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New cytosine derivatives as inhibitors of DNA methylation

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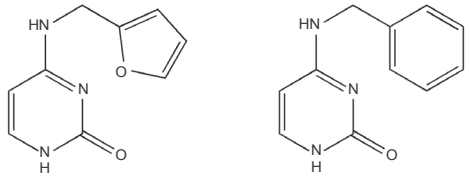
Neoplastic transformation is associated with alteration in DNA methylation, includes both global hypomethylation and gene specific hypermethylation. Great effort has been directed on towards development of novel strategies that can change the inappropriate gene methylation pattern in cells and thus, to redirect cell fate in human cancers.

It is believed that the creation of conditions for restoration of the pattern of epigenetic modification that is proper for every organism is an opportunity to reverse cancerogenesis.

DNA cytosine methylation catalyzed by DNA methyltransferase 1 (DNMT1) is an epigenetic route to gene expression regulation and development. Changes in methylation pattern lead to carcinogenesis. Inhibition of DNMT1 activity could be a good strategy of safe and efficient epigenetic therapy.

We present a novel group of cytosine analogs as inhibitors of DNA methylation, new methods of their synthesis and their effect on *in vitro* reaction of DNA methylation.

Inhibitory activity of each compound was analyzed in *in vitro* DNA methylation reaction catalyzed by DNA methyltransferase from *Spiroplazma*. Cytosine derivatives were divided into three groups according to modification at exocyclic amino group. 4-N-furfurylcytosine (I) and 4-N-benzylcytosine (II) (K_i 70 and 10 μM, respectively) were further modified.



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The best obtained inhibitors were 4-N-furfuryl-5-azacytosine, 4-N-benzyl-5-methylcytosine, 4-N-furfuryl-5-methylcytosine with K_i 0.7, 3.6 and 15 μM, respectively. These compounds cause a much greater reduction in the level of DNA methylation in cancer (HeLa) than in normal (HEK293) cells. Derivatives substituted with aliphatic chains at 4-N acted as uncompetitive inhibitors.

Almost all of analyzed compounds inhibit DNA methyltransferase activity in the competitive manner.

The results covered by patent co-financed by the European Regional Development Fund under the Operational Programme Innovative Economy.

SERRATE and CBC: two important factors involved in pre-mRNA splicing and microRNA biogenesis

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MicroRNAs (miRNAs) are small non-coding RNAs of about 21 nt in length, which take part in a wide variety of physiological and cellular processes. In plants, primary transcripts of miRNA genes, called pri-miRNAs, are synthesized by RNA polymerase II (RNAPII), and contain characteristic hairpin-like secondary structures in which sequences of mature miRNAs are embedded. These pri-miRNAs are processed by DCL1 into pre-miRNAs that consist only of miRNA-containing stem/loop structures which are then processed into miRNA/miRNA* duplexes. The duplexes are exported from the nucleus to the cytoplasm, where mature miRNAs are loaded into RNA silencing complexes (RISC) that induce mRNA cleavage or translational inhibition. Although the conversion of pri-miRNAs into mature miRNAs is catalyzed in the plant nucleus by one enzyme, DCL1, other proteins are also involved in the process. These are: CBC (a nuclear cap-binding complex, which is composed of two subunits, CBP20 and CBP80), a dsRNA binding protein HYL1 (HYPONASTIC LEAVES1) and a zinc-finger containing protein SERRATE (SE). It has been reported that these DCL1 partners are required for the efficient and correct excision of miRNA from pri-miRNA. Moreover, SERRATE and CBC have dual roles: in miRNA biogenesis and pre-mRNA splicing. Therefore, we compared the level of pri-miRNAs in *cbc, hyl1-2* and *se-1* mutants with the level observed in wt Arabidopsis plants. The results showed that the expression level of more than 50% *A. thaliana* pri-miRNAs was changed in the *se-1* and *cbc* mutants, and 40% in *hyl1-2*. In the case of the *se-1* and *cbc* mutants, we observe changes in the same pri-miRNAs, and in the *hyl1-2* mutant, the changes appear also in other precursors. In order to understand the processes in which the SERRATE protein is involved, we have decided to search for RNAs and proteins that interact with SERRATE. To this end, we have constructed the *Arabidopsis thaliana* transgenic plant, in which in the genetic background of the *se-1* mutant the FLAG-tagged version of the SE gene is integrated into the genome. We chose two stable lines with the highest expression level of FLAG-SE. Next, we carry out immunoprecipitation against the FLAG epitop of the fusion protein to find interactions between SERRATE and proteins and RNAs. Using Western blot, we confirmed the interaction of SE with CBP80 and HYL1. The interaction between CBP80 and SE has been previously detected by us in transfected Arabidopsis protoplasts using the BiFC method. Identification of novel protein partners of SE will be performed by mass spectrometry. In addition, we will also sequence RNA molecules present in the complexes precipitated with SERRATE.

