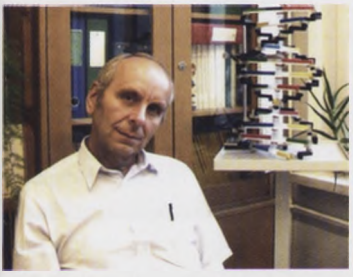
LABORATORY OF tRNA BIOCHEMISTRY

Head: ***Jan Barciszewski***

MOLECULAR BIOLOGY GROUP

Head: ***Jan Barciszewski***



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Research support:	*Iwona Gawrońska*
Ph.D. Students:	*Marta Gabryelska, Monika Nowak*

Keywords: • *catalytic RNAs* • *ribozymes* • *leadzyme* • *brain tumor* • *epigenetics* • *nonprotein coding RNA* • *high pressure*

Nucleic acids data bases: 5S rRNA and ncRNA

Ribosomal 5S RNA (5S rRNA) is an integral component of the large ribosomal subunit in all known organisms with the exception of only mitochondrial ribosomes of fungi and animals. It is thought to enhance protein synthesis by stabilization of a ribosome structure. The updated database of 5S rRNA and their genes (5S rDNA) was prepared. It contains 2280 primary structures of 5S rRNA and 5S rRNA genes. These include 536 eubacterial, 61 archaebacterial, 1611 eukaryotic and 72 organelle sequences. The database is available on-line through the World Wide Web at http://biobases.ibch.poznan.pl/5SData/.

The noncoding RNAs database is a collection of currently available sequence data on RNAs which have no protein-coding capacity and have been implicated in the regulation of cellular processes. RNAs included in the database form very heterogeneous group of molecules that act at different levels of information transmission in a cell. It includes RNAs which effect chromatin structure, influence transcriptional and translational regulation of gene expression, modulate protein function, and regulate subcellular distribution of RNAs and proteins. These RNAs with potential regulatory functions have been identified in prokaryotic, animal and plant cells.

The database can be accessed at http://biobases.ibch.poznan.pl/ncRNA/. Currently, the database includes over 30000 sequences from over 100 organisms a majority of which represent noncoding mammalian transcripts from the large scale cDNA sequencing projects. These sequences account for over 90% of the data. Certain groups of RNAs (microRNAs and snoRNAs), present in previous editions, were removed to avoid the redundancy with other specialized databases. Individual nucleotide sequence of ncRNAs can be retrieved in

FASTA format as separate entries or downloaded as batch files. The data can be searched using transcript names, accession numbers or organism names. In addition to the direct access to the database records, search results are also linked to full GenBank entries. In the current version of the database, we included BLAST server which allows to perform sequence similarity analysis using the full ncRNA database (approximately 64 Mb).

Identification of trans-antisense RNAs (ncRNAs)

Although thousands of noncoding RNAs have been discovered in the last years, for most of them functions remain unknown. Recent efforts have been mostly concentrated on the well defined RNA families, like snoRNAs or microRNAs. However, one of the most obvious ways of RNA actions – antisense mechanism – remains untouched.

The analysis of the human ncRNAs with unknown function revealed that 118 of them can interact with different mRNAs. We have identified more than 1100 *trans*-antisense ncRNAs. One of the most interesting findings was the fact that among the proteins expression of which can be regulated by antisense RNA, the majority fulfilled the regulatory tasks in the cell. This shows that the RNA-dependent regulatory signals can be multiplied at protein level. Currently, we are implementing our method in the worldwide available web service – hybridBLAST.

Molecular basis for kinetin-dependent missplicing reversal in Familial Dysautonomia

Familial Dysautonomia is a disease caused by a single nucleotide mutation located at 5' splice site of exon 20 of IKBKAP (I-κ-B kinase associated protein) mRNA. It results in increased rate of exon 20 skipping. Since IKBKAP plays important role in activation of transcription factors any deregulation of its expression leads to severe developmental defects (diseases).

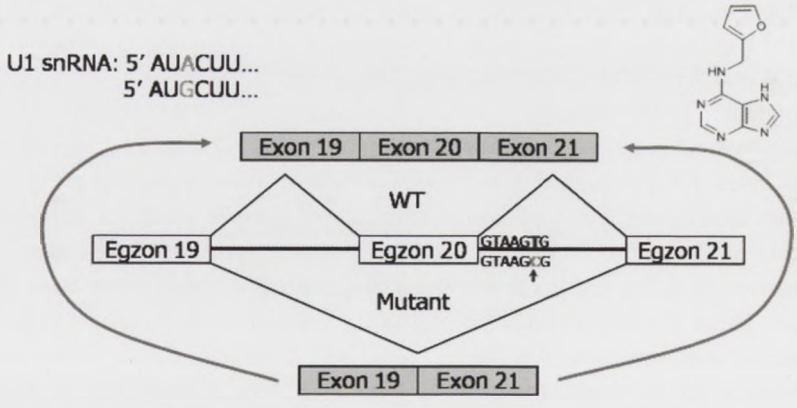

Figure 1. The mechanisms of IKBKAP missplicing reversal. Two methods have been indicated – by compensatory mutation in U1 snRNA (left) or by kinetin (right).

The correct IKBKAP splicing pattern can be re-established *in vitro* in two distinct ways: by introduction of the compensatory mutation in U1 snRNA, which restores the origi-

nal base pairing pattern with the splice site and by application of kinetin (6-furfuryladenine) – a plant hormone (cytokinin). The exact mechanism of kinetin action remains unknown.

We have applied bioinformatics tools, such as molecular dynamics and tertiary structure modeling to propose a molecular mechanism of kinetin action. The results of calculation have been verified experimentally. It seems, that kinetin have a great potential in therapy of splicing aberrations.

A search for cytokinin receptors

Crystal structure of the complexes of mistletoe lectin I with phloretamide

The X-ray structure of the complex of the European mistletoe lectin I (*Viscum album*, ML-I) with the plant growth hormone, 3-(p-hydroxyphenyl)-propionic acid amide (phloretamide, PA) has revealed the binding of PA in so far not described hydrophobic cavity located between the two subunits of the protein. No such cavity is observed in related lectins from non-parasitic plants. The binding of PA is achieved through interactions with Val228A, Leu230A, Arg388B and the C-terminal Pro510B. It is conceivable that binding of PA to ML-I is a part of defence mechanism of the parasite against the host, whereby the parasite prevents the growth hormone of the host from interfering in its own regulatory system.

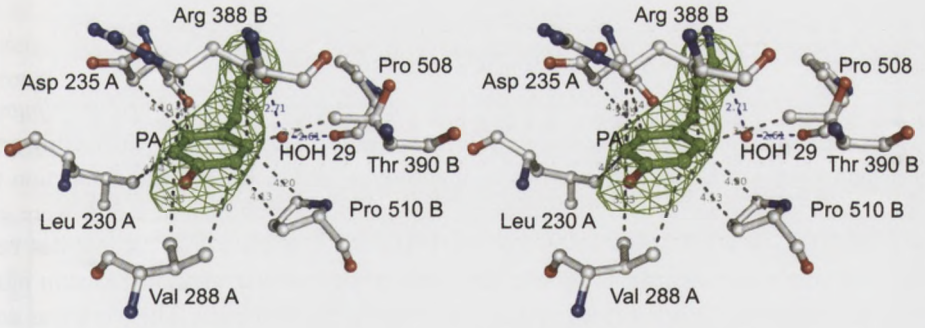

Figure 2.

The current finding about the specific binding of PA to ML-I clearly indicates that hetrodimeric RIPs (ribosome inactivating proteins) are multifunctional proteins whose functions in the cell have not yet been fully recognized and analysed. The work was done together with C. Betzel, V. Erdmann and W. Rypniewski (*Meyer et al., 2007*).

Crystal structure of the complexes of mistletoe lectin I with zeatin

The crystal structure of ML-I in complex with zeatin has been refined to a final resolution of 2.57 Å. X-ray diffracting crystals of ML-I were obtained with the counter diffusion method using the Gel Tube R Crystallization Kit (GT-R) on board the Russian Service Module on the international space station ISS at the Japanese aerospace exploration agency (JAXA) GCF flight number 6. Hexagonal bipyramidal crystals were grown during three months under microgravity conditions. Before data collection, crystals were soaked with saturated solution of zeatin. Diffraction data to 2.57 Å were collected applying synchrotron

radiation and cryo-techniques. The structure was refined subsequently to investigate whether a binding has taken place in a pocket close to the C-terminus of the ML-I B-subunit with an ability to bind a plant hormone. A clear F_o-F_c signal has been found and interpreted as a single trans-zeatin molecule at the hormone binding site of ML-I. The work was done together with C. Betzel, V. Erdmann and W. Rypniewski (*Meyer et al. 2008, submitted*).

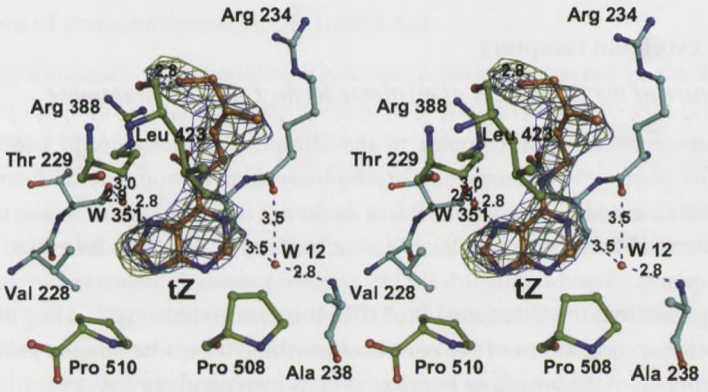

Figure 3.

Crystal structure of the complexes of mistletoe lectin I with kinetin

The crystal structure of mistletoe lectin I of the European mistletoe *Viscum album* in complex with kinetin has been refined to a final resolution of 2.5 Å. X-ray diffracting crystals of ML-I were obtained similarly as in the case of zeatin. Before data collection the crystals were soaked in saturated solution of kinetin. Diffraction data to 2.5 Å were collected applying synchrotron radiation and cryotechniques. A clear F_o-F_c signal has been found and interpreted as a single kinetin molecule as well as two additional kinetin molecules in a hydrophobic pocket close to the C-terminus of the B-subunit. The work was done together with C. Betzel, V. Erdmann and W. Rypniewski (*Meyer et al. 2008, submitted*).

Human brain tumor therapy through interference in RNA intervention (iRNAi)

Astrocytic tumors are the most frequent primary brain neoplasias affecting children and adults. They represent a heterogeneous group of tumors that may differ in their localization, clinical findings, proliferation rate, invasive potential and tendency to progression. On the basis of clinicopathological criteria, they are classified into pilocytic astrocytomas (WHO I) with slow growth and rarely undergoing malignant transformation and three groups of diffusely infiltrative: astrocytomas comprising diffuse astrocytomas (WHO II), anaplastic astrocytomas (WHO III) and glioblastoma (WHO IV). Glioblastoma multiforme (GBM), the most devastating primary human brain tumor is a rapidly growing malignant astrocytic tumor with a high morbidity and mortality. The current management of GBM is based on cytoreduction through surgery, radiotherapy and chemotherapy. Patients with glioblastoma invariably fail to achieve long-term survival time, which is thought to be a result of acquired resistance to chemotherapeutics or radiation. Despite that broad ap-

proach to treatment, median patient survival is approximately 6-8 months although about 40% of patients with GBM die within 6 months after the diagnosis. A mechanism of glioma cell invasion is based on the attachment of tumor cells to extracellular matrix, its degradation and subsequent penetration into adjacent brain structures.

It is known that tenascin-C (TN-C), the extracellular matrix protein, is highly expressed in tumor tissue of the majority of malignant tumors including brain tumor. TN-C commonly elevated in high-grade gliomas increases the invasiveness of glioma cells. It is the dominant epitope of glioblastoma multiforme. GBM represents an excellent target for RNA interference (RNAi) because these cells are among a few rapidly proliferating ones in CNS and only microglia and endothelial cells have the ability to divide.

The key therapeutic advantage of using RNA interference lies in its ability to specifically and efficiently knock down the expression of disease-causing genes of known sequence.

The unique feature of RNAi is its exquisite sequence specificity, which enables the use of RNAi to selectively knock-down expression of alleles carrying point mutations, insertions or deletions, and thereby to produce cells with reduced expression of essential genes.

RNAi relies on a multistep intracellular pathway which can be roughly divided into two phases. In the first one, endogenous or exogenous dsRNA molecules present within the cell are processed through the cleavage activity of RNase III-type activity (Dicer) into short 20-30 nucleotide fragments called siRNAs. In the second step, siRNAs as well as many proteins including nucleases and helicase, form RNA-induced silencing complex (RISC). Through unwinding of double stranded siRNA, the complex becomes activated with single–stranded, noncoding siRNA which guides the RISC complex to its complementary target RNA causing its endonucleolytic cleavage.

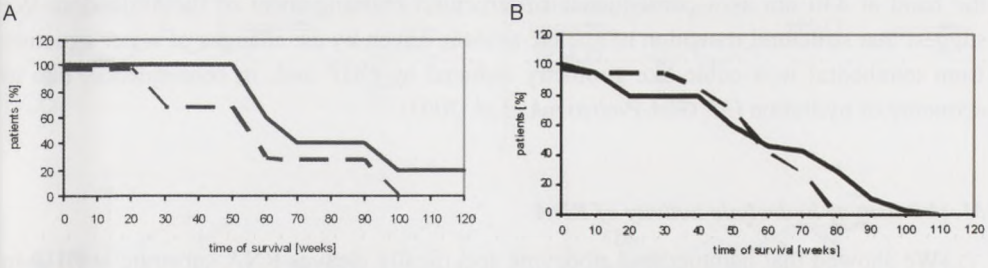

Figure 4. Differences in overall survival time of the patients with astrocytoma WHO Grade III (A) and glioblastoma multiforme WHO Grade IV (B) treated with ATN-RNA. Primary tumor (continuous line), recurrent tumor (dashed line).

We developed a novel strategy of brain tumor therapy based on RNAi which when applied during surgery inhibits neoplasia cells through a specific down-regulation of tenascin-C. We used double stranded (dsRNAs) interfering RNAs (ATN-RNA), having a complementary sequence to mRNA of tenascin-C, to reduce the protein expression in brain tumor cells. RNAi was injected into postoperative area of 46 patients. The follow-up study with MRI and CT clearly showed increased survival (Fig. 4) and better quality of life (QOL) of the treated patients. This novel therapy shows big therapeutic potential (Fig. 5). We called the technology an intervention with RNAi (iRNAi). The results of the treatment of the first 11 patients with ATN-RNA have been recently published (*Zukiel et al. 2006; Nowak et al. 2008, submitted*).

NUCLEIC ACIDS GROUP

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Ph.D. Students: *Mirosława Skupińska, Beata Plitta, Marek Żywicki*

Keywords: • *brain tumor* • *DNA methylation* • *catalytic RNAs* • *leadzyme* • *epigenetics* • *noncoding RNA*

Inhibition of tobacco mosaic virus infection in Nicotiana tabacum with leadzyme

We developed a new method for inhibition of Tobacco mosaic virus (TMV) infection in tobacco plants based on specific RNA hydrolysis induced by a leadzyme. The leadzyme has been identified as a small RNA motif consisting of six- or five-nucleotide-long asymmetric purine-rich loop within RNA duplex. Such a motif is capable of RNA cleavage in the presence of Pb^{2+} ion. We identified a leadzyme target sequence in genomic TMV RNA and designed a 16-mer oligoribonucleotide which forms a specific leadzyme motif having five-nucleotide catalytic loop. The synthetic 16-mers RNA was applied together with non-toxic, catalytic amount of lead to infected tobacco leaves. We observed inhibition of TMV infection in tobacco leaves due to specific TMV RNA cleavage effected by a leadzyme.

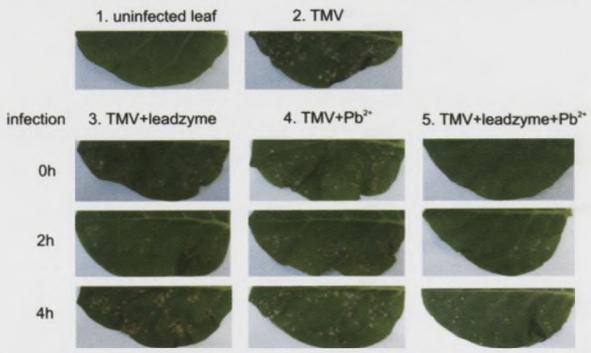

Figure 8. Effect of leadzyme on TMV expression in Nicotiana tabacum leaves in a time-dependent experiment. Catalytic RNA (leadzyme) and/or Pb^{2+} were applied together with TMV at 0h, 2h or 4h after TMV infection. Leadzyme at concentration 2.5 μM, Pb^{2+} at 5 μM and TMV at 5 μg/ml were used for inoculation. Inhibition of TMV infection with the leadzyme in a presence of Pb^{2+} was manifested as a decreased number of local lesions.

The optimal activity of the leadzyme *in vivo* was observed at TMV/Pb^{2+}/RNA co-inoculation and at 2-fold Pb^{2+} excess over the catalytic RNA. Significant reduction of TMV was observed even when the leadzyme was applied up to two hours after inoculation of leaves with TMV. This was manifested by decreased number of local lesions on infected tobacco leaves treated with catalytic RNA and Pb^{2+}.

Both macroscopic observation of tobacco leaves and RT-PCR analysis confirmed effectiveness of exogenously applied ssRNA for inhibition of viral expression. This process called leadzyme interference is determined by specific recognition and cleavage of target site by RNA catalytic strand in the presence of Pb^{2+} (*Wyszko et al. 2006*).

5S rRNA as Pb-zyme

We have shown that D-loop of 5S rRNA can function as a leadzyme. It consists of D loop nucleotide sequence (3')GCCAGAGGGUG(5') of human, calf, and rat liver 5S rRNA that binds an oligoribonucleotide (5')CGGUCGAGCCAU(3') in a very specific way, and hydrolyzes it *in trans* at C-G but not G-G or U-G linkages. The leadzyme has an asymmetric pentanucleotide purine rich loop flanked with two short double-stranded RNAs. Mammalian 5S rRNA, to our knowledge, is the first native leadzyme working *in trans,* in addition to yeast $tRNA^{Phe}$ that is the *cis* ribozyme.

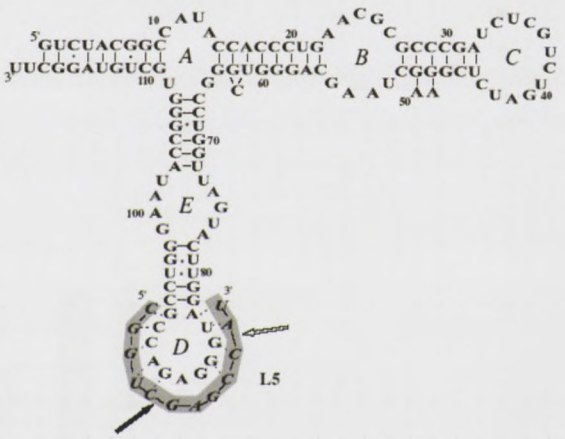

Figure 9. Model of secondary structure of calf liver 5S rRNA (leadzyme) with L5 oligonucleotide complex. Heavy arrow indicates specific cleavage with leadzyme, open arrow – non specific cleavage.