Medicago ABCG10 transports (iso)liquiritigenin

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Full size ABC proteins belonging to the ABCG subfamily were identified mainly in plants and fungi. They are influencing different physiological processes, and play a particular role in defense response to biotic and abiotic stresses. There is growing number of evidence that ABCG transporters could be responsible for transport of antifungal, antimicrobial secondary metabolites and signaling molecules (Goossens et al., 2003).

The ABCG10 from *Medicago truncatula* was proposed as a modulator of isoflavonoid levels during the defense response associated with *de novo* synthesis of medicarpin (Banasiak et al., 2013). Expression analyses revealed that *MtABCG10* transcript is present in the vascular tissue of different organs and the corresponding protein has been found in the plasma membrane.

Treatment of roots with fungal cell wall oligosaccharides (general elicitor) resulted in a strong induction of *MtABCG10* expression together with genes coding enzymes from phenylpropanoid pathway namely: phenylalanine ammonia – lyase (PAL) and isoflavone synthase (IFS). Silencing of *MtABCG10* in Medicago hairy roots resulted in lower accumulation of phenolic compounds, among them were precursors of Medicago phytoalexin medicarpin.

Interestingly exogenous application of such precursors as liquiritigenin and isoliquiritigenin resulted in the induction of *MtABCG10* expression. Loading/transport experiment performed with liquiritigenin and isoliquiritigenin in *MtABCG10* silenced hairy roots has shown a significant differences in the transport efficiency of these compounds between wild type and *MtABCG10* silenced lines. We postulate that MtABCG10 is a transporter of liquiritigenin and isoliquiritgenin free aglycones.

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High-resolution crystal structures of complexes of plant AdoHcy hydrolase

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S-Adenosyl-L-methionine (AdoMet) is the most common donor of methyl group in cellular methylation of a wide range of substrates, from small-molecule compounds, such as norepinephrine, catecholamines or phospholipids, to macromolecules, including proteins, nucleic acids and polysaccharides. AdoMet-Dependent methylation generates equimolar amounts of *S*-adenosyl-L-homocysteine (AdoHcy), which is a strong inhibitor of AdoMet-dependent methylases. By removal of AdoHcy, *S*-adenosyl-L-homocysteine hydrolases serve as an important regulator of AdoMet-dependent methylation reactions. The enzyme controls the AdoMet:AdoHcy ratio, which is perceived as an indicator of transmethylation activities of the cell. AdoHcy hydrolase inhibition results in accumulation of AdoHcy in the cell. As a consequence, crucial AdoMet-dependent processes are stopped. *S*-adenosyl-L-homocysteine hydrolase catalyzes the reversible breakdown of AdoHcy to adenosine (Ado) and homocysteine (Hcy). The equilibrium of the reaction is shifted far into the direction of AdoHcy synthesis. In addition, the enzyme activity is inhibited by adenosine, which is a product of the hydrolysis reaction. Under physiological conditions, removal of Ado and Hcy is rapid and the net result is AdoHcy hydrolysis.

We present the first crystal structure of AdoHcy hydrolase of plant origin, from the legume yellow lupine (*Lupinus luteus*). The structures have been determined for three complexes of the enzyme, with a reaction byproduct/substrate (adenosine), its nonoxidizable analog (cordycepin), and a product of inhibitor cleavage (adenine). In all three complexes, the enzyme has a closed conformation. In addition to the adenosine, adenine or cordycepin ligands found in the substrate-binding domain, each subunit contains a tightly bound NAD^+ molecule in the cofactor-binding domain. A sodium cation is found near the active site, coordinated by residues from a conserved loop that hinges domain movement upon reactant binding. An insertion segment present in all plant AdoHcy hydrolases is located near an substrate-pocket access channel and participates in its formation. In contrast to bacterial and mammalian AdoHcy hydrolases, the channel is open when adenosine or cordycepin is bound, and is closed in the adenine complex. Contrary to *S*-adenosyl-L-homocysteine hydrolases from other organisms, which form tetrames, the plant enzyme is active as a homodimer.