Diagnosis and classification of different tumors

A new, simple and reliable method for diagnosis of various tumors is described. It is based on a thin layer chromatography (TLC) quantitative determination of 5-methylcytosine (m^5C) in relation to its damage products of DNA from a tumor tissue. Currently, there is strong evidence that oxidative stress through reactive oxygen species (ROS) plays an important role in the etiology and progression of a number of human diseases. Oxidative damage of DNA, lipids and proteins is deleterious for the cell. 5-Methylcytosine, along with other basic components of DNA is the target for ROS, which results in the appearance of new modified nucleic acid bases. If so, an m^5C residue constitutes mutational hotspot position, whether it occurs within a nucleotide sequence of a structural gene or in a regulatory region. We have analyzed 82 DNA samples taken from brain tumor tissues. DNA was isolated and hydrolyzed into nucleotides which, after labelling with [γ-^{32}P]ATP, were separated on TLC. The chromatograms were evaluated using phosphoimager and the amounts of m^5dC were calculated as a ratio (R) of [$m^5dC/m^5dC+dC+dT$]100 spots intensities. The R

value could not only be a good diagnostic marker for brain tumors, but also a factor differentiating low and high grade gliomas. Therefore, a DNA methylation pattern might be a useful tool for primary diagnosis of brain tumor or as a marker for the early detection of the relapse of a disease. This method has several advantages over those existing nowadays.

Breast and colon cancers are among the leading causes of cancer-related deaths in population of Europe and North America. Early detection of these malignancies could improve the chances for successful treatment and recovery. Due to its chemical nature and biochemical role in the cell, 5-methylcytosine (m^5C) can be a sensitive marker of progress of the tumor formation induced by the oxidative damage reactions. The analysis of global m^5C content in DNA can show neoplasia in the different light. The aim was to analyze the genomic DNA cytosine methylation status from tissues of patients with breast and colon cancers. We have analyzed the amount of m^5C in DNA of 26 patients. The material for biochemical analysis was collected with thick-needle biopsy from postoperative preparations. For the group with breast carcinoma, owing to many indications, 7 patients underwent neoadjuvant therapy with different sets of chemotherapeutics. Two-dimensional thin layer chromatography (TLC) has been used to monitor 5-methylcytosine level in RNA free DNA extracted from breast and colon cancer tissues.

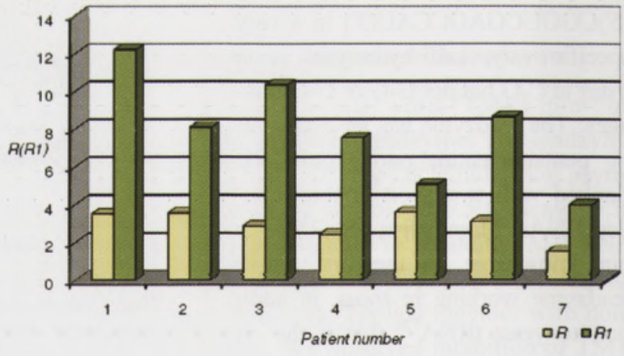

Figure 10. Diagram of DNA methylation (m^5C) status in tissues from patients with breast cancer and preoperative chemotherapy.

The methylation of cytosine at C-5 position in DNA from breast cancer patients correlates well with the malignancy of tumors. It reflects the stage of disease and can be used as a good method for diagnostics of these tumors. Interestingly, a very high amount of m^5C in DNA for the breast cancer patients treated with different chemotherapeutics, which are not methylating compounds, suggests an activation of DNA methyltransferase and a genomic suppression of the DNA repair genes expression. These differences clearly reflect the health conditions of the patients. We have noticed that the R coefficient is better than $R_1=[m^5C/m^5C+C]$ in diagnosis of these neoplasias. The quantitative global analysis of m^5C in DNA can be used as a good marker for diagnosing neoplasia in clinical practice (*Barciszewska et al. 2007*).

DNA methylation in aging research

A TLC-based fast and simple method for quantitative determination of total genomic levels of m^5C in DNA can be applicable to aging research with respect to rapid and high throughput screening and comparison. The analysis of global alternations of m^5C in serially

passaged human skin fibroblasts shows age-related global hypomethylation during cellular aging *in vitro*. This method can be useful for screening potential modulators of aging at the level of epigenetic alterations (*Barciszewska et al. 2007*).

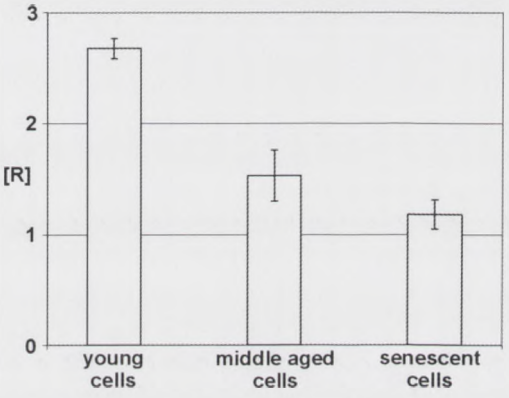

Figure 11. A comparison of amount of m^5C in DNA samples from 3 age groups of serially passaged human skin fibroblasts: young (less than 15% lifespan completed), middle aged (between 50 and 80% lifespan completed) and senescent (more than 98% lifespan completed). R is the coefficient, which is equal to the ratio of $[m^5C / m^5C+C+T] \times 100$.

Structure of DsrA RNA

Although the non-protein-coding part of the bacterial genome is relatively small (ca 25%), some ncRNAs capable of posttranscriptional gene silencing have been found. One of the most extensively studied bacterial small ncRNAs is DsrA RNA. It is an 87-nucleotide regulatory noncoding RNA, a key component of coldshock response machinery in *Escherichia coli* which acts by binding two mRNAs, thus controlling translation of two major regulatory proteins: RpoS: global transcription activator and its antagonist – H-NS. Two different secondary structures of DsrA RNA have been proposed. Both of them show three hairpins, but the difference concerns the location and structure of the second hairpin loop which binds H-NS mRNA, as well as Hfq – ubiquitous and unspecific RNA binding.

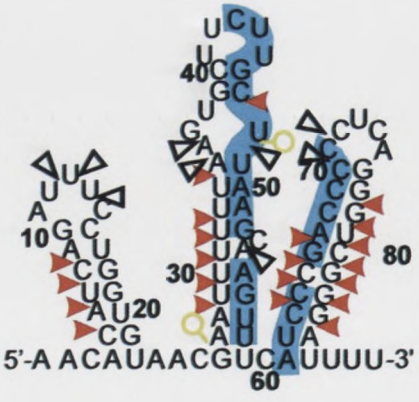

Figure 12. New secondary structure of DsrA RNA. V1 hydrolysis – red arrows, S1 – white arrows, RNaseH – yellow square, regions protected by the Hfq from the hydroxyl radicals ana lead- marked.

The complementary sequences in hairpin loops are crucial for an increased rate of complex association and propagation. Our structural data obtained with RNase H, DNAzyme, hydroxyl radicals and lead-induced hydrolysis, enable us to verify the existing models and propose the new one. It differs mostly on the second hairpin which has a dynamic structure

interacting with protein and target RNAs. These results bring new insights into molecular mechanism of DsrA RNA action (*Rolle et al. 2006*).

Aminoacyl-tRNA synthetases: target for new anti-infectives

Aminoacyl-transfer RNA synthetases (AARS) catalyze the attachment of the amino acid to its corresponding tRNA during translation. This reaction occurs in two steps: the formation of aminoacyladentylate which is bound to the enzyme and transfer of activated amino acid on 3'-terminal adenosine of tRNA. These enzymes are universal and essential for protein biosynthesis and cell viability.

Increasing rates of bacterial resistance and slow progress in new antibiotic development have led to a progress of infectious diseases of humans. The key of its usefulness lies in an ability to find a drug that inhibit a pathogen AARS but not its human counterpart. These targets have been validated in nature as AARS inhibition and have been shown as the specific mode of action for many natural antimicrobial agents synthesized by bacteria and fungi. Therefore, AARS have the potential to be the target for novel anti-infectives from natural sources or synthetic.

By the screening and analysis of natural compounds, we have identified several potential candidates as inhibitors of bacterial aminoacylation e.g. EGCG (epigallocatechin gallate, $IC_{50} = 4{,}6 \times 10^{-6}$, $Ki \approx 5 \times 10^{-6}$ M), morin and sanguinine.

PROTEIN BIOSYNTHESIS GROUP

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Ph.D. Students:	*Marta Sikora, Anna Kietrys, Aleksandra Szopa, Joanna Suszyńska, Dorota Gurda*

Keywords: • *regulation of translation – plant system* • *anti-sense oligonucleotides* • *natural products* • *homocysteine* • *N-homocyste-inylation of protein* • *aptamers* • *biotechnology legislation* • *biosafety* • *public perception*

Introduction

The structure of nucleic acids plays a critical role in functioning of biosynthesis machinery. Our projects focus on the correlation between the mechanism of action, function and structure of different biomolecules during protein synthesis process.

Ribosome for thought

The interaction and conformational relationships between rRNAs and ribosomal proteins are responsible for the ribosome activity. We tested 9 different antisense oligodeoxynucleotides complementary to the selected, highly conserved sequences of *Lupinus luteus* 26S rRNA which are engaged in the interactions with tRNA molecules. The ribosomes were converted either to pre- or to post-translocational states, with or without pre-hybridized oligonucleotides, using tRNA or mini-tRNA molecules (Fig. 13). The activity of those ribosomes was tested *via* the so-called binding assay.

We showed that:

- before and after translocation, fragments of domain V between helices H70/H71 and H74/H89 don't have to interact with nucleotides 72-76 of the acceptor arm of A-site tRNA;
- helix H69 doesn't have to interact with DHU arm of tRNA in positions 25 and 26 after forming the peptide bond, but before translocation;
- helices H69 and H70 interact weakly with nucleotides 11, 12, 25 and 26 of A-site tRNA before forming a peptide bond in the ribosome;
- interactions between helices H80, H93 and single stranded region between helices H92 and H93 and CCA-end of P-site tRNA are necessary at all steps of elongation cycle;
- before and after translocation, helix H89 doesn't have to interact with nucleotides in positions 64-65 and 50-53 of A-site tRNA TΨC arm.

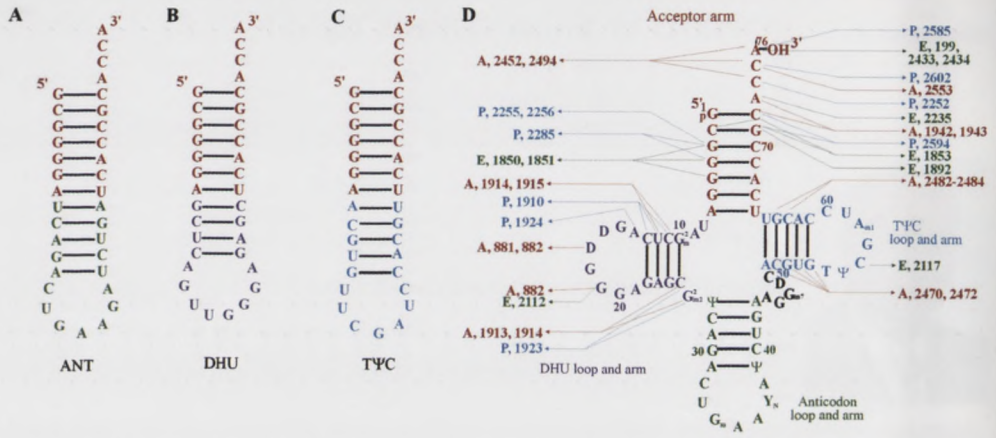

Figure 13. Secondary structures of mini-tRNA molecules (A-C) and tRNA^{Phe} (D); the interactions with 26S rRNA are shown in different colours for A-, P- and E-site tRNAs.

We observed well defined structural changes of ribosome's conformation during different steps of the elongation cycle of protein biosynthesis (Fig. 14).

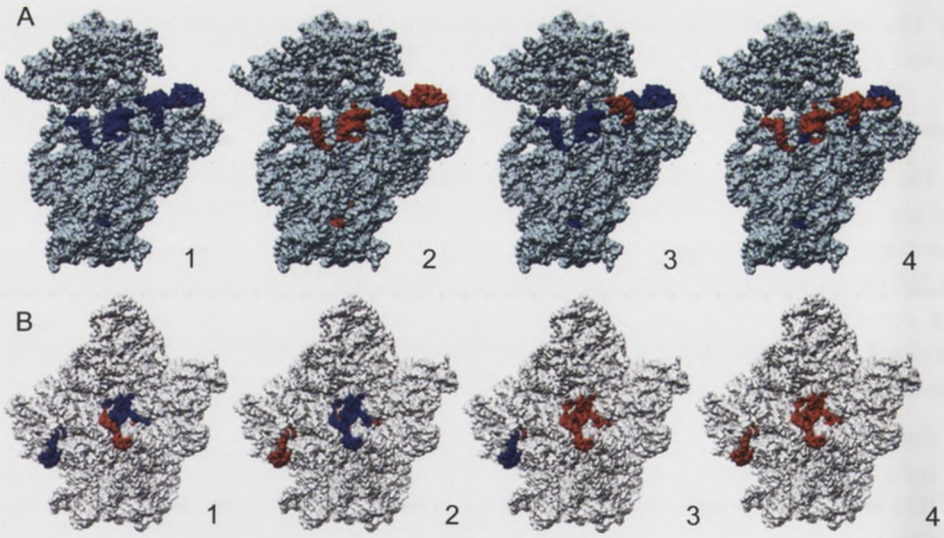

Figure 14. Conformational changes in the small (A) and large (B) ribosomal subunits during the course of protein synthesis.

Numbers correspond to four main translocational states before (1,2) and after (3,4) translocation step of polypeptide synthesis. Colours indicate rRNA tendency to open (red) or close (blue) the ribosome structure.

The structure of the ribosome is more "open" when ribosomal A and P sites are occupied in pretranslocational state. On the contrary, we observed "closed" conformation at posttranslocational state with P site occupied. We have concluded that the oscillation of the ribosome between these two main states during the elongation cycle causes the exposition

of individual functionally important sites of small ribosomal RNA. This allows for highly coordinated cooperation between ribosomal domains during the protein biosynthesis.

Selection of RNA aptamers and their application for testing of biological functions of HIV-1 RT and Dicer

SELEX (*Systematic Evolution of Ligands by Exponential Enrichment*) is a method of identification of high affinity aptamers for a surprising variety of molecular targets, including nucleic acid binding proteins, non-nucleic acid binding proteins, as well as small organic molecules. This procedure is the way for finding new drugs that specifically and effectively block viral replication. We used the SELEX method to obtain oligoribo-nucleotides that specifically inhibit the RNase H activity of HIV-1 RT. We also showed that the previously selected aptamers against HIV-1 RT may not inhibit different variants of the same viral protein. Human ribonuclease Dicer is our second target using the SELEX procedure. Dicer processes dsRNA into siRNA and miRNA which direct an RNA-induced silencing complex (RISC) to cleave mRNA or block its translation. Dicer homologues are found in many organisms like *D. melanogaster*, *C. elegans*, *A. thaliana*, yeast, mouse and humans. Until now the regulation of its function is unknown. We show that Dicer could be regulated by short uncleaved dsRNA (around 60 nt).

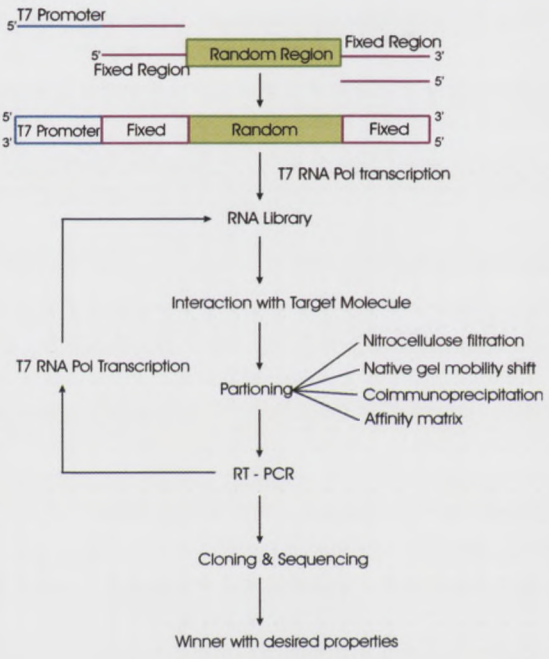

Figure 15. The SEXEX procedure.

Anti-Nε-Hcy-Lys-protein antibodies

N-Homocysteinylated proteins are structurally different, comparing with native proteins, and are thus likely to be recognized as neoself antigens and induce an autoimmune response.

We developed the new method for obtaining and purification of anti-*N*ε-Hcy-Lys-protein antibodies. Previous studies suggest that anti-*N*ε-Hcy-Lys-protein antibodies recognize *N*ε-Hcy-Lys epitope on *N*-homocysteinylated proteins. To prepare affinity matrix for selective binding of anti-*N*ε-Hcy-Lys-protein antibodies, we have modified *N*ω-aminohexyl-Agarose with Hcy-thiolactone, which resulted in the attachment of Hcy via its carboxyl group to amino group of *N*ω-aminohexyl residue, yielding *N*ω-Hcy-aminohexyl-Agarose.

The immune serum was obtained from rabbits inoculated with *N*ε-Hcy-Lys-keyhole limpet hemocyanine and IgG fraction prepared by chromatography on protein A-Agarose.

The anti-*N*ε-Hcy-Lys-protein IgG was adsorbed on *N*ω-homocysteinyl-aminohexyl-Agarose column at pH 8.6 and eluted with a pH 2.3 buffer. The enzyme-linked immunosorbent assays demonstrate that the antibody recognizes specifically *N*-homocysteinylated variants of hemoglobin, albumin, transferrin, and antitrypsin. Anti-*N*ε-Hcy-Lys-protein antibodies may be an essential tool for identification of *N*ε-Hcy Lys-protein in biological samples, including human serum, and may facilitate the studies of the role of *N*ε-Hcy Lys-protein in human disease.

The identification of the sites of posttranslational Hcy incorporation in *N*-Hcy-albumin provided us with the hypothesis that *N*-homocysteinylation affects physiological function of albumin. We will examine the effects of *N*-homocysteinylation on proteolytic turnover of albumin in human cells.