Crystal structure of methylthioadenosine phosphorylase from Antarctic soil metagenomic library

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The rhodamine specific fluorescent protein (RSFP) comes from an Antarctic soil metagenomic library, which was screened for lipolytic active clones. One of the obtained clones lacked lipolytic activity, but surprisingly it showed a pink fluorescent phenotype in the presence of rhodamine B (RB) (Cieśliński et al., 2009). The gene responsible for this phenotype, named *rsfp,* encodes the enzyme with unknown function. Enzymatic and structural study allowed identification of its function as a putative methylthioadenozine (MTA) dependent phosphorylase (EC2.4.2.28) – the key enzyme of the methionine salvage pathway in most eukaryotes, Archaea and some bacteria. The *rsfp* gene was cloned and expressed in an LMG194 *E. coli* strain using an arabinose promoter. The obtained RSFP protein was purified in a two step procedure using the FPLC system. The crystal structure of phosphorylase in *apo* form and in the complex with RB were determined by us (Bartasun et al., 2013).

In order to crystallize a complex of RSFP with MTA the native protein was mixed with five times molar excess of MTA in presence of sulfate or phosphate. Diffraction data for RSFP/MTA complexes with sulfate and phosphate anions were collected at resolution 1.98 Å and 2.57 Å, respectively, using synchrotron radiation at BESSY Berlin.

The Fourier transformation (rigid body procedure) using RSFP *apo* (PDB ID : 4GLF) (Bartasun et al., 2013) as a starting model was applied to solve the crystal structures of the above complexes. The consistent indexing of all diffraction data was maintained in XDS and the structure refinement was performed using *REFMAC*. The crystal structures of RSFP/MTA/sulfate and RSFP/phosphate were compared to its *apo* form and to the other known MTA phosphorylase structures.

The monomer of RSFP has a mixed α-β architecture. The active form of the enzyme is a trimmer, which is reflected in the crystal symmetry. The trimmer assembly is built on the three fold component of the crystallographic 6_3-fold axis. The crystal structure of the RSFP/MTA/sulfate complex revealed one active site per monomer located in the vicinity of the highly flexible D222-D236 loop near the interface between monomers.

In summary, the crystallographic studies of RSFP revealed that this protein is able to bind a single MTA molecule in a deep binding pocket located in the cleft between neighboring monomers creating a trimmer only in the presence of a sulfate anion, which is isostructural to the physiological one – phosphate. In the presence of a phosphate anion there was no molecule of MTA in the active site of enzyme.

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Phytohormone-binding proteins in legume plants

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Plant pathogenesis-related proteins of class 10 show an ability to bind phytohormones. They are cytosolic proteins present in various plant tissues (Fernandes et al., 2008, 2009, 2013). Some their homologues are developmentally regulated. PR-10 proteins are also involved in the plant defense reactions. Among them, two major protein subclasses can be distinguished: very abundant and not abundant revealing less than 20% sequence identity to the first subclass. The low abundant protein subclass specifically binds cytokinins (CSBP, cytokinin-specific binding protein) (Pasternak et al., 2006). They evolved from a very abundant PR-10 protein class.

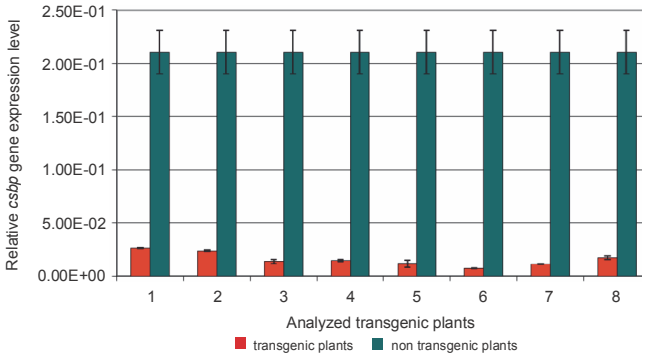

Fig. 1. RNAi-mediated *Medicago truncatula csbp* gene silencing in transgenic plants

In order to recognize physiological function of the MtCSBP, we have applied RNAi-mediated gene silencing strategy in a transgenic *Medicago truncatula*. The *Mtcsbp* coding DNA fragments (1-443nt and 1-130nt) were subcloned into pHellsgate 12 vector, introduced into *Agrobacterium tumefaciens* GV3101, and used for plant transformation. The transgenic plants were obtained *via* direct shoot organogenesis.

We have analyzed the expression pattern of *csbp* gene and other genes of the cytokinin transduction pathway in transgenic plants. As a consequence of *csbp* gene silencing (Fig. 1) a significant drawdown expression of the gene coding for cytokinin receptor *cre1* was pointed out. A visible decrease of the *pr-10.1* gene was also observed.