Analysis of sites susceptible to N-homocysteinylation in human blood proteins

Clinical studies have shown that elevated plasma total Hcy is associated with an increased risk of the development of vascular and neurological diseases. An underlying mechanism of Hcy pathogenicity may involve Hcy incorporation into protein, which occurs in the human body. The pathway involves metabolic conversion of Hcy to Hcy-thiolactone catalyzed by methionyl-tRNA synthetase during protein biosynthesis. Hcy-thiolactone is chemically reactive and forms adducts with protein, *N*-Hcy-protein, in which the carboxyl group of Hcy is linked by an amide bond to ε-amino group of a protein lysine residue. This reaction impairs protein structure and function. Major pathophysiological consequences of protein N-homocysteiny-lation include induction of anti-*N*-Hcy-protein auto-antibodies andthrombogenesis in humans, which contribute to atherosclerosis in humans. The bulk of Hcy circulating in human blood is N-linked to hemoglobin and albumin. Lys525 is a predominant site of N-homocysteinylation in human serum albumin *in vitro* and *in vivo*.

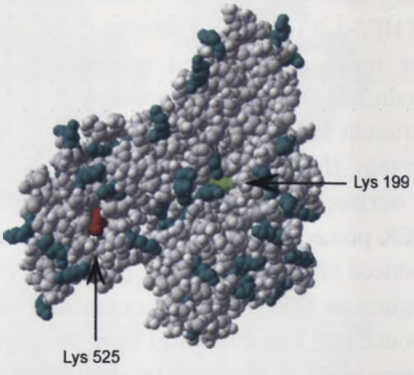

Figure 16. The structure of human albumin. The green color indicates lysine residues, the red Lys 525 as predominant site of N-homocysteinylation and the light green N-Hcy-Lys 199.

We analyzed native and Hcy-thiolactone-modified human serum proteins by proteomic approaches. Protein samples were digested with trypsin; the resulting peptides were purified by HPLC and subjected to MALDI-TOF mass spectrometric analyses. We found that Lys4, Lys12, Lys137, Lys159, and Lys99 are sites for the modification of human serum albumin by Hcy-thiolactone. Two sites in hemoglobin susceptible to the modification of Hcy-thiolactone, βLys18 and αLys16, were also identified. Furthermore, we have found that a peptide containing *N*-Hcy-Lys525 is easily detected in MALDI-TOF mass spectrum

of a tryptic digest of human serum proteins. Based on this finding, we have developed a method that allows direct monitoring of *N*-Hcy-albumin in human serum.

Legislation of biotechnology

We are also engaged in the problems of legal and social aspects of biotechnology (bioinformation, intellectual property, legislation and public perception of biotechnology), as well as in general analysis of Polish botechnology. Professor Tomasz Twardowski was a delegate of Poland in OECD and UNEP, he is a President of Polish Federation of Biotechnology. He is also a member of advisory bodies for Government and Parliament. Our activities include publishing the Polish journal dedicated to biotechnology "BIOTECHNOLOGIA" (editor-in-chief: T. Twardowski), under the auspices of the Polish Biotechnology Committee.

Figure 17. Quarterly "Biotechnologia" in 2007.

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LABORATORY OF CANCER GENETICS

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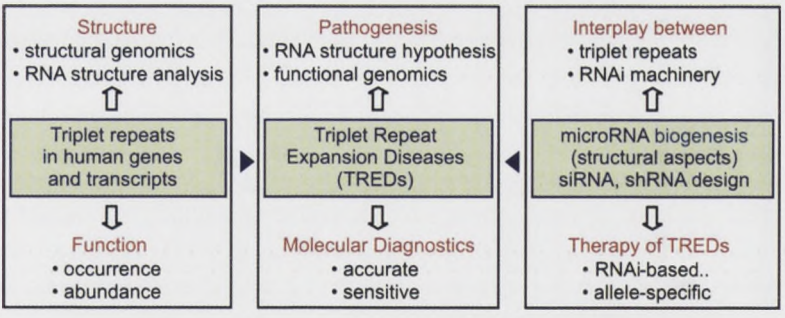
Keywords: • *mutation detection technology* • *repeat expansion diseases* • *microRNA biogenesis* • *RNA interference technology* • *RNAi of triplet repeats*

The research conducted in this laboratory continues to be medically-oriented, but its focus has changed in recent years from cancer genetics to various aspects of human hereditary neurological disorders known as Triplet Repeat Expansion Diseases (TREDs).



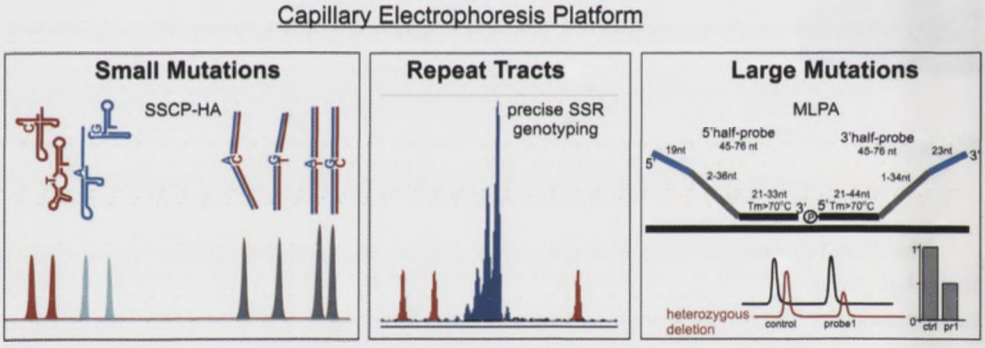
Our goal is to define the role of mutant transcript in the pathogenesis of these diseases. To better understand the RNA-mediated pathomechanisms, we learn about normal functions of triplet repeats in transcripts by analyzing their occurrence, abundance and structures. Our another major objective is to develop causative therapies for TREDs by taking advantage of RNA interference technology. We investigate the RNA interference of repetitive sequences and intend to achieve discrimination between mutant and normal transcripts using various approaches. To use the RNAi technology effectively, we study how this process occurs naturally with microRNAs. We also continue our efforts to improve DNA genotyping and mutation detection methods.

Current research activities and major results

More efficient mutation detection and genotyping methods

Various sequence alterations of human genome include small base substitutions, insertions and deletions, repeat number polymorphisms of mini- and microsatellites, and copy number variations (CNV). Their analysis remains an indispensable part of many research projects in molecular genetics, and reliable DNA-based diagnostics is becoming an important element of modern medical practice.

Among the various techniques developed for small mutation detection, single-strand conformation polymorphism (SSCP) and heteroduplex analysis (HA) are widely used owing to their simplicity, high sensitivity and low cost. The SSCP detects base changes in sin-



gle-stranded DNA, whereas HA does the same in double-stranded DNA when subjected to electrophoresis in non-denaturing conditions. Recently, we have characterized and optimized experimental conditions for the combined SSCP and HA by capillary electrophoresis – the method developed earlier in our laboratory. We also identified two major structural factors responsible for the reduced mutation detection rate in long amplicons. The first one is increased contribution from other secondary structure domains in longer DNA fragments, which mask the structural change induced by the mutation. The second factor is the higher number of SNPs including common polymorphisms present in longer fragments. In another study, we have established the capillary electrophoresis conditions allowing for rapid mutation detection by heteroduplex analysis.

We have developed a capillary electrophoresis method for a more accurate and sensitive analysis of triplet repeat expansions that may occur in a number of human genes and cause TREDs. This method takes advantage of the easily available DNA size standards calibrated with gene specific allelic ladders prior to analysis. We have also optimized the conditions for the amplification of repetitive sequences that significantly decrease the contribution from stutter products.

Recently, we have extended the spectrum of our genetic tools by including the multiplex ligation-dependent probe amplification (MLPA) method that allows for an analysis of large genomic deletions and insertions. The important modification of this method is a new strategy of assay design. This strategy uses short MLPA probes that can be easily generated by chemical synthesis and allows for the analysis of any genomic region of interest.

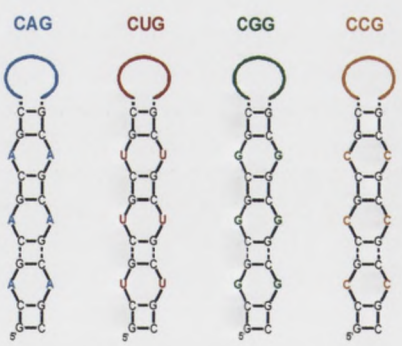
Triplet repeats in human mRNAs – occurrence, polymorphism, and structure

The short tandem repeats (STRs) are defined as tandemly repeated tracts of DNA composed of 1-6 base pair long motifs. Focusing on triplet repeats in the human transcriptome, we have addressed several detailed questions regarding their frequency and localization in human mRNAs. To answer these questions, the GenBank database was trawled for all 20 different triplet repeat tracts composed of at least six repeats, and the 718 repeat tracts were found in 619 mRNAs. The frequency of identified tracts reversely correlated with their length. The most frequently occurring motifs were CAG, CGG, CCG, CUG, AGG and ACC. Considering different mRNA regions, most of the repeats were located in the ORF (67%), followed by 5'-UTR (24%) and 3'-UTR (9%).

An important feature of the short tandem repeats is their length polymorphism. To determine STR polymorphism in human mRNAs, we genotyped the CAG and CTG repeats present in the coding regions of more than 100 human genes. We found that the number of consecutive uninterrupted triplets in the most frequent repeat allele is the major determinant of its length polymorphism, and that repeat tracts in TREDs-related genes are significantly longer and more polymorphic than the tracts in TREDs-unrelated genes. We also found that the CAA triplets are predominant interruptions within the CAG repeat tracts (both coding for glutamine). This suggests that selective pressure acts stronger against long CAG tracts in genes and transcripts than against long polyglutamine tracts in proteins.

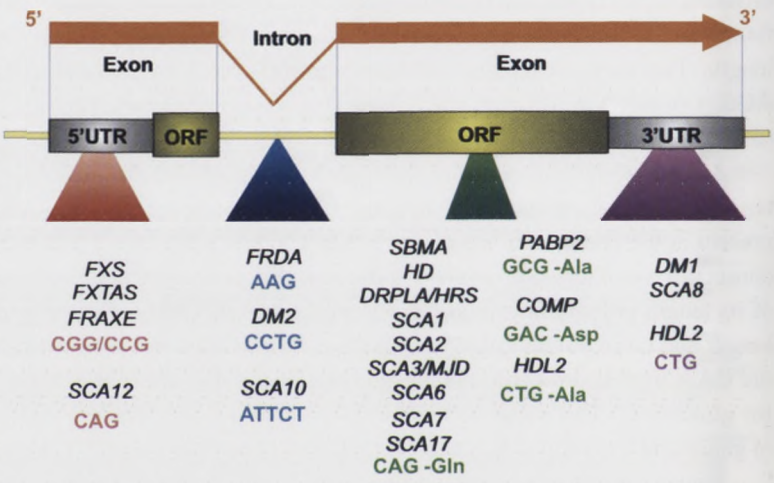
Regarding the barely known functions of transcripts containing triplet repeats, we performed the bioinformatic expression characteristics of numerous such transcripts in multiple human tissues. The mRNAs which had the highest contributions to the observed levels of transcripts containing different types of the CNG repeats were identified. A more extensive analysis was done for the transcripts containing the CUG repeats and transcripts encoding the repeat binding proteins. The results of these expression studies have considerable implications for the mechanism of the RNA pathogenesis of TREDs.

We have performed a systematic structure analysis of trinucleotide repeats in transcripts using a battery of chemical and enzymatic probes. These experiments, beside answering important questions concerning the structure formation abilities of different repeats, also provided unique information regarding the specificity of different structure probes acting on highly regular RNA sequences. Out of the investigated 20 model transcripts composed of all possible triplet repeats, 6 (CGG, CCG, CAG, CUG, CGA, CGU) formed stable hairpins, 2 (AGG, UGG) formed quadruplexes, 6 unstable hairpins and the remaining were single-stranded. The quasistable stem structure of a triplet repeat hairpin is composed of the periodically occurring base pairs and single mismatches, and the repeats show a tendency to assume several variant alignments. Another characteristic feature of hairpins formed by the repeats is the rigidity of their structure which increases with repeat length.



Human neurological diseases caused by triplet repeat expansions

Since 1991, more than 20 genetic disorders caused by the expansion of unstable repeats have been identified. These diseases include fragile X syndrome (FXS), myotonic dystrophy (DM), Huntington disease (HD) and a number of spinocerebellar ataxias (SCAs). TREDs result from the repeat expansions in the specific single genes. The expandable repeat may be present in any part of the implicated gene, in most cases in its ORF, but also in 5'-UTR, 3'-UTR or intron.



Our research in this field is focused on understanding the molecular pathomechanisms of these diseases and on determining the role of RNA in pathogenesis. Since more recently, we have been also strongly involved in research related to the RNA-directed therapy of TREDs.

The structures of triplet repeat regions in the transcripts of TREDs genes

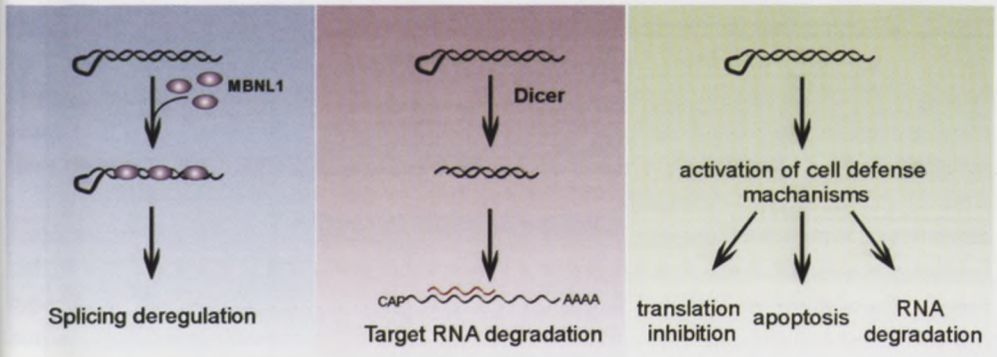
After defining the RNA structures formed by the isolated triplet repeat sequences, it was necessary to find out what is the architecture of the repeat regions within their natural sequence context. For most of TREDs-related transcripts, we experimentally characterized these structures. We found that in FMR1, SCA1, SCA6 mRNAs the repeat flanking sequences contribute significantly to the stability of the repeat hairpins. We observed that the polymorphic CCG repeat located in HD transcript near the expandable CAG repeat participates in the formation of a single hairpin structure by the repeat region. Also, the hairpin structure formed by the polymorphic CAG repeat tract of AR mRNA is stabilized at its base by the base-paired CUG and CAG repeats. In other studied TREDs-related transcripts, the repeats have more freedom of alignment and form a number of slipped hairpin variants.

In 4 out of the 20 TREDs-related genes: the *SCA1*, *SCA2, FMR1 and TBP*, their normal variants contain specific interruptions (base substitutions) within the repeat tracts, and these interruptions are absent in the expanded alleles. We have determined the structural role of various interruptions present within the repeat tracts of transcripts. It turned out that the role of the interruptions in mRNA is either the weakening or effective shortening of hairpins formed by pure repeats. Different structure destabilization strategies may be undertaken depending on the type, number and localization of the repeat interruptions.

RNA-mediated pathogenesis in TREDs

The repeats present in the translated regions are thought to exert their pathogenetic functions on the level of proteins which, in most cases, contain the expanded polyglutamine tracts. It has been argued, however, that toxic effect of the expanded polyglutamine containing proteins may not be the only mechanism of toxicity and mutant transcripts may contribute to the pathogenesis of TREDs.

Originally, this idea springs from the studies of myotonic dystrophy. The mechanism of RNA pathogenesis proposed for DM1 implies that long CUG repeats confiscate specific proteins from their normal binding sites in other transcripts, and compromise their function. Although most clearly demonstrated for DM1, DM2 and FXTAS, the growing list of diseases that might involve the pathogenic RNA mechanism include spinocerebellar ataxia type 8 (SCA8), SCA10, SCA12 and Huntington's disease like 2 (HDL2).



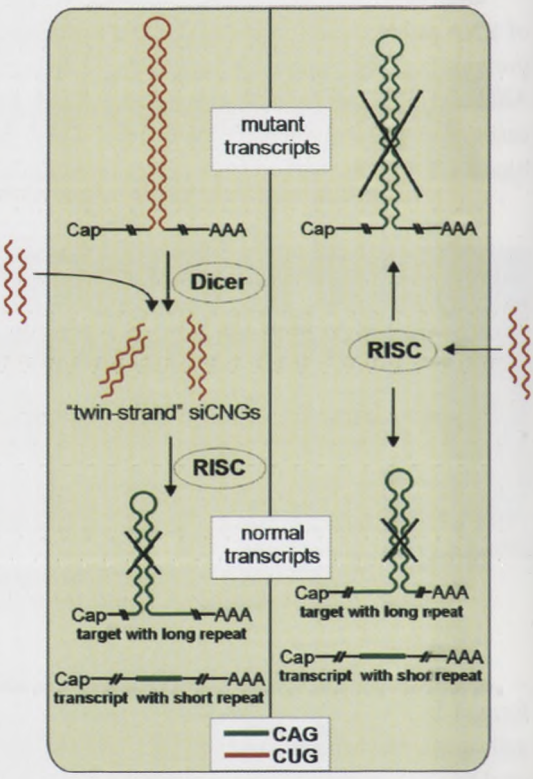
We have shown that CAG repeats in transcripts form hairpin structures similar to those formed by CUG repeats, and we hypothesized that this could imply a similarity of the pathogenic mechanism triggered by CUG and CAG repeats. Thus, the RNA toxicity attributed to DM1 could possibly contribute to the pathogenesis of polyglutamine diseases. We subjected this hypothesis to experimental verification.

Regarding the protein sequestration mechanism, we could demonstrate that, indeed, some splicing aberrations caused by the expanded CUG repeats in DM1 cells could be also observed in the cellular models of selected polyglutamine diseases in which mutant transcripts containing expanded CAG repeats were expressed.

Although widely accepted, the protein sequestration model is not the only one that can contribute to the pathogenesis of TREDs. We have shown that transcripts containing expanded CUG and CAG repeats are partially processed by ribonuclease Dicer and the products of these Dicer-induced cleavages could silence genes that contain long complementary repeats in their transcripts. We also hypothesize that the presence of long hairpin structures composed of triplet repeats in transcripts may activate sequence-unspecific signaling pathways in cells and also contribute in this way to the pathogenesis of TREDs.

Interplay between triplet repeat hairpins and RNAi machinery

Long hairpin structures formed by trinucleotide repeats can mimic miRNA precursors. We have shown that transcripts composed of long tracts of CAG and CUG repeats can be cleaved by Dicer in vitro and also in patient-derived cell lines. Long double-stranded RNA formed by repeats is processed into short interfering RNAs. We detected such siRNAs in cells from HD, SCA1 and DM1 patients. Moreover, we proved that the products of the cleavage of DMPK transcript: ~21 nt long CUG RNAs act as siRNAs and silence transcripts containing long complementary repeats. This observation allowed us to propose a new RNAi-based strategy for the therapy of TREDs.



Generally, RNA interference is a potentially powerful tool for the therapy of many human diseases. The causative therapy of TREDs should involve the specific silencing of mutant allele only. Potential targets for RNAi reagents could be the triplet repeat region itself or SNPs linked to the mutation.