The *csbp* gene silencing causing visible changes in the *cre1* and *pr-10.1* genes expression level indicates that cytokinin perception in the cell is weaker and plant becomes less sensitive to phytohormone stimulus.

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Antibiotic bacitracin induces degradation of nucleic acids

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Recently, we have discovered that bacitracin induces degradation of nucleic acids, being particularly active against RNA (Ciesiołka et al., 2012). This observation is highly unexpected since several antibiotics from different therapeutic groups, which have been earlier tested in our laboratory, do not show such properties. Bacitracin is a polypeptide antibiotic active against Gram-positive strains. It is produced as a mixture of related compounds by *Bacillus subtilis* var *Tracy*. Since its discovery in the 40s of the former century bacitracin has been extensively applied against numerous bacterial infections. Despite some its analogs are applied in treatment of viral infections, bacitracin is advisable to be used, like other antibiotics, only against bacterial infections, especially the methicillin-resistant *Staphylococcus aureus* (MRSA) (Chapnick et al., 1996). It is believed that the mechanism of bacitracin action consists in disturbing cell wall synthesis by inhibiting dephosphorylation of the lipid carrier (Epperson et al., 2000; Ming et al., 2002). We have decided to characterize nucleolytic properties of bacitracin.

It turned out that RNA at concentration of 0.01 mg/ml in the presence of 10 mM bacitracin is completely degraded after 2 minutes while with 1 mM bacitracin 15% of the initial RNA was degraded. Cleavages occurred at guanosine residues, preferentially in single-stranded RNA regions. Complexation of the antibiotic with Cu(II) and the addition of H_2O_2 did not change the cleavage patterns suggesting hydrolytic mechanism of the reaction. Bacitracin was also able to degrade DNA to some extent but comparable effects to those observed with RNA required its 10-fold higher concentration; 250 mM bacitracin cleaved DNA while with 25 mM antibiotic no degradation products were detected. The phosphate groups at the cleavage sites were present at the 3′ ends of RNA products while at the 5′ ends of DNA fragments. Interestingly, the presence of 2 mM EDTA did not have any influence on RNA degradation but completely inhibited degradation of DNA. For DNA degradation divalent metal ions, like Mg(II), Mn(II) or Zn(II), were absolutely necessary. The presence of Mg(II) and Na(I) ions at high concentration affected RNA cleavage only slightly. Finally, two bacitracin samples from different commercial suppliers did not change their properties in the presence of nuclease inhibitors or after heat treatment. In summary, the experimental data support newly discovered nucleolytic properties of bacitracin.

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Influence of the SWI/SNF complex on alternative splicing in *A. thaliana*

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The SWI/SNF complex is an ATP-dependent chromatin remodeling factor which plays a key role in the regulation of eukaryotic chromatin structure. This dynamic multiprotein complex has the ability to remodel nucleosomes, preferentially at the promoter region of genes. The SWI/SNF complexes are recruited to their substrates by acetylated histone tails, and they use energy from ATP hydrolysis to modify DNA-protein contacts in the nucleosome. As a result, the processivity of RNA polymerase II can be changed, which, subsequently, influences processing of primary transcripts made by RNA pol II.

Using the RT-PCR panel based on fluorescently labeled primers we have analyzed alternative splicing in Arabidopsis *brm* and *swi3c* mutants (BRM and SWI3c are two subunits of the *A. thaliana* SWI/SNF complex). The results showed a significant effect of both the proteins studied on selection of pre-mRNA splice sites. Among the 265 splicing events analyzed, significant changes were observed in about 10% of all analyzed cases. Most of the changes were observed in the *brm* mutant, or were common to both analyzed swi/snf mutants. Among different splicing events tested exon skipping and intron retention were the most frequently effected in the mutants. Additional experiments carried out on the *brm* point mutant (the mutation disrupts only the ATPase activity of BRM), and the double *brm x swi3c* mutant clearly show two different mechanisms of the SWI/SNF influence on alternative splicing in *A. thaliana*: one that depends on SWI3c and the BRM ATPase activity, and another one which seems to be independent from the BRM enzymatic activity. More detail analyses of the results obtained in the *brm* point mutant revealed a preferential usage of splicing sites which are synthesized as first in a sequence of pre-mRNA. Our data prove a role of BRM and SWI3c in regulation of alternative splicing, most likely through changing the elongation rate of RNA polymerase II.