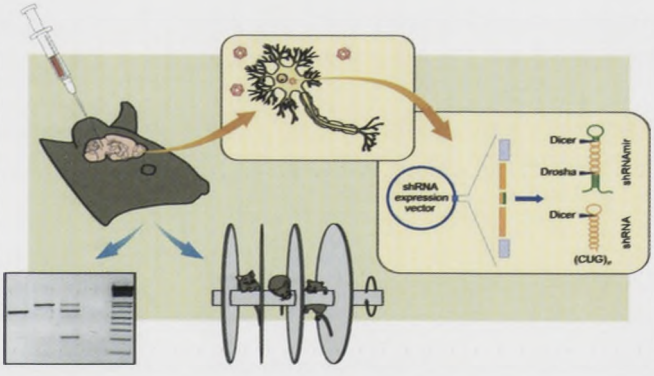
In spite of earlier discouraging results with targeting the triplet repeat region, we have shown that allele-specificity in the silencing of mutant transcripts can be achieved with this approach. We applied our observations from the cellular models of TREDs to demonstrate that synthetic siRNAs composed of CUG repeats transfected into cells silence, preferentially transcripts from the expanded, mutant alleles containing CAG repeat tracts. Our further studies with patient-derived cell lines revealed that the preferential silencing of mutant alleles with siCUG is a more general phenomenon and applies to all investigated cellular models of HD, SCA1, SCA3, SCA7, DRPLA.

We compared the activity of siCUG reagents with duplexes composed of CAG/CUG repeats to refer to the previously described approaches for targeting the repeat region in TREDs transcripts. Only siRNAs composed of one type of repeat turned out to be allele-specific, and duplexes silenced transcripts from normal and mutant alleles equally. Further characteristics of siCUG revealed that longer reagents are even more effective and for each length of the reagent preferential silencing of mutant allele could be achieved.

We have also tested the SNP-targeting approach for endogenous HD, SCA1 and SCA3 transcripts and showed that siRNAs designed to target SNP in mRNA can also distinguish between two alleles, provided some specific requirements are fulfilled.

Towards the RNAi-based therapy of TREDs

Stable, allele-specific silencing of the mutant copy of the target gene is critical for the curative treatment of neurodegenerative disorders, such as TREDs. The usage of chemically synthesized siRNAs results in a potent but only short-term effect, and the delivery of siRNA into specific brain tissue is cumbersome especially when this has to be done repeatedly. The disadvantages described above may be overcome by using vector-encoded shRNA reagents. shRNA are hairpin RNA structures endogenously expressed in cells, where they enter the RNAi pathway. Moreover, the shRNA reagents are reported to silence genes more effectively than siRNAs. We have designed two types of reagents for the allele-specific in vivo silencing of TREDs genes. The first type are shRNAs composed of CUG repeats with different stem length and loop sequence, expressed from plasmid vector driven by polymerase III promoter. Our shRNAs mimic pre-miRNA precursors and are cut by ribonuclease Dicer to form siRNA reagents in cells. The second type of reagents we are developing are hairpins composed of CUG repeats additionally containing flanking sequences derived from natural pri-miRNA precursor expressed from plasmid vector driven by polymerase II promoter. In cells, such structures need to be cleaved by ribonuclease Drosha and then shorter precursors are further processed by Dicer. The selected expression cassettes will be cloned into rAAV vectors and tested first in neuronal cell cultures. Finally, their therapeutic potential will be evaluated in the mouse model of SCA3.



We have recently generated a knock-in mouse model of spinocerebellar ataxia type 3 expressing expanded human ataxin 3 gene containing 69 CAG repeats. We will use these mice for experimental therapy that selectively targets the mRNA expressed from the expanded human allele, leaving the normal copy of ataxin 3 mRNA intact. The cassettes that express shRNA targeting the expanded repeats, packed into the rAAV particles, will be delivered into the mouse brain by stereotactic injections. The injections will reach the primary pathology regions in the mouse brain including the cerebellar nuclei, pons and the neurons therein. After injections into the brains of affected animals, we will screen the mice in order to investigate the changes in phenotype and the progression of the disease. The phenotypical studies will involve behavioral tests such as an open field test as well as motoric tests on the rotarod and string test. The mice will also be examined for the level of expression of human mutated versus normal ataxin 3 allele by RT-PCR and western blotting, as well as for the alterations in brain structures affected by the disease. In particular, we will examine the brain for neurodegeneration and neuronal loss, reactive gliosis and the presence of inclusions in affected neurons by immunocytochemistry.

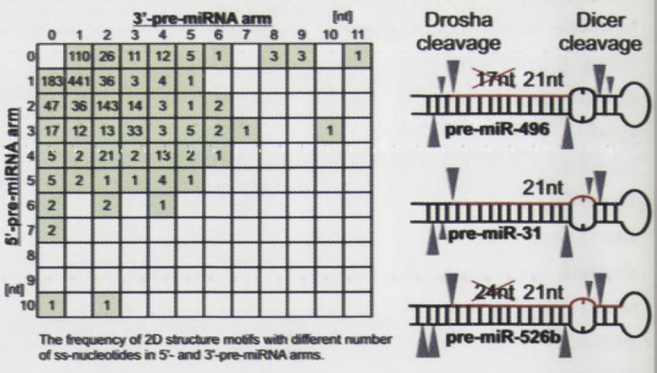
Structural aspects of miRNA biogenesis

MiRNA biogenesis in human cells consists of two RNA processing steps: first, in the cell nucleus the primary precursors (pri-miRNAs) are recognized and cleaved by the Microprocessor complex composed of ribonuclease Drosha and DGCR8 protein to form pre-miRNAs. Next, the pre-miRNAs are further processed in cytosol by the ribonuclease Dicer which generates duplexes containing mature miRNAs. Among the questions that remain to be answered are: to what extent are these processing events involved in the regulation of miRNA expression? How do the diverse structures of miRNA precursors contribute to this regulation? The aim of our research is to shed more light on the structural aspects of miRNA biogenesis and take advantage of this knowledge in the construction of more efficient miRNA and siRNA expression cassettes useful for RNAi technology.

Bioinformatics related to miRNA biogenesis

We have analyzed in detail the predicted structures of human pre-miRNA precursors and revealed their characteristic features including types, distribution, sequence, location and orientation of RNA secondary structure motifs present in the precursor hairpin.

Moreover, in search of pri-miRNA structures most deviant from the postulated consensus structure, we have predicted and analyzed the secondary structures of hundreds of human pri-miRNAs. We have found that structures of a substantial portion of pri-miRNAs strongly deviate from the consensus. Representatives of these precursors were subjected to detailed structural and functional analysis, to determine how they are processed in cells.



The frequency of 2D structure motifs with different number of ss-nucleotides in 5'- and 3'-pre-miRNA arms.

Experimental approach to miRNA biogenesis

We analyzed the RNase Dicer reactions with a number of pre-miRNA precursors and their mutants, the structures of which were experimentally determined. We found how the individual structural motifs present within precursor hairpins influence the specificity of Dicer cleavage. The results of our experiments may provide a structural explanation for the common observations that individual miRNAs show different degrees of length heterogeneity and that miRNAs excised from different precursors differ considerably in their length.

Our results may also contribute to the practical knowledge regarding the construction of pri-miR- and pre-miR-based siRNA expression cassettes. The relaxed specificity of Drosha and Dicer cleavages should be considered very carefully when designing allele-specific siRNA reagents.

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LABORATORY OF PROTEOMICS AND METABOLOMICS

Head: ***Maciej Stobiecki***

Professors:	*Krzysztof Gulewicz, Maciej Stobiecki, Przemysław Wojtaszek*
Research Staff:	*Łukasz Marczak*
Research Support:	*Danuta Ciesiołka, Wojciech Wysocki, Małgorzata Zielińska*
Ph.D. Students:	*Dorota Muth, Agnieszka Szuba*

Keywords: • *abiotic and biotic stress* • *Arabidopsis thaliana* • *cell wall (glyco)proteins* • *electrospray* • *flavonoids* • *α-galactosides* • *glycoconugates* • *liquid chromatography* • *Lupinus* • *Medicago truncatula* • *mass spectrometry* • *metabolomics* • *metabolite profiling* • *metabolite fingerprinting* • *pathogenesis* • *plant-microbe interactions* • *post-translational modifications* • *proteomics* • *secondary metabolites*

The main scientific interest of the group focuses on the problems within areas of plant biology and biochemistry (projects focused on genera *Lupinus*, *Arabidopsis thaliana*, *Medicago truncatula*): 1) the identification and biological activity of plant secondary metabolites, 2) the mechanisms of various plant responses to pathogenic or symbiotic microorganisms, 3) the role of cell wall (glyco)proteins in plant development and in plant interactions with microorganisms, 4) proteomic studies of molecular and cellular mechanisms of plant responses to stress, and 5) identification of post-translational modifications of proteins of WMC (cell Wall-plasma Membrane-Cytoskeleton) continuum caused by nitric oxide. The second goal is directed to medical applications of mass spectrometry: 1) searching for marker (diagnostic) proteins in specific cancer disease, and 2) studies of blood proteins homocysteinylation in order to elucidate the role of this chemical modification of proteins in cardiovascular diseases. The third area of activities is directed to: 1) nutraceutical value (prebiotic properties) of α-galactosides isolated from seeds of *Fabaceae* family plants and 2) the identification and biological activity of *Uncaria tomentosa* compounds.

Current research activities

- application of chromatographic techniques combined with mass spectrometry for profiling of plant secondary metabolites;
- structural elucidation of natural products with mass spectrometric techniques – creation of database of flavonoid conjugates mass spectra;

- analysis of changes in the secondary metabolism of plants (genus *Lupinus*) in response to pathogenic and symbiotic microorganisms or in genetically modified plants (*Arabidopsis thaliana, Solanum tuberosum* and *Medicago truncatula*);
- identification of post-translational modifications in proteins of WMC (cell Wall-plasma Membrane-Cytoskeleton) continuum, caused by nitric oxide;
- proteomics of plant WMC continuum under mechanical stress;
- mass spectrometry approach for protein biomarker discovery;
- characterization of protein N-homocysteinylation using mass spectrometry;
- prebiotic properties of α-galactosides isolated from seeds of *Fabaceae* family plants;
- identification and biological activity of *Uncaria tomentosa* compounds.

Major recent results

Metabolic profiling

The application of objective methods for detection and/or identification of secondary metabolites isolated from plant material is of highest importance in biological and medical sciences, especially for systems biology approaches. Contemporary gas and liquid chromatographic methods are used for separation of different classes of natural products. They are combined with various types of detectors which ensure variable level of certainty of their identification. In the course of our studies, we elaborated an efficient procedure for extraction of the phenolic secondary metabolites from plant tissues. Application of mass spectrometric techniques combined with liquid chromatography systems (HPLC or UPLC), additionally with UV and MS (qTof) detections assures reasonably good level of certainty during identification of the natural products. However, there exist some differences in a number of identified compounds between two modes of analysis: targeted and untargeted. The targeted profiling approach is used for qualitative and quantitative identification of flavonoid glycoconjugates in plants (*Arabidopsis thaliana*, *Medicago truncatula* and various species of genus *Lupinus*) exposed to biotic (pathogenic and symbiotic microorganisms) and abiotic (UV light, temperature, and chemical) stimuli. The main concern is directed to the role of phenolic compounds. Changes in quinolizidine alkaloids during interactions of lupine species with environmental factors are also analyzed.

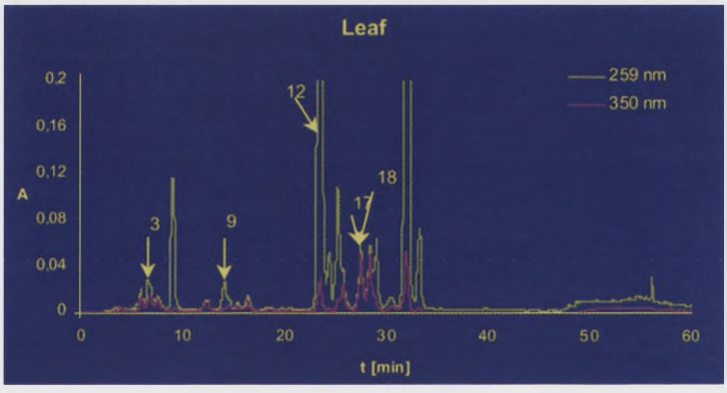


Figure 1. HPLC/DAD analysis of phenolic compounds from leaves of blue lupine (Lupinus angustifolius).

We are elaborating methods of Maldi Tof applications for high throughput fingerprinting of basic secondary metabolites, such as: anthocyanins in plant extracts. These methods may be applied for fast analyses of numerous samples obtained from biological material without necessity of time consuming LC separation. The second group of secondary metabolites that we are interested in, are glycoalkaloids synthesized in potato tubers of genetically modified plants. LC/MS approach was elaborated for quantitative analysis of target glycoalkaloids in the extracts obtained from potato plants. In our laboratory, we can use GC/MS and LC/MS systems for metabolic profiling of primary and different classes of secondary metabolites from various plant sources.

Utilization of different ionisation modes (positive and negative) with CID MS/MS permits to obtain structural information about analysed flavonoid glycoconjugates. Registration of CID mass spectra from $[M+H]^+$, $[M+Na]^+$ and $[M-H]^-$ ions allows to differentiate isomeric compounds.

Lupine phytoalexins

Plants react to pathogenic infection with a broad array of defence responses. The best characterised reaction is the production of low molecular weight anti-microbial compounds, termed phytoalexins. In legume plants, these molecules are mainly isoflavonoids with the pterocarpan-type structures. Profiling of flavonoid conjugates in control and infected with pathogenic microorganisms (*Pleiocheta setosa – L. albus*. *Colletotrichum lupini – L. angustifolius*) lupine seedlings demonstrated that active compounds against microorgansmes are prenylated isoflavones (luteone and wighteone). No isoflavonoids of pterocarpan-type structure were found thus opening the possibility for simple isoflavones to act as phytoalexins in lupines. The role of the above mentioned compounds in defence reaction of lupine are studied in 87 lines of *L. angustifolius* for which genetic mapping population exists.

Role of cell wall peroxidases and phenolic secondary metabolites in defence reaction

Phenolic compounds secreted into the apoplast play an important role in the architecture of plant walls. Mechanical properties of the walls are regulated developmentally or in response to various stimuli by changes in the extent of intra- or intermolecular interactions and/or covalent cross-links; the latter usually involving hydroxycinnamic acids. Phenolic acids might occur in the walls as side groups of hemicelulloses or, putatively, proteins. Thus formation of ferulic dehydrodimers might be one of the mechanisms of cross-linking wall components, and such molecules have been found to be linkage units, especially between arabinoxylans and between hemicelluloses and lignin. Formation of phenolic cross-links is tightly regulated during cell wall development and might also be activated during defence responses. The reaction is catalysed by isoforms of wall peroxidases and/or laccases, which may have different substrate specificities, and depend on the redox conditions and availability of co-substrates in cell walls. We established an *in vitro* model system to study the process of dimerisation of phenylpropanoid acids, it has been evidenced that isoflavonoids might act as anti-oxidants in plant walls, thus affecting the formation of phenolic cross-links.

Proteomics and cell biology analysis of plant WMC (cell Wall-plasma Membrane-Cytoskeleton) continuum

Plant cell walls together with plasma membrane and cytoskeleton form a structural and functional continuum spanning the whole plant cell. As the cell walls of individual cell are also a part of an apoplast, this continuum functions as an element providing not only cellular, but also organismal integrity. We are analysing the cell wall proteome in plants grown under normal conditions and subjected to stress. The most interesting to us is the mechanical stress which places the whole WMC continuum under tension. Osmotic stress, evoked by compounds of variegated characteristics, is a major model system that we are utilizing. Through application of various hydrolytic enzymes, we selectively destroy covalent linkages in the walls which enables us to draw conclusions on their importance for the functioning of the continuum. Comparative analysis of electrophoretic 1D and 2D protein patterns gives us information on the intricacy of intertwined assembly of the walls. Proteomic analysis with the use of LC/ESI/MS/MS and MALDI TOF MS systems allows us to identify the proteins involved in the maintenance of the plant cells integrity.

Identification of post-translational modifications of the WMC continuum proteins caused by nitric oxide

Although NO is mainly considered as a signalling molecule, it can also exerts its functions through modification of proteins due to its radical properties. Reacting directly, or indirectly, with proteins NO might form post-translational modifications (PTM) such as S-nitrosylation (addition NO to sulfhydryl groups of cysteines) and nitration (addition of a NO_2 group) of protein tyrosines. These are to variable extent reversible, thus creating a regulatory system slightly similar to protein phosphorylation. As almost nothing is known about NO-related PTMs in plant proteins, we have started our research into NO-modified amino acid residues among WMC proteins, especially in the cell wall proteome. As a model system, we are utilizing various NO donors and/or modulators of NO function or NO signaling pathways.

Mass spectrometry recently has become an increasingly important technique for the determination of proteins' PTMs. The combination of protein separation by PAGE and Western immunoblotting with MS techniques is a very powerful tool, also useful in our studies. We are focused on the detection of both NO-induced PTMs by MALDI TOF mass spectrometry. To investigate relatively labile S-nitrosylated cysteines, we utilize a highly specific biotin switch assay. Nitrotyrosine seems to be a more stable NO-derivative, and this is analyzed through different approaches. One of them is based on the observation that nitrated proteins reveal specific shifts in m/z values when analyzed with MS. For example, addition of NO_2 group causes a shift by +45m/z. Moreover, operation of the laser during MALDI analyses induces photodecomposition and generation of the product ions, which create a unique pattern of MALDI-MS peaks in the examined spectra and constitutes evidence for the presence of a nitrotyrosine.

As far as it is known from research of animal models, the level of nitration is significantly enhanced under various pathological conditions, especially under oxidative stress. We are investigating the relations between tyrosine nitration and osmotic stress in the sus-

pension-cultured tobacco BY-2 cells subjected to short-term stress and in cells adapted to such stress.

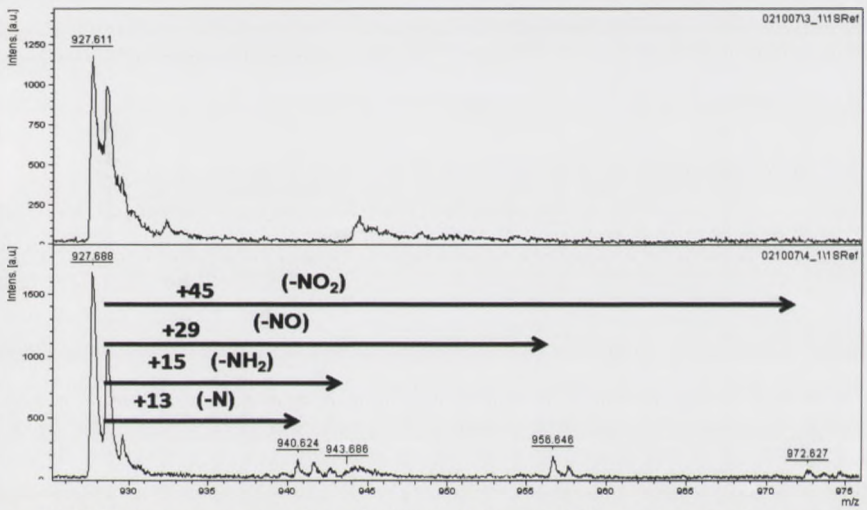
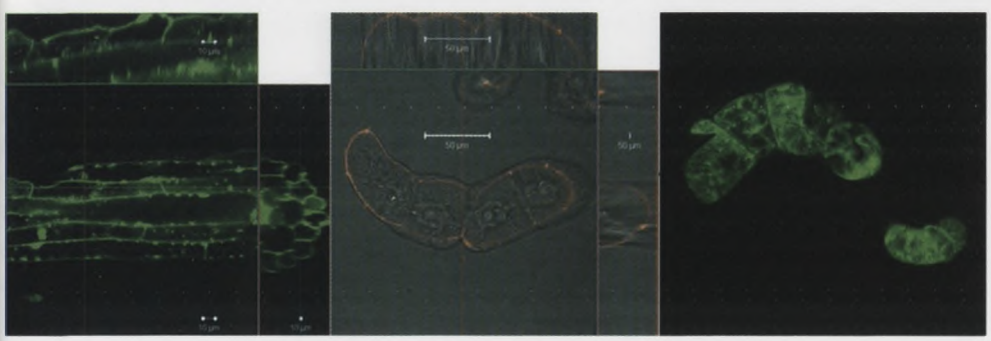


Figure 2. Maldi Tof mass spectrum of tryptic digest of nitrated BSA, nitrotyrosine is present in one of the peptides in the sample. Control – above, and nitrated BSA-below (products of photodecomposition of nitrotyrosine are noticeable).

Confocal Microscopy

Proteomic studies are complemented with the microscopic analysis. The aim of this work is to identify changes in the cytoskeletal complement of WMC continuum evoked by the NO action. To this end, the organization of the actin and microtubulin cytoskeleton has been analyzed in plant cells grown in the presence of various NO modulators (fig. 3a – cortical microtubules in fixed BY2 cells).



a) b) c)

Figure 3. Various confocal images. a) distribution of NO in Arabidopsis root (DAF 2FM), b) visualization of nitrotyrosine residues in apoplastic proteins of BY2 cells (immunolocalization in vivo), c) organization of cortical microtubules in BY2 cells (whole mount analysis).

Furthermore, we investigate the effects exerted by NO donors and modulators of NO activity on the localization of PTM's. We utilize *in vivo* immunolocalization and analysis of fixed microscopic preparations of S-nitrosotiols and nitrotyrosines in roots and cell suspension culture (Fig. 3b – nitrotyrosines in apoplastic proteins of BY2 cells).

Finally, DAF-2-FM diacetate is used to localize subcellular source of NO generation in various plant cells (Fig. 3c – NO in *Arabidopsis thaliana* root).

Mass spectrometry approach for protein biomarker discovery

During recent years, mass spectrometry have become very advanced and sensitive method of protein detection and analysis. The limit of proteins detection has been considerably lowered. Proteins are the basic determinant of tissue and cell function. Due to instant changes of physiological status of organism, the composition of proteins content must also change, so in opposition to genome, proteome is a dynamic structure illustrating the present state of organisms, tissues or cells. Clinical proteomics is a branch of proteomics and its aim is analysis of protein changes during disease development and treatment. State or quantity of factors or molecules (in many cases proteins), which express the disease risk, illness or stage of disease are called biomarkers. The goal of clinical proteomics is then identification and characterization of protein biomarkers.

In our research, we concentrate on the analysis of low molecular weight proteins present in blood plasma and serum of controls and patients suffering from different kinds of cancer diseases. For the proteins' profiling, we use MALDI-TOF mass spectrometer working in an linear and positive ions acquisition mode. Specific conditions for protein concentration and extraction prior to the analyses were worked out and utilized for our purposes, which enabled us to enrich the mass spectra in measurable peaks, therefore giving better chance not to overlook important information. The data obtained from hundreds of samples are analyzed with advanced statistical calculations (bioinformatics) and potential biomarker proteins will be further identified and characterized in detail.

Characterization of protein N-homocysteinylation using mass spectrometry

It is known that high level of cholesterol in blood is the main factor of arteriosclerosis, but also N-homocysteinylation of proteins is a process leading to many harmful effects, especially for human cardiovascular system. Homocysteine is produced in living organisms as side effect of activity of several enzymes. In human organism, homocysteine is usually synthesized from methionine delivered together with eaten food. It is an indirect product of methylation and is generally undergoing two further conversions: remethylation back to methionine and transsulfuration to cysteine. Two enzymes utilizing B12 and B6 vitamins are engaged in transformations of homocysteine: they are methionine synthase and cystationine β-synthase, respectively. Mutations of genes coding these enzymes and deficit of folic acid and mentioned vitamins may result in increased concentration of homocysteine and, in consequence, serious problems leading to cardiovascular diseases.

Mass spectrometry is widely used for the identification of proteins and determination of protein modifications. We developed a method for determination of lysine N-homocysteinylation sites of blood proteins such as albumin or hemoglobin utilizing MALDI-

TOF mass spectrometry. Now, we are working on a new method of quantification of N-homocysteinylated proteins. Since the MALDI mass spectrometry is a problematic technique in manner of quantitative analysis, we are testing new matrices and new labeled and non-labeled peptides as the internal standards for direct quantification of tryptic peptides derived from digestion of chosen blood proteins. The results obtained so far give hope that this approach will result in a reliable diagnostic method for diseases caused by enhanced level of homocysteine in human blood.

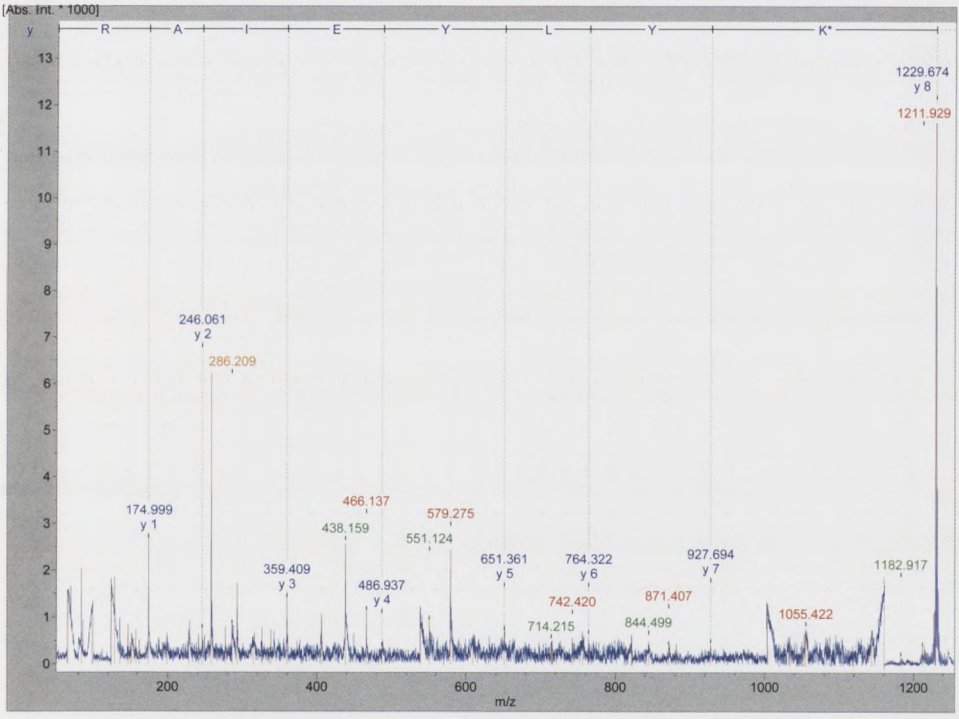


*Figure 4. Post Source Decay spectrum of 1229 m/z tryptic peptide from albumin (K*YLYEIAR) indicating that Lys 137 in albumin sequence undergoes N-homocysteinylation in presence of homocysteine thiolactone.*

Prebiotic properties of α-galactosides isolated from seeds of Fabaceae family plants

The main component of extract remaining after the debittering process of lupine seeds are α-galactosides considered, up to now, as antinutritional factors. However, recent studies have shown that these sugars applied to diet as a supplement beneficially influence the development of bifidobacterium population in the colon which, in turn contributes to human health in many ways. The aim of our investigation is assessment of effect of the lupine α-galactosides, applied during chicken embryogenesis, on hatchability, intestine bacterial profile, development and meat trials.

Identification and biological activity of Uncaria tomentosa compounds

The last aim is biological activity of preparation obtained from *Uncaria tomentosa*. This Peruvian liana has been recently a subject of scientific interests due to its wide range of pharmacological properties. Our studies include determination of chemical composition of raw material (bark and leaves), water and alcoholic extracts. Standardized preparations of different extracts are tested for their apoptotic and necrotic activity on various human cancer cells (HL-60, K-562, Raji) by using cytometric methods and statistical evaluation of the results.

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CENTER FOR BIOCRYSTALLOGRAPHIC RESEARCH

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X-Ray crystallography has been and continues to be the major source of information about the three-dimensional structure of macromolecules. Typically, crystallographic studies of the structure of a macromolecule are divided into several steps. First, the biomolecule under study has to be obtained in crystalline form, which is often a success-limiting step. Next, the crystals are exposed to X-rays and the diffraction pattern is recorded. The diffraction pattern is then the basis for the structure determination process.

The Center is equipped and prepared to carry out all the steps necessary for macromolecular structure determination, from protein purification and crystallization through low-temperature diffraction experiments, to structure evaluation and analysis. The biomolecules studied in the Center include both proteins and nucleic acids.

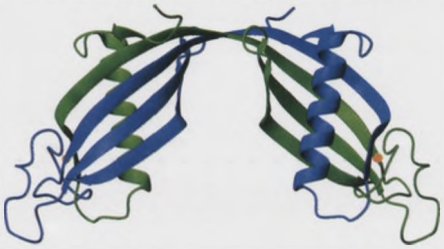
The Center was created in 1994 with financial support from the Foundation for Polish Science as a joint initiative of the Department of Crystallography, A. Mickiewicz University and the Institute of Bioorganic Chemistry.

Current research activities and major results

3D Domain swapping and amyloidogenesis

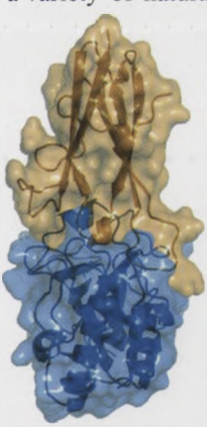
Amyloid-forming proteins attract attention because of their role in the pathogenesis of a number of diseases, like Alzheimer's disease and the prionoses. The pathophysiological processes involve abnormal conformational changes, followed by aggregation. Human cystatin C (HCC), a potent and abundant inhibitor of cysteine proteases, changes its structure on prolonged incubation to form inactive dimers. The tendency to oligomerize may explain why HCC forms amyloid deposits in the brain arteries at advanced age. Formation of HCC amyloid is accompanied by the presence of HCC dimers in the cerebrospinal fluid. The

amyloidogenic property of HCC is drastically amplified in the endemically occurring L68Q mutant that causes amyloidosis and brain hemorrhage leading to death in young adults. We have reported, for the first time, the crystal structure of HCC and demonstrated that the protein aggregates to form dimers through an exchange of structural units. This phenomenon of 3D domain swapping is a mechanism for forming oligomeric proteins from their monomers. The same mechanism of 3D domain swapping has been found for N-truncated HCC, which is the dominating material isolated from cystatin C deposits of patients suffering from HCC amyloidosis. The structure of 3D domain-swapped HCC dimers suggests possible mechanisms of amyloid fibril formation and explains the role of the L68Q mutation. It also has implications for other diseases involving conformational pathologies. In recent experiments, we have demonstrated that by strategic placement of cysteine residues in the HCC sequence, we can introduce new disulfide bridges, allowing red-ox control of domain swapping, and in consequence – control of dimerization, oligomerization, and amyloid fibril formation.



Cysteine proteases and their inhibitors

Several serious diseases related to tissue degeneration, such as osteoporosis or muscular dystrophy, are linked to abnormalities in the functioning of cysteine proteases. There is a variety of natural inhibitors of these enzymes, ranging from sizeable proteins, such as cystatins, to relatively small molecules, such as E-64. The latter compound contains an oxirane ring that blocks the enzyme by forming a covalent bond with the catalytic thiol group. We have studied a number of complexes of the model cysteine protease papain with synthetic inhibitors containing a peptide sequence modeled after the binding epitope of HCC, and a reactive group, such as oxirane or diazomethylketone. Cysteine proteases are also involved in pathogen invasion. We have determined the crystal structure of the wild-type zymogen (known as superantigen B) of the enzyme from the highly virulent bacterium *Streptococcus pyogenes*. In another project, we focus on the cysteine protease inhibitor, called chagasin, from the protozoan responsible for Chagas disease, *Trypanosoma cruzi*. Although chagasin has the same size as cystatins, our crystal structure has demonstrated that it has a completely unrelated fold. Nevertheless, the enzyme-binding epitope has the same three-loop architecture, as revealed by the crystal structure of a complex between chagasin and the human enzyme cathepsin L.



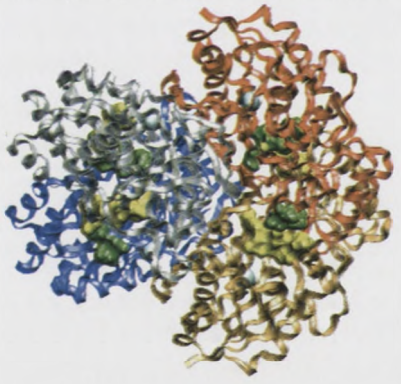
Retroviral enzymes

Retroviral protease is a key enzyme in the replication cycle of the HIV-1 retrovirus, the causative agent of AIDS. Since the determination of the three-dimensional structure of the enzyme and the discovery that it is a symmetric, homodimeric aspartic protease, it has

become the major target in the rational design of drugs for the treatment of AIDS. Our current interest in this field focuses on new-generation HIV-1 protease inhibitors as potential drugs, on complexes of the enzyme with autodigestion products, and on proteases from other retroviruses. We are also studying proteases from other retroviruses. The recently determined structure of the enzyme from the leukemia-causing HTLV virus has revealed important differences in the active-site architecture between the HIV-1 and HTLV enzymes, thus explaining the failure of successful anti AIDS drugs in the treatment of HTLV infections. We have also been studying the structure of retroviral integrase, which is responsible for the integration of the retroviral genetic material into the host cell genome.

Antileukemic bacterial asparaginases

The interest in periplasmic bacterial L-asparaginases has been instigated by their antileukemic activity. Asparaginases of this type, for example *Escherichia coli* L-asparaginase II (EcAII), are homotetramers with four active sites. Each active site is created by amino acids from two monomers, including amino acids from conserved motifs. The mechanism of the asparaginase reaction is not fully understood. It could be a variant of the reaction catalyzed by serine proteases, but with a threonine in the role of the nucleophilic serine. The other residues of the putative catalytic triad could be D90 and K162. The antitumor activity of these enzymes is the effect of their high affinity for the substrate. Depletion of L-asparagine in the circulating pools starves the tumor cells, which have reduced levels of L-asparagine synthesis. We have studied a number of EcAII mutants to shed more light on the enzymatic mechanism of these enzymes. We have discovered that EcAII binds zinc cations, which may be of importance as far as its role as a drug is concerned.



Plant-type asparaginases

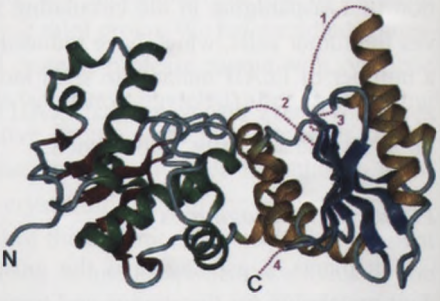
In plants, L-asparagine is the most abundant metabolite for the storage and transport of nitrogen that is utilized in protein biosynthesis. Asparagine hydrolysis in plants is catalyzed by asparaginases with no homology to the bacterial-type enzymes. The most studied enzymes in this class, from legume plants, are involved in metabolic pathways connected with assimilation of atmospheric nitrogen. We have cloned, sequenced, expressed, and crystallized the enzyme from *Lupinus luteus* (LlA). We have also shown that the *E. coli* genome encodes a highly homologous enzyme, EcAIII. The structure of EcAIII has been solved demonstrating that it is an N-terminal nucleophile

(Ntn) hydrolase that undergoes autoproteolytic activation to liberate the N-terminal threonine (subunit β) nucleophile. The active protein is an $(\alpha\beta)_2$ heterotetramer. The structure and maturation pattern place EcAIII in one class with aspartylglucosaminidases. However, enzymatic and kinetic studies show that both LlA and EcAIII are predominantly active as isoaspartyl aminopeptidases, and that their L-asparaginase activity is of secondary importance. In this light, LlA and EcAIII gain in relevance as enzymes responsible for the degradation of malformed proteins, in which a peptide bond occurs at the side chain of asparagine or aspartic acid.

Regulation of methylation processes in plants

S-adenosylhomocysteine (SAH) is a by-product, and strong inhibitor of all cellular methylation reactions that depend on S-adenosylmethionine (SAM). Therefore, efficient removal (by SAH hydrolase, or SAHase) of SAH is necessary to keep the cell going. We have recently determined the structure of SAHase from *Lupinus luteus*, providing the first glimpse of the enzyme from plants. Plant SAHases have a long insert (about 40 residues) in their sequence which distinctly differentiates them from the animal homologs.

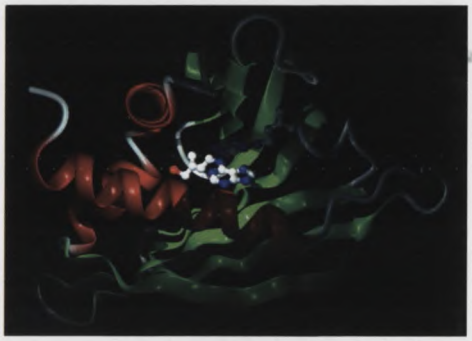
Proteins involved in plant-bacterium symbiosis

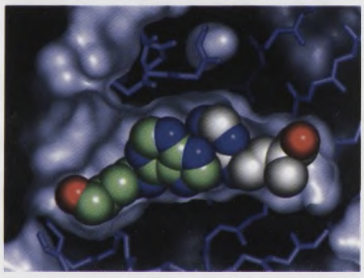


Symbiosis between legume plants and nitrogen-fixing bacteria depends on exchange of precise molecular signals. This process results in the formation of root nodules in which the bacteria assimilate atmospheric nitrogen. In addition to plant signals, bacterial signals, called Nod factors, are also necessary. A prominent role in this respect is played by the bacterial NodZ protein, which catalyzes fucosylation of the Nod factor chitin oligosaccharide. We have cloned, overexpressed, and crystallized the NodZ enzyme from *Bradyrhizobium*, also in a selenomethionyl form, and the structure has been solved by the MAD method. The crystal structure reveals a two-domain folding pattern, with one of the domains folded according to Rossmann motif. This is consistent with the fact that GDP-fucose is the sugar donor in the fucosylation reaction.

Plant pathogenesis-related and hormone-binding proteins

The pathogenesis-related class 10 (PR-10) proteins have been detected in nearly all studied plants but never in non-plant organisms. The PR-10 class includes numerous ho-

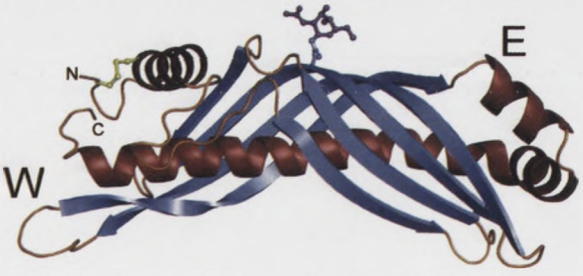




mologs with expression patterns responding to pathogens and other stress factors. Tree pollen allergens are also in this class, similarly to the distantly related cytokinin-specific binding proteins (CSBP). In contrast to CSBP, the function of classic PR-10 proteins remains unknown despite their high cytosolic content. We have determined the crystal structure of a CSBP protein in complex with the plant hormone zeatin, confirming that its fold is consistent with PR-10 classification. This fold consists of a seven-stranded antiparallel β-sheet wrapped around a long C-terminal helix. Between the sheet and the helix there is a large internal cavity, where the ligands are bound. The C-terminal helix is unusual since it shows high sequence variability and geometrical distortions. These distortions determine the shape and size of the cavity and may, therefore, control the ligand specificity of the protein. Recently, we also managed to crystallize a classic PR-10 protein in complex with zeatin. In both complexes there are multiple copies of the hormone in the binding cavity (CSBP – two, PR-10 – three) but the modes of binding are different.

Insect hormone binding proteins

The complicated insect development program is orchestrated by hormones, such as Juvenile Hormone (JH). This very labile and highly hydrophobic molecule is transported in the insect hemolymph by a specialized protein called Juvenile Hormone Binding Protein (JHBP). Recently, we solved the structure of this mysterious protein from *Galleria mellonella* revealing its hot-dog-like fold, in which a long α-helix is almost completely wrapped in a highly curved β-sheet. The same folding motif can be found in two low-homology human proteins, which also bind hydrophobic ligands, but it is present there in tandem repeats, indicate gene duplication. Surprisingly, the relatively small JHBP protein contains two hydrophobic pockets, one at each pole. Analysis of the interior of those pockets together with other biochemical experiments leaves no doubt which of them harbors the hormone binding site.

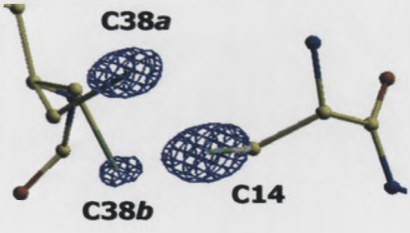


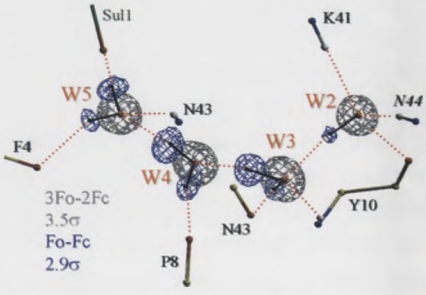
Nuclear receptors

The functional nuclear receptor for the steroid hormones responsible for molting and metamorphosis in insects is an unusual heterodimeric molecule. The crystal structure of the DNA-binding Domains (DBDs) of the two partners (EcR and Usp) in complex with their natural DNA response element, *hsp27*, reveals a novel element (an α-helix) in the C-Terminal Extension (CTE) of the EcRDBD domain. The location of this helix in the minor groove of the DNA target does not match any of the locations reported previously for nuclear receptors. Mutational analyses suggest that this α-helix is a component of an EcR-box, a novel element indispensable for DNA-binding.

Protein structure at ultrahigh resolution

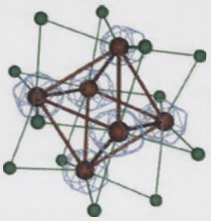
The crystal structure of a mutant of BPTI (Bovine Pancreatic Trypsin Inhibitor) has been refined using synchrotron data extending to 0.86 Å resolution to an R-factor of 0.1035. From full-matrix least-squares refinement the accuracy of C-C bond distances is 0.010 Å. Many H-atoms can be seen in difference electron density maps, including those in water molecules. The structure reveals a double-conformation disulfide bridge (C14-C38) and a salt bridge between the N- and C-termini. It also provides examples of asparagine residues in unusual conformations and illustrates the importance of N-H...π hydrogen bonds for the stabilization of the molecular structure. At this resolution, the refinement is highly overdetermined allowing for the removal of main-chain restraints and for unbiased verification of the stereochemical standards used in protein structure refinements at lower resolution. This reveals, for instance, that deviations up to 20° from peptide planarity are quite possible.





Crystallographic methodology

The crystal structure of a Z-DNA hexamer has been solved from the anomalous signal of the P atoms at copper wavelength. The multiplicity of the diffraction data was the most crucial single factor for the solution of the phase problem. The structure was refined to an R factor of 0.089 at 0.95 Å resolution. In another study, we have demonstrated the power



of the $(Ta_6Br_{12})^{2+}$ complex for phasing protein structures and determined the precise geometry and molecular interactions of this cation in a protein crystal context. We have an active Training, Implementation and Dissemination (TID) program within the European BioXHIT project, with emphasis on remote access to crystallographic synchrotron beamlines.

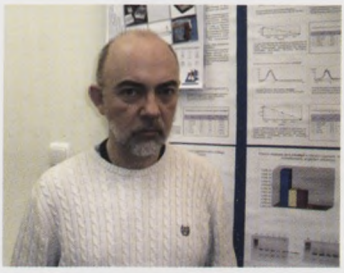
We also develop Internet tools for teaching crystallography: www.man.poznan.pl/CBB/ → Teaching.

Crystal engineering

Our interest in this area is on hydrogen bonds as determinants of supramolecular organization, with focus on both extremely strong (i.e. short) and very weak hydrogen bonds. In the latter category, we are interested in C-H…X and Y-H…π interactions. We have discovered interaction mimicry utilizing C-H…O bonds and leading to one-dimensional isostructurality. Also, we have used the N-H...N and C-H...O synthons to engineer a supramolecular helix. When applied to nucleoside salts, the concept of supramolecular synthons has revealed preferred interaction patterns, also including C-H donors. Our current focus is on co-crystals as potential vehicles for Active Pharmaceutical Ingredients (APIs).

STRUCTURE-FUNCTION RELATIONSHIP IN BIOLOGICAL MOLECULES GROUP

Head: ***Wojciech Rypniewski***



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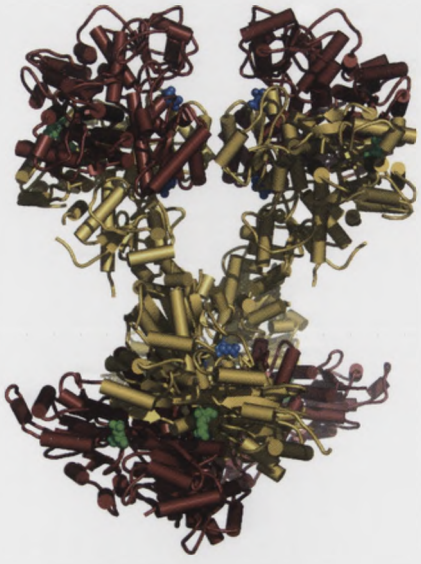
Ph.D. Students: *Katarzyna Banaszak, Joanna Raczyńska, Agnieszka Kiliszek*

Keywords: • *key enzymes in metabolism* • *trinucleotide repeats in RNA* • *biomoecules at atomic resolution* • *hairpin-duplex RNA transition*

Current research activities and major results

Enzymes of key significance in metabolism

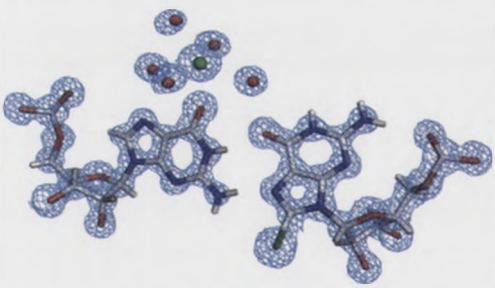
Glycolysis is the foundation of both anaerobic and aerobic respiration and occurs in nearly all organisms. It is the main energy source in many prokaryotes and in the eukaryotic cell types devoid of mitochondria or functioning under low oxygen or anaerobic conditions. The rate of glycolysis is tightly regulated subject to the cell's needs for energy and building blocks for biosynthetic reactions. Phosphofructokinase (PFK) is a key control point in the glycolytic pathway, just downstream of the entry point for hexose sugars. It catalyses the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate and ATP to ADP. PFK is the enzyme with the most complex regulatory mechanism in the glycolytic pathway. The major isozyme of PFK is PFK1, a multi-subunit allosteric enzyme whose activity is modulated by a number of effectors. The sophistication of the control mechanism of PFK in eukaryotes is matched by its complex evolutionary history. Subunits of eukaryotic PFKs are a result of tandem gene duplication of the prokaryotic precursor with the redundant parts having evolved to acquire new functionalities, allowing the enzyme to become responsive to an even larger range of allosteric effectors than the bacterial enzyme.



We have been studying the crystal structures of two eukaryotic PFKs: from baker's yeast (*Saccharomyces cerevisiae*) (shown) and from the skeletal muscle of rabbit, in complex with their ligands to determine the structure-function relationship of this key glycolytic enzyme: the enzymatic reaction mechanism and the allosteric control, and to understand the evolution of this complex enzyme in terms of the 3D structure. The control of metabolic pathways in eukaryotic microorganisms, in particular fungi, has a major impact on economy and medicine. Yeast is ubiquitous in nature and is used on a large scale in industry (e.g. in fermentation of sugar) and in biotechnology (e.g. as eukaryotic systems for protein expression), and for the production of organic compounds - semiproducts in the synthesis of therapeutics. Some species of yeast are pathogenic. Mutations in human PFKs have been linked to several genetic diseases.

RNA fragments relevant to pathogenesis and control processes in biological systems

"Molecular biology is undergoing its biggest shake-up in 50 years, as a hitherto little-regarded chemical called RNA acquires an unsuspected significance" (*The Economist*). This is due to the recent discoveries that in addition to the traditionally recognised roles in ferrying the genetic code across the nuclear membrane and in protein synthesis, RNA also plays major roles in biological processes which had not been ascribed to it previously: the catalysis and regulation of gene expression (miRNAs and siRNAs). There is also a growing interest in applying RNA technologies in therapeutics (ribozymes, RNAi). RNA possesses structural richness that matches its newly discovered functions and the knowledge of its structure, like with other types of biological molecules, is the key to understanding its properties, function and interactions with the environment. Yet RNA is by far the least studied type of macromolecule in terms of three-dimensional structures.

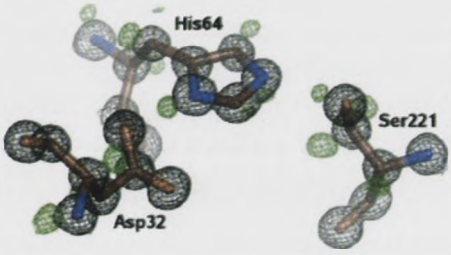


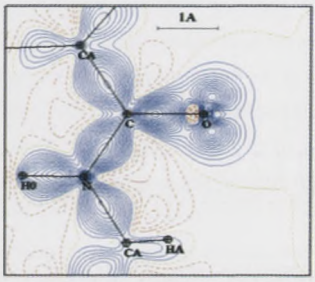
We carry out crystallographic studies of physiologically relevant non-canonical RNA duplexes, including their interactions with solvent and small ligands, and RNA fragments that undergo hairpin-duplex transition. Recently, we have become very interested in RNA sequences containing CNG repeats (where N stands for any nucleotide). Such structures are implicated in about 20 neurological diseases. In these studies, we collaborate with our colleagues from the Laboratory of Structural Chemistry of Nucleic Acids, the Laboratory of RNA Chemistry and the Laboratory of Cancer Genetics.

Ultra-high resolution studies of biological molecules

Another area of our interest are the fundamental properties of proteins and their interactions with ligands and substrates. We have done a series of atomic resolution studies of

serine proteases and protease/inhibitor complexes. Atomic resolution (ca. 1 Å) data obtained using synchrotron radiation reveal information previously inaccessible to crystallographers. Whereas conventional crystallography yields little more than the fold and approximate disposition of residues, with high resolution studies it is possible to examine in





detail the anisotropic thermal motions of atoms, visualise hydrogen atoms (left), the details of protein hydration and the solvent structure. Unrestrained (unbiased) refinement of atomic coordinates results in molecular models accurate enough to address issues like differences in substrate specificity and detailed reaction mechanisms. At resolution much higher than 1 Å it is possible to examine the detailed electronic structure of the molecule and observe features such as bonding electrons and free-electron pairs (right).

PROTEIN BIOCHEMISTRY GROUP

Head: *Michał M. Sikorski*

Professors: *Michał M. Sikorski*

Research support: *Alina Kasperska*

Ph.D. Students: *Wiesława Woronowicz, Agnieszka Ciesielska, Özgür Çakıcı*

Keywords: • *plant pathogenesis-related proteins* • *allergens* • *DNA microarray analysis* • *cytokinin-specific binding proteins* • *gene expression* • *gene silencing* • *recombinant proteins*

Current research activities

- studies on the physiological role of cytokinin-specific binding proteins (CSBP) by gene silencing approach in transgenic *Medicago truncatula*
- functional and structural studies of PR-10 proteins in the complex with natural and synthetic cytokinins (determination of the specificity of PR-10 proteins towards binding plant hormones: evolution of PR-10 proteins to CSBPs)
- application of microarray analysis to study plant gene expression
- immunochemical determination of IgE level in allergic patients – diagnosis of human food and pollen allergy
- microarray analysis of selected genes expression in case of IgE-mediated Type I allergy

Major recent results

Multigene family of PR10 proteins in yellow lupine

PR-10 proteins (pathogensis-related) are usually encoded by multigene families widespread in higher plants. Despite their ubiquity within plant kingdom, the function of PR-10 proteins still remains unclear. Many of the PR-10 class members were recognized as major food and pollen allergens. The constitutive expression of some *pr-10* genes suggests more general biological role of PR-10 proteins in plant developmental program.

We have identified to date a set of eleven homologous *pr-10* genes in yellow lupine cDNA and genomic library. It includes the genes encoding cytokinin-specific binding proteins (*csbp*), classified together with *pr-10* due to similar tertiary structure. Finally, three subclasses of PR-10 proteins: LlPR-10.1, LlPR-10.2 and LlCSBP were distinguished in yellow lupine, according to the level of amino acid sequence identity (Table 1). The highest identity values at the nucleotide level were noticed within the subclass *Llpr-10.2,* where the two pairs of very close homologues were identified: *Llpr-10.2a/Llpr-10.2d* and *Llpr-10.2b/Llpr-10.2c.* Expression of *Llpr-10.2* genes: *LlYpr-10.2.a*, *LlYpr-10.2.b* and *LlYpr-10.2.e* was also monitored in yellow lupine leaves during development, in wounded leaves

and leaves infected with pathogenic bacteria *P. syringae*. It has to be noticed that in legumes, PR-10 proteins can be additionally considered in a symbiosis context (symbiotic interaction of legume plant with soil bacteria *Bradyrhizobium* sp. (*Lupinus*).

Table 1. Yellow lupine pathogenesis-related proteins of class 10

cDNA	Gene	Protein	Amino Acids*	MW (kDa)	pI**
subclass LIPR-10.1					
Llpr-10.1a	*LlYpr-10.1a*	**LlPR-10.1A**	**156**	**16 859**	**5,19**
Llpr-10.1b	*LlYpr-10.1b*	**LlPR-10.1B**	**156**	**16 655**	**5,35**
Llpr-10.1c	*LlYpr-10.1c*	**LlPR-10.1C**	**156**	**16 749**	**5,08**
subclass LIPR-10.2					
Llpr-10.2a	*LlYpr-10.2a*	**LlPR-10.2A**	**158**	**16 904**	**4,95**
Llpr-10.2b	*LlYpr-10.2b*	**LlPR-10.2B**	**158**	**16 888**	**4,81**
Llpr-10.2c	*LlYpr-10.2c*	**LlPR-10.2C**	**158**	**16 805**	**4,81**
Llpr-10.2d	*LlYpr-10.2d*	**LlPR-10.2D**	**158**	**16 812**	**4,85**
Llpr-10.2e	*LlYpr-10.2e*	**LlPR-10.2E**	**157**	**16 869**	**4,85**
Llpr-10.2f	*LlYpr-10.2f*	**LlPR-10.2F**	**157**	**16 821**	**4,85**
subclass LlCSBP (cytokinin-specific binding proteins)					
Llcsbp1	*LlYcsbp1*	**LlCSBP1**	**158**	**17 875**	**4,81**
Llcsbp2***	*LlYcsbp2*	**LlCSBP2**	**155**	**17 399**	**4,73**

*including the initial Met; **theoretical values, based on amino acid contents (program COMPUTE pI/Mw; http://expasy.hcuge.ch/); ***with 15nt-lack in 5' end of the coding region

Yellow lupine (*Lupinus luteus*) PR-10 proteins present in root extracts seem to protect the bacteroid tissue against pathogen invasion.

The expression profiles of five *Llpr-10 genes* belonging to both *pr-10* subclasses were determined in roots and leaves of yellow lupine plants treated with salicylic acid. An elevated levels of expression in leaves in response to salicylate were observed for different studied *Llpr-10* gene homologues. It corresponds with the fact that SA-response elements, such as TCA-like sequences described in tobacco, are present in all known yellow lupine *pr-10* gene promoters. *pr-10* genes from other plant species also respond to salicylate, however, the kinetics of response often differs. In addition, the effect of *Llpr-10.2* gene homologue silencing in yellow lupine plants was investigated. However, no phenotype changes were observed. It might be due to the presence of the *pr-10* gene homologues in the studied plant, where they take over the function of the silenced gene.

An example of PR-10 genes expression profiles' analysis is shown in Fig. 1 where the DNA microarray technique was applied. The comparison of the tertiary structures of different homologues suggests their function as ligand binders/carriers.

Our structural studies on PR-10 protein complexes with small ligand molecules clearly demonstrate their ability to bind plant hormones – cytokinins.

Structural studies on CSBP and PR-10 proteins

The results of atomic resolution structural studies on cytokinin-specific binding protein (CSBP) from legume plant *Vigna radiata* confirmed the classification of CSBPs in the PR-10 protein folding class, despite low amino acid sequence identity (less than 20% identity).

Figure 1. An example of lupine gene expression profile analysis using DNA mi-croarray technique.

It suggests that CSBPs have evolved from classic PR-10 class proteins to specifically bind plant hormones. The low level of sequence conservation between the CSBP and PR-10 protein group presumably results from early divergence or fast mutation rate. The evolutionary pressure has left the protein core intact, which implies its relevance to functional/biological role for PR-10 proteins

Yellow lupine proteins involved in plant hormone binding

Functional studies on two yellow lupine *csbp* genes revealed their differential expression in selected organs as shown by real-time PCR analysis (Fig. 2).

Immunochemical detection of CSBPs showed essential differences in the accumulation of LlCSBP1 and LlCSBP2 protein homologues in the analyzed yellow lupine organs (Fig. 3). It has to be noticed that CSBPs are present in the analyzed plant organs at relatively low level (in comparison to the PR-10 protein content). The low CSBPs content in plant might suggest their regulatory function or involvement in a signal transduction pathway.

The physiological function of CSBP is currently studied in transgenic *M. truncatula* with application of gene silencing techniques. We expect a phenotype effect when a *csbp* gene expression will be blocked and its protein product will not

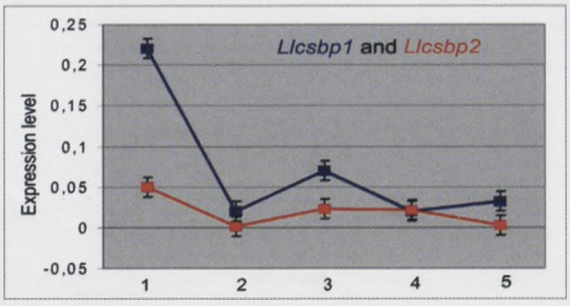


Figure 2. Real-time PCR analysis of the Llcsbp genes expression in yellow lupine organs: 1 – young leaves; 2 – old leaves; 3 – pods; 4 – seeds; 5 – roots.

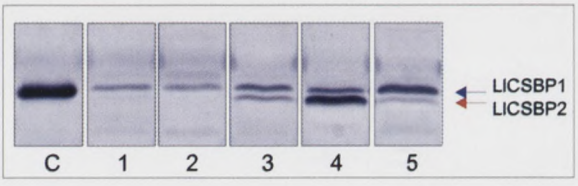


Figure 3. Immunochemical detection of yellow lupine CSBPs in various plant organs: C – recombinant LlCSBP1; 1 – petioles; 2 – leaves; 3 – pods; 4 – seeds; 5 – hypocotyls.

be synthesized in transgenic plant. A real-time PCR will be also applied to show the changes in expression patterns of selected plant marker genes. The effect of *csbp* gene silencing on nodulation process in legume plant will also be examined. We expect to get some data on the involvement of cytokinin-binding protein in functioning of root nodule.

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LABORATORY OF BIOINFORMATICS

Head: ***Jacek Błażewicz***

Professors: *Jacek Błażewicz, Piotr Formanowicz, Marta Kasprzak*

Research Staff: *Piotr Łukasiak, Marta Szachniuk, Aleksandra Świercz*

Keywords: • *computational biology* • *computational complexity* • *models of parallel computing* • *graph theory* • *sequencing by hybridization* • *searching for motifs* • *phylogenetic trees* • *RNA and protein structure analysis* • *peptide sequencing* • *modelling of biological processes* • *DNA microarrays* • *restriction mapping* • *DNA computing*

The research area of the laboratory involves many branches of computational biology focusing on combinatorial methods of analyzing and solving biological problems. Among our main areas of interest, there are: sequencing by hybridization, assembling, nucleotide and amino acid sequence alignment, searching for motifs, analysis of evolutionary history, discovering 2- and 3-dimentional structures of protein and nucleotide chains.

The general objective of the research is to explore the area of science where molecular biology meets mathematics and computer science. This allows to work on mathematical formulae of biological problems, to classify their computational complexity and to develop appropriate algorithms based on complexity analysis.

Most of recent research activities have concentrated on combinatorial aspects of sequencing by hybridization as well as on development of new algorithms for this approach. The algorithms proposed take into account hybridization errors, being the main drawback of the SBH method. To analyze the problem, a graph theory is used and the interaction of ideas between biology and mathematics resulted in new models of sequencing (on the biological side) and a new class of graphs called *DNA graphs* (on the mathematical side), for example.

The DNA sequencing is a procedure used on the lower level of nucleic acids sequence determination. The higher levels are assembling and mapping. The research is carried out also on these levels. A new method for restriction map construction, as well as a new approach to genome assembly, have been proposed. The latter resulted in a reconstruction of the coronavirus causing SARS.

The three research areas mentioned above concern the reading of genetic information that is usually the first stage in molecular biology research. The second one is an analysis of this information which, in general, is a much more difficult task. In this area, the research in

the laboratory concentrates on determining protein and RNA structures. In the former case, the secondary structure is predicted on the basis of amino acid sequences, using a method called *Logical Analysis of Data*. Moreover, domains in protein structure are also analyzed. In the second case, the NMR data is an input for a combinatorial problem of NOE assignments, being the first step of the tertiary structure determination. Moreover, new methods are being developed for sequence alignment. The other field of research is phylogeny analysis, where, among others, gene duplication events are analyzed.

The team is also involved in the research on developing new vaccines for HCV, being responsible for a construction of an intelligent database for storing and analyzing the results of the project.

Current research activities:

- computational complexity analysis and algorithms for sequencing by hybridization with the presence of errors;
- non-classical approaches to sequencing by hybridization;
- simplified partial digest mapping;
- prediction of protein secondary structure using Logical Analysis of Data;
- domain analysis in protein structures;
- algorithms for RNA structure analysis based on NMR data;
- algorithms for a phylogenetic tree construction;
- gene duplication analysis;
- designing structural databases;
- algorithms for peptide assembly;
- modeling of the human body iron homeostasis process;
- designing of DNA microarrays;
- analysis of DNA microarrays data.

Major recent results:

Simplified partial digest mapping

The new approach for the DNA restriction mapping problem, named Simplified Partial Digest, was invented in our Institute and is an alternative for the well-known Partial Digest approach, not very useful in laboratory implementation. The new approach is much easier to realize and needs only two stages of the experiment performed. For the new method, theoretical analysis has been done and new algorithms have been proposed and tested. Current research in this area goes toward designing new algorithms solving the problem for data containing experimental errors of several types (measurement errors, false negatives, etc.).

Sequencing by hybridization

The DNA sequencing by hybridization has been investigated in the case of two instances: the standard SBH with oligonucleotides of constant length and the isothermic SBH with oligonucleotides of constant temperature of melting oligonucleotide duplexes. The

latter approach was invented in our Institute. For both approaches, theoretical analysis has been done and new algorithms have been proposed and tested. The two approaches have been also tested concerning their robustness for errors coming from repetitions of oligonucleotides within an original sequence. The standard approach appeared to be better fit for sequencing repetitive fragments of DNA.

Resonance assignment problem in a determination of RNA tertiary structure with NMR

Liquid state Nuclear Magnetic Resonance (NMR) spectroscopy has been now well established as a method for RNA tertiary structure determination. Most steps of the determination process for RNA molecules are performed with the use of computer programs which, however, do not apply to a resonance assignment, being the starting point of the whole procedure. Magnetization transfer pathway, which determines the assignments, is constructed during an analysis of possible connections between selected resonance signals within multidimensional NMR spectra. It has been proved that resonance assignment in 2D NMR spectra is strongly NP-hard, thus computationally intractable. The same concerns an assignment performed within 3D NMR spectra. On the basis of the proposed combinatorial graph models of the problem, one for 2D and one for 3D spectra, several algorithms have been designed, being alternative tools for an automatization of the pathway construction. Exact algorithms have been proposed to generate all the feasible solutions to the problem for 2D and 3D spectra. Two heuristics, tabu search and genetic algorithm, have been implemented to perform the search of the most valuable solutions within 2D NMR spectra. All of the procedures have been applied to the experimental and simulated spectral data for RNA molecules.

Prediction and analysis of protein secondary structures

The study of protein structures is now one of the most fundamental tasks in biochemistry. Its results influence the development of new products and processes in medicine (drug design), agriculture (crops development and modification, treating plants diseases), industry, etc. However, current protein sequence analysis is still a compromise between what is desired and what is possible. To help in solving this problem, the structural features of proteins have been divided into levels. Secondary structure prediction has been one of the most important aspects of structural analysis of proteins. A necessity of creating a tool that could be helpful in the prediction has been followed by considering the Logical Analysis of Data, a new, high accuracy, rule-based method. Thus, a new system has been proposed which generates secondary structure model based on the primary structure, being the input and finds rules responsible for prediction effect. The approach has led to relatively high prediction accuracy for certain protein structures. Subsequently, the research has been extended for developing models of simplified protein structures and an analysis of different aspects of protein chains.

Protein structure analysis and prediction in context of CASP

As a result of a collaboration with Protein Structure Prediction Center at the University of California in Davis, an important part of a robust system for automated processing of the

protein models, their evaluation and visualization of the results have been proposed and implemented. The system has been successfully used during the seventh edition of CASP (Critical Assessment of the Techniques for Protein Structure Prediction) experiment. CASP aims at establishing the current state of the art in protein structure prediction, identifying the progress that has been made, and highlighting future directions of the research in this area. The predictions are evaluated by independent assessors who are considered experts in the field. We have been cooperating with Protein Structure Prediction Center since 2004. Based on experience gathered during CASP experiments, we also try to find our own, preferably efficient and reliable methods for delineating domain boundaries and protein structure prediction. Our approach to the first problem resulted in the method that was the best in 30% of the cases in CASP7 (Fig. 1 presents two examples of the best domain predictions for, respectively, PDB id:2H28, and PDB id:2IVX proteins).

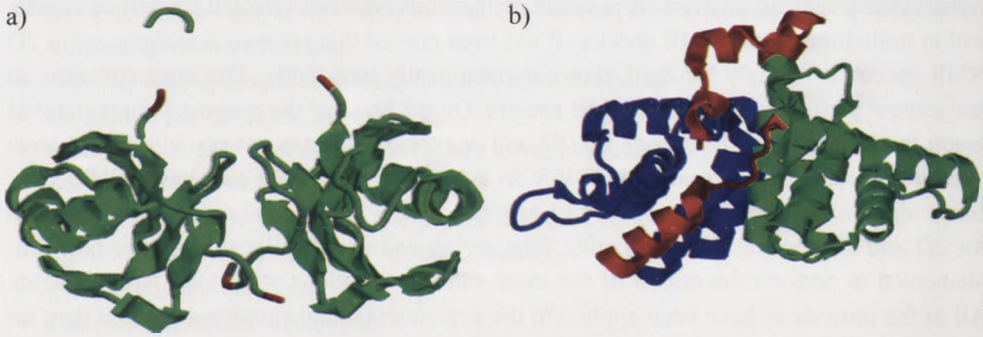

Figure 1. Exemplary best domain predictions in CASP7 found by the algorithm designed in our Labratory: a) CASP target T0304 (PDB id:2H28) – the green color denotes ideally predicted domain, red denotes prediction error; b) CASP target T0338 (PDB id:2IVX) – the green and blue colors denote ideally delineated parts of domains, red denotes prediction error.

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POZNAŃ SUPERCOMPUTING AND NETWORKING CENTER

Director
Jan Węglarz

Technical Director
Maciej Stroiński

Chairman of the Council of Users: ***Tomasz Łodygowski***

Keywords: • *NREN* • *Géant connectivity* • *Polish Optical Internet* • *PIONIER* • *optical networks* • *metropolitan area networks* • *ATM network* • *gigabit network* • *distributed processing* • *parallel computing* • *portals* • *Grids* • *security* • *data management* • *reliability*

Poznań Supercomputing and Networking Center (PSNC) was founded at the end of 1993 by the State Committee for Scientific Research (KBN) which supports the whole enterprise. Owing to the decision of the Conference of Rectors of the City of Poznań, PSNC has been affiliated to the Institute of Bioorganic Chemistry of the Polish Academy of Sciences (PAS).

Main fields of networking and supercomputing activities cover:

- operating the Polish Optical Network – PIONIER (Fig. 1), which connects 21 MANs using DWDM technology with 10 Gigabit Ethernet transmission system,
- operating Poznań Metropolitan Area Network (POZMAN), which bases upon own fiber optic cables and 10 Gigabit Ethernet technology,
- operating the International Connection to the Géant network basing upon 10 Gbit/s connectivity (primary link realized in POS technology and backup link in 10GE) and dedicated point-to-point links based on 10 Gbit/s lambda,
- supporting High Performance Computing and distributed computing between geographically distant domains,
- securing IT infrastructure,
- providing archivers and data backup environments.

PSNC is responsible for the research and development of:

- Polish Optical Internet project,
- technologies for next generation networking,
- Grid technologies,
- portals,

- IT security,
- eLearning and eContent services and applications.

One of the statutory tasks of PSNC is to research, test and disseminate new Internet technologies and ideas and to promote their use in the society. In order to fulfill this obligation, PSNC has been organizing conferences and workshops on networking and distributed computing since 1994.

Rapidly increasing number of Internet users and the vision of a future demand for terabit bandwidth have led to the proposal of the Polish Optical Internet – PIONIER project (see the detailed information on http://www.kbn.gov.pl/en/pionier/).

The concept of the project envisages the realization of three basic objectives:

- to develop the information sciences infrastructure in Poland up to the level which facilitates conducting research in the area of challenges of the contemporary science, technology, services and applications,
- to produce and test pilot services and applications for the information society which forms the basis for implementations in science, education, administration and economy,
- to let Poland compete in the area of software development for new applications.

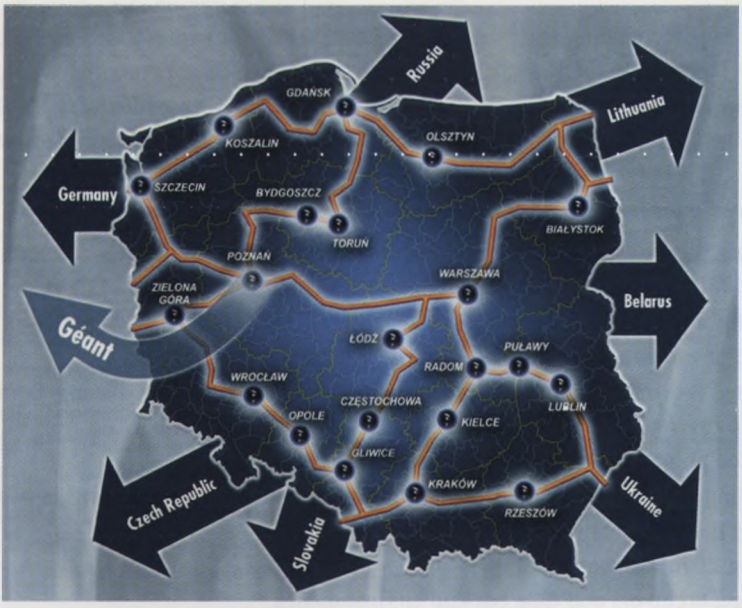

Figure 1. Polish Optical Network – PIONIER

The achievement of PIONIER concept is based on three paradigms:

- the Optical Intelligent Network basing upon own fiber optic cables,
- the network services organized in the structures of Grids,
- the access to the applications and the Grids via specialised portals.

Applications, services and technologies developed in the framework of PIONIER project will be implemented in all areas of life. Therefore, during the realization of the program, the costs of implementation will be incurred by the interested entities (ministries, government, telecommunication operators, hardware and software vendors, as well as other entrepreneurs).

In order to accomplish the tasks mentioned above, there are 190 employees in PSNC working in four main departments and in one for logistic, financial and administrative purposes.

International co-operation in European Framework Programmes: 5, 6 and 7

PSNC is engaged in over 20 international projects, mainly founded by the European Commission in the 6th and 7th Framework Programmes. Five of the projects have been coordinated by PSNC: DORII (www.dorii.eu), GridLab (www.gridlab.org), Phosphorus (www.ist-phosphorus.eu), Porta Optica (www.porta-optica.org) and RINGrid (www.ringrid.eu). In addition, PSNC is becoming the Microsoft Innovation Centre in co-operation with Microsoft Poland and Sun Centre of Excellence in co-operation with Sun Microsystems.

Our knowledge is used in security area of the POLISH PLATFORM FOR HOMELAND SECURITY (www.ppbw.pl). This Platform was established with the aim of creating integrated technology and computer tools to support activities enhancing public security. At the same time, it fosters cooperation in the area of security among the fields of science, research and development, and education.

Another example of R&D activity in co-operation with other HPC centres is the National Data Storage project, which aims to deliver a nation-wide remote backup service supported by the PIONIER network and MAN operators.

NETWORK DEPARTMENT

Manager: ***Artur Binczewski***

The main role of the department is to manage the countrywide **Polish Optical Network – PIONIER**. The network is based on own optical fibres and connects Metropolitan Area Networks in Poland, giving access to the Internet services and to the advanced telematic services. It also connects the supercomputing centers. The PIONIER network is built with multi-λ DWDM and 10 Gbit/s Ethernet technologies.

The PIONIER external links include: 2x10 Gbit/s connectivity to GÉANT – Pan European Research Network, 7.5 Gbit/s connectivity to the world Internet and a number of connections to the main telecommunication operators in Poland: TP S.A., Tel-Energo, PKP, and Exatel. PSNC manages the crossborder links to neighbouring countries as Germany, Czech Republic, Slovakia, Ukraine, Belarus, Lithuania or Russia. PSNC is responsible for the management and operations of complete PIONIER network and all of its external connections.

The Network Department is also responsible for the management of Poznań Metropolitan Area Network POZMAN which currently connects all universities, institutes of the Polish Academy of Sciences as well as research and development institutes within Poznań region directly via own fibre optic cables. It also integrates services for the business and administration areas, e.g. developing and maintaining the virtual network of the City Hall.

Additional activity of Network Department focuses on introduction of new networking technologies and the network expansion. These challenges are now being realized within the framework of the "PIONIER project: "Polish Optical Internet – Applications, Services and Technologies" which started in 2001. The project aims at the delivery of advanced and intelligent optical infrastructure for the academic society in Poland (over 5000 km of own fibers connecting all metropolitan area networks), provision of new services with guaranteed transmission quality and the development of advanced applications for the Information Society.

PSNC represents Polish scientific community in GÉANT2 – the European research network and it has acted in TERENA – the Trans-European Research and Education Networking Association. PSNC has been an international member of the Internet 2 Consortium and it was registered as a Local Internet Registry in the international organization - RIPE.

Apart from operational day-to-day services, the Network Department plays an active role in the European research activities, including projects like PHOSPHORUS, PORTA OPTICA, SEQUIN, ATRIUM, 6Net, MUPBED, TF-NGN, Cisco NGN Lab, IPv6 TF.

PHOSPHORUS project (coordinated by PSNC) is addressing some of the key technical challenges to enable on-demand e2e network services across multiple domains. The Phosphorus network concept and test-bed will make applications aware of their complete Grid resources (computational and networking) environment and capabilities, and able to

make dynamic, adaptive and optimized use of heterogeneous network infrastructures connecting various high-end resources.

Phosphorus will enhance and demonstrate solutions that facilitate vertical and horizontal communication among applications middleware, existing Network Resource Provisioning Systems, and the proposed Grid-GMPLS Control Plane.

PORTA OPTICA STUDY (coordinated by PSNC) was performed within the 6th Framework Programme. The main aim of the project is a collection of all the necessary information and initiating the development of the research networking in the Eastern Europe (Belarus, Moldavia, Ukraine), and baltic countries (Lithuania, Latvia and Estonia) as well as south Kaukas (Armenia, Azerbaijan, Georgia) which is enabling their connection to the GÈANT2 infrastructure.

SEQUIN, an acronym standing for "Service Quality Across Independently Managed Networks", involved eight partners from seven countries. The objective of SEQUIN was to define and implement the end-to-end approach to Quality of Service (QoS) that would operate on multiple management domains and exploit a combination of IP and ATM technology. PSNC has led "Proof of concept testbed" workpackage, where the work focused on the testbed setup and a proof-testing solution in European environment.

ATRIUM project was aiming at the exploitation of MPLS and terabit technologies. During this project, a testbed of terabit IP routers running MPLS over DWDM was set up in order to study advanced mechanisms providing high QoS for end users. Again – PSNC was responsible for a design of application test suite and final acceptance tests with the use of advanced GRID applications.

6NET is a joint enterprise of Cisco Systems and major leading research organizations in Europe. 6NET project has built an international, experimental IPv6 network which served as the testbed for all IPv6 related technologies. This project is supported by PSNC by the means of the development of new IPv6 management tools, testing existing DHCP solutions (http:// www.6net.org/).

Another project from the area of optical networking is called **MUPBED**. This multipartner European testbed is being built for validation of ASON/GMPLS technologies and its integration in multi-domain environment. In this project PSNC provides the study and practical solution to the problem of extending ASON/GMPLS mechanisms towards end users (http://www.ist-mupbed.org/).

There are some NoE Projects in the networking area as well – one of them is **EMANICS**. The main aim of the project as the network of excellence is the promotions and supporting the development in: research activities, integration, coordination the efforts required for the deployment of innovative network management solutions.

PSNC also hosts **Cisco Networking Academy** and **Cisco NGN laboratory,** where it provides training of next generation Internet technologies for home and office use, such as IP telephony, videoconferencing, wireless networking, etc. The training sessions are provided for SME in the Wielkopolska region.

PSNC has also established **Polish IPv6 Task Force**, the first Polish initiative to bring together those interested in the implementations of IPv6 protocol in Poland. This forum includes the representatives of both industry and science (http://www.pl.ipv6tf.org/).

TF-NGN – the task force of TERENA is composed of representatives of the National Research Networks and of research institutions. The Task Force aims to study and develop technologies that are viewed as strategically important for the GÉANT project and the NRENs. The responsibilities of PSNC there include:

- the leadership of next generation network equipment test group,
- the leadership of new transport protocols research group.

SUPERCOMPUTING DEPARTMENT

Manager: ***Norbert Meyer***

The activities of the Supercomputing Department cover several issues. The main one concerns the delivery of the HPC services, including the support for the end user and developing middleware for improving distributed computations. Other topics, not less important, concern the handling of appropriate IT security level (secure access to HPC infrastructure and the security of the national network PIONIER), and a delivery of storage services. The PIONIER-CERT is the Computer Security Incident Response Team that has been established to provide effective incident response service to the members and users of Polish Optical Network (http://cert.pionier.gov.pl/). We actively take part in R&D, especially in the development of middleware tools for the Grid environment, security in the open network environment and large data sets management, e.g. in the Polish Platform in Homeland Security or in co-operation with Microsoft under the Microsoft Innovation Center.

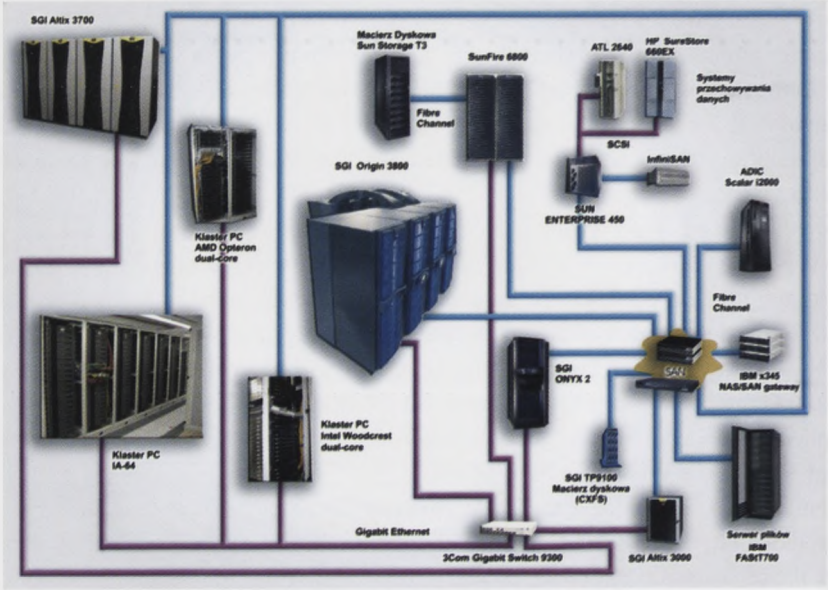

Figure 3. The HPC and storage infrastructure in PSNC.

Complex computations cannot be done on a single system because of the limitation of single computing node ability. PSNC remains consistent in carrying out the project of building a local HPC infrastructure covering various demands of our end users from universities and other R&D institutions. Therefore, the hardware infrastructure we currently have includes scalar systems and new generation clusters of PCs. This infrastructure allows solving important and difficult computational problems owing to its significant computational power (performance).

Nowadays, the local computational resources contain scalar systems: SGI Altix 3700, SGI Origin 3800, SunFire 6800, and tightly coupled clusters: Intel IA-64, Intel Xeon, AMD Opteron64, data exploration systems and visualization laboratory (SGI Onyx2). The summary peak performance of all these computers is about 11 Tflops. The storage infrastructure is based on SAN technology, including IBM FAStT700 Storage Server with a capacity of 150 TB, three e-servers xSeries IBM x345 with IBM Tivoli SANergy software and two IBM 2109 F-32 switches. Additionally, HSM software is used to enhance the storage space using the ADIC Scalar i2000 tape library. The entire capacity of the archive is 280TB (Fig. 3).

The main users of our computational systems are universities, research institutes, governmental institutions, and departments of the Polish Academy of Sciences. The users from Poznań generate over 60% of supercomputers load. The other amount of our system performance is used by scientific community from the biggest Polish universities in Wrocław, Gdańsk, Kraków, Łódź, Szczecin and Zielona Góra.

The group takes part in national R&D projects focused on:

- Virtual Laboratory (http://vlab.psnc.pl),
- Grids (BalticGrid, EGEE-2/3, int.eu.grid, RINGrid, DORII, gEclipse, CoreGRID, etc.),
- Security (Polish Platform for Homeland Security, Microsoft Innovation Center projects).

We actively take part in IST projects: BalticGrid (http://www.balticgrid.org), EGEE (http://www.eu-egee.org), IntEuGrid (http://www.interactive-grid.eu/) and CoreGrid (http://www.coregrid.net/), PRACE and e-IRG-SP2. We also coordinate DORII and RinGrid (http://www.ringrid.eu) projects. We lead an important role in designing graphical user interfaces to the Grid infrastructure for the mobile users. The solution (Migrating Desktop, developed within CrossGrid project) has been put into practice into the international grid testbed and was successfully tested by the scientific community. For the domestic scientific community, we offer the cluster of computational nodes which is a part of multi-clusters installation across Poland with more than 1000 processors. The set of tutorials are available for the wide user audience regarding usage of grid resources and PSNC resources in general.

The research activity of the Supercomputing Department covers also security in open network environments, support for the users in terms of the remote laboratory instruments' access (http://vus.psnc.pl/), and building software toolkits that will be useful for the users of the Grid computational environment. These software tools will increase the Grid environment capabilities and optimal utilization of the supercomputers performance by the Polish scientific users. In the scope of HPC, we also have considerable accomplishments in the research of the intrusion detection systems and the backup systems that allow to automate the process of making backup copies of the file systems.

NETWORK SERVICES DEPARTMENT

Manager: ***Cezary Mazurek***

The Network Services Department (NSD) is responsible for network services in POZMAN and PIONIER networks for research, education, local administration and business. These services include maintenance and provisioning of email accounts, discussion lists, ftp and virtual web servers. PSNC also provides DNS, NTP and News services for regional and country-wide customers and operates w3cache service. Moreover, the department operates 24x7 streaming services for regional and academic radio stations and provides ad-hoc streaming services for live events. All of the network services are continuously monitored for availability and performance.

Other activities of NSD concentrate on developing and managing advanced Internet services. The main activity areas include, but are not limited to:

- digital libraries,
- content delivery networks,
- telemedicine,
- information grids and data management,
- services for E-Government,
- audio and video streaming,
- education services,
- high definition videoconferencing,
- system interoperability and open source.

The works in this scope cover research projects aiming at design and development of the emergent services as well as deployments of the products being developed in this way. Some examples of the products and services are following.

In the scope of digital library, NSD is developing the dLibra framework. It is designed to be used for maintaining multiple collections of various digital assets, like digital books, video and audio files. dLibra supports the OAI-PMH standard which allows to interconnect independent digital libraries and share digital resources between the users of the libraries. dLibra is home to about 20 regional and institutional digital libraries, most of which are federated in the country-wide Federation of Digital Libraries (http://fbc.pionier.net.pl). This special service, built with the use of the OAI-PMH protocol gives access to over 100,000 digital objects.

NSD maintains the multimedia content distribution system which is used by Telewizja Polska S.A. (TVP – Polish National Television) to offer interactive television services (http://www.itvp.pl). The system is a result of the iTVP project, which was co-funded by TVP and the Ministry of Science and aimed at providing access to the TVP's archives and live broadcasts. It covered the issues of content digitalization, production, encoding, distribution, delivery and access in a secure and scalable way. Under this project, the Content

Distribution System Platform, which is structured in a two-level hierarchical way, has been developed and deployed. The system has been positively verified during internet broadcasts of live events such as music festivals and Olympic games.

As far as telemedicine is concerned, the works of NSD focus on supporting the regional healthcare in Wielkopolska with advanced services utilizing the capabilities of the optical network. To this end, NSD launched an internal project in cooperation with Division of Trauma, Burns and Plastic Surgery of the Poznań University of Medical Sciences, and the Institute of Computing Science of the Poznań University of Technology, which has aimed to design and develop a pilot system of telemedical services. The "Telemedycyna Wielkopolska" project developed a prototype of the medical teleconsultations service which has been tested with several hospitals in the region. Further works focus on creation of the Medical Digital Library that would allow to build value-added services such as medical teleeducation, clinical decision support and reporting.

In the area of data management, NSD has been involved within European project Gèant2 since September 2006 in the development of Common Network Information System (cNIS). Its aim is to harvest and store information about the computer network topology within a central repository, and further make it available to other network services. NSD issued version 1.0 of the cNIS platform in autumn 2007. It is planned that the software will be deployed in National Research and Education Networks in 2008.

In the area of e-Government services, NSD cooperates with the Poznań City Hall in developing the MIM – Multimedia City Guide (http://www.city.poznan.pl/), which has been online since 1995. It is a web-based service providing Poznań inhabitants and guests with useful information about the city and delivering them a group of electronic services. The MIM provides dedicated services for establishment of new SME business entities, obtaining certified copied from the Civil Registry, applying for the identification card and other that support Poznań citizens in day-by-day operations. The MIM provides also a set of vortal thematic services on education, natural environment, quality of life, tourism, sports, and services built on the basis of geographical information such as the city map, Old Town guide or the grave search engine for communal cemeteries.

As far as education services are concerned, the NSD deployed the Polish Educational Portal Interkl@sa group (http://www.interklasa.pl). It currently contains almost 10 various portals and services, including such services as Virtual Classroom (http://klasa.interklasa.pl), School Portals (http://ski.szkoly.interklasa.pl) and Frantice (http://frantice.interklasa.pl). They are a source of educational content and a forum for information exchange in the Polish education. NSD provides the Portal Framework, its services (e.g. e-mail, news, groupware tools, document management, virtual classroom, etc.), and tools for the remote content management.

High definition videoconferencing is the subject of work within the HDVIPER project. This project, realized under the CELTIC programme, aims to develop and deploy videoconferencing services utilizing high definition technology capabilities for use within business, medicine and education environments. NSD is responsible for usage scenarios in medicine and education.

NSD strongly supports establishment of open standards and usage of open source software. It participates in the FP6 Qualipso project (http://www.qualipso.org/), which aims to

provide a platform for trustworthy open source software to promote its usage in business and industry. NSD is involved in various activities, including supporting interoperability between systems and design of the open source software development framework.

NSD employs over 50 people involved in day-to-day operations of network services as well as in research and development works.

APPLICATIONS DEPARTMENT

Manager: ***Jarosław Nabrzyski***

The main role of the Applications Department is to support the users in using and developing scientific applications. Other activities of the Applications Department include developing tools and services for Grid computing. The main research activities of this department are focused on resource management in distributed environments, Grid portals, Grid authorization systems and mobile user services. The Department is responsible for development and integration of the Gridge Toolkit. Gridge Toolkit is an open source software initiative aimed to help users to deploy ready-to-use grid middleware services and create productive Grid infrastructures. All Gridge Toolkit software components have been integrated together and form a consistent distributed system following the same interface specification rules, license, quality assurance and testing. Gridge aims to be a Grid-In-The-Box solution that can be easily deployed on any distributed infrastructure.

Gridge Toolkit components have been successfully tested with different versions of Globus Toolkit as well as other core Grid middleware solutions. The Gridge Toolkit software is available for free with a full commercial support. In addition to Gridge, the Application Department offers to users:

- Technical support, consulting, training and development for Gridge Toolkit and Globus Toolkit,
- Assistance in design, deployment and configuration of grid middleware software,
- On-site installation and integration of Gridge Toolkit and Globus Toolkit,
- Workshops and hands-on training on 'Grid-Enabled' technologies.

The Applications Department is involved in numerous EU, nationally and comercially funded research and development projects. These include: EU-funded QoSCoSGrid, BREIN, ACGT, OMII-Europe, BeInGrid, Challengers, CoreGrid, Phosphorus, Federica and OGF-Europe, national "Multicriteria Grid Resource Management", or funded by France Telecom: "Autonomic Grid Computing".

Apart from research software implementations, the Application Department is responsible for maintenance and administration of scientific databases and HPC applications. It also provides wide support for PSNC application users. The Applications Department closely cooperates with other PSNC departments, especially in the field of Grid computing.

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CENTER OF EXCELLENCE FOR NUCLEIC ACID-BASED TECHNOLOGIES (CENAT)

Head: ***Marek Figlerowicz***

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Research Staff:	*Magdalena Alejska, Luiza Handschuh, Zbigniew Michalski, Agnieszka Żmieńko*
Ph.D. Students:	*Michał Góralski*

Keywords: • *functional genomics* • *proteomics* • *DNA microarrays* • *2D protein electrophoresis* • *mass spectrometry* • *gene expression* • *transcriptome analysis* • *microRNA* • *human genome* • *cancer research* • *plant genome*

At the end of 2004, the Center of Excellence for Nucleic Acid-Based Technologies (CENAT) was established at the Institute of Bioorganic Chemistry, PAS. The major goal of CENAT is introduction of new nucleic acid-based technologies in medicine, biotechnology and agriculture. Thus far, the most significant CENAT initiative has been the organization of a Regional Genomics Center (RGC). This laboratory, which is unique to Poland, was created as a joint venture of the Institute of Bioorganic Chemistry, Karol Marcinkowski University of Medical Sciences and Poznań University of Technology.

The broad fields of study of the Regional Genomics Center are functional genomics, proteomics and metabolomics of human, animals, plants, fungi and microorganisms. Proteomic studies are performed with the use of a 2D electrophoresis system and two mass spectrometers: LC/ESI/q-Tof equipped with nano-LC pump and Maldi-Tof. For the metabolomic studies, we employ GC/Tof with electron and chemical ionization sources. The laboratory is also equipped with instruments and software allowing for complex DNA microarray experiments from microarray designing and construction to its hybridization and analysis. We developed repeatable procedures of total RNA/mRNA/miRNA labeling and hybridization applicable to both self-constructed and commercial oligonucleotide or cDNA microarrays. Close cooperation with Laboratory of Bioinformatics provides professional support at the designing/analysis stage. Consequently, during the last three years, CENAT has become an advanced technological platform promoting interdisciplinary research and collaboration among different institutes in Poland.

Major research projects currently underway at the RGC:

Functional genomics and proteomics in cancer research. Model studies on the molecular mechanism of acute myeloid leukemia.
Grant from: Ministry of Science and Higher Education, 2006-2009.
Collaboration between: Institute of Bioorganic Chemistry PAS, Poznań University of Medical Sciences, Poznań Supercomputing and Networking Center and Poznań, University of Technology.

A new, gene expression-oriented method of mucopolysaccharidosis treatment.
Grant from: Ministry of Science and Higher Education, 2007-2009.
Collaboration between: Institute of Bioorganic Chemistry PAS and Gdańsk University.

The influence of the prebiotic bacteria on the gene expression pattern in human intestine cells.
Grant from: Ministry of Science and Higher Education, 2007-2009.
Collaboration between: Institute of Bioorganic Chemistry PAS and Agricultural University in Poznań.

Protein homocysteinylation as a factor affecting the profile of human gene expression.
Grant from: Ministry of Science and Higher Education, 2007-2009.
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Cross-reactive allergy induced changes in the profile of gene expression in human blood cells.
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Collaboration between: Institute of Bioorganic Chemistry PAS, Poznań University of Medical Sciences and Medical University of Białystok.

Identification of genes involved in *Bacillus subtilis* response to stress.
Grant from: Ministry of Science and Higher Education, 2006-2008.
Collaboration between: Institute of Bioorganic Chemistry PAS and University of Gdańsk.

Identification of genes involved in plants in response to osmotic stress.
Grant from: Ministry of Science and Higher Education, 2005-2008.
Collaboration between: Institute of Bioorganic Chemistry PAS and Institute of Biochemistry and Biophysics PAS, Warszawa.

Comprehensive analysis of the cucumber chloroplast genome trancriptional activity.
Grant from: Ministry of Science and Higher Education, 2007-2009.
Collaboration between: Institute of Bioorganic Chemistry PAS and Warsaw University of Life Sciences.

Identification of genes involved in *Brassica* plants response to pathogen infection.
Grant from: Ministry of Science and Higher Education, 2007-2009.
Collaboration between: Institute of Bioorganic Chemistry PAS and Łódż University

Diagnostic protein markers of breast cancer.
Grant from: Ministry of Science and Higher Education, 2006-2009.
Collaboration between: Institute of Bioorganic Chemistry PAS and Maria Sklodowska-Curie Cancer Research Center in Gliwice.

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