Variable length sequence variants of the 5′ untranslated region of p53 mRNA and their impact on the efficiency of translation initiation

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The p53 protein is involved in cell cycle control, DNA repair and induction of apoptosis. The synthesis of the full-length p53 protein is initiated from the AUG1 codon within exon 2. In the cell, there is also present ΔNp53 isoform, which lacks N-terminal transactivation domain and which is synthesized from the AUG2 codon localized in exon 4. Recently, it has been proposed that both proteins can be synthesized in the cap-independent mode using an IRES element present in the 5′ untranslated region (5′UTR) of the p53 mRNA. Moreover, it appears that p53 also makes use of several promoters and undergoes alternative splicing to generate multiple protein isoforms.

The aim of our study was to investigate the impact of different variants of p53 5′UTR on the efficiency of translation initiation of model mRNAs *in vitro* and *in vivo*. It turned out that all of the p53 5′UTR variants downregulated translation of *Renilla luciferase* reporter protein *in vitro* in Rabbit Reticulocyte Lysate (RRL). The presence of intron 2 sequence severely inhibited translation of reporter protein and it occurred only from AUG2 codon for ΔNp53 isoform. We also observed a significant decrease of translation efficiency for 5′UTR variants that included the region between two transcriptional promoters P0 and P1. In the case of variants containing both initiation codons, translation efficiency was much higher from AUG1 than from AUG2. Moreover, translation initiation from AUG1 was stimulated by the presence of 5′ cap structure and severely inhibited in the presence of increasing concentration of the cap analog. However, the presence of the 5′ cap structure and the addition of cap analog did not change the level of initiation from AUG2. These results suggested that the translation initiation from AUG1 codon was mostly cap-dependent while initiation from AUG2 showed rather cap-independent character. The results were similar when examined *in vivo* in HeLa and MCF-7 cell lines.

Structural mapping of p53 5′UTR variant that begins from P1 promoter and contains AUG1 and AUG2 codons showed that the two characteristic hairpin structures G56-C169 and U180-A218 were preserved *in vitro* and in RRL. Secondary structures of two extended variants of 5′UTR of p53 mRNA that corresponded to transcription initiation at promoter P0, as well as retention of intron 2, were also determined. It turned out that the P0-P1 region was folded independently in relation to the downstream sequences and was arranged into several small, relatively unstable hairpin motifs. Retention of the intron 2 sequence in p53 mRNA caused disappearance of important structural motifs and creation of new interactions. However, the large hairpin motif G56-C169 with the p53 initiation codon was still present.

The toe-printing analysis was also applied to investigate the formation of the ribosomal complex on the model mRNA constructs. The toe-print pattern was detected downstream of the AUG2 codon for ΔNp53 isoform. The lack of toe-print for the AUG1 codon might be caused by its localization within the highly-structured hairpin G65-C169.

Studies of influenza virus vRNA secondary structure

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Influenza A viruses (IAV) are members of the Orthomyxoviridae family. They constitute a heterogeneous virus population containing subtypes, which are defined as any combination of their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) (Bouvier et al., 2008).

IAV is the causative agents of annual epidemics and occasional pandemics of respiratory disease in human. It is estimated that infection with seasonal strains of influenza virus results it the death about 250 000-500 000 people per year (Neumann et al., 2009).

Influenza A virus possesses an eight segment (–) sense RNA genome, which codes for at least eleven proteins. The viral RNAs are associated with the polymerase complex and nucleoprotein (NP), forming ribonucleoproteins (RNPs). RNPs show a double-helical conformation in which two NP strands of opposite polarity are associated with each other along the helix. The fraction of the RNA sequence is exposed at the surface of the RNP. That could indicate a specific spatial disposition in the RNP, which determine its interaction with other(s) RNP(s) (Arranz et al., 2012).

Knowledge about secondary structure of influenza virus RNA is limited. The influenza virus genome segments contain highly conserved sequences at the 5′- and 3′-termini that are complementary to each other. These 5′- and 3′- ends can form a structural motifs: „panhandle", „fork", „hook", „corkscrew" and it depends on replication cycle phase. This fact can suggest a significant role of secondary structure in regulation virus life cycle (Gultyaev et al., 2010). As well there was revealed existence of hairpins in mRNA NP and NS1, which are necessary for the efficient gene expression (Ilyinskii et al., 2009). Bioinformatics studies indicate existence of conserved structural motifs (pseudoknt, hairpin) in mRNA, which could regulate a translation virus proteins (Moss et al., 2012).

Here we present secondary structure of segment 7 and 8 vRNA influenza virus A/Viet Nam/1024/2004 (H5N1). In our research SHAPE method (*Selective 2′-Hydroxyl Acylation analyzed by Primer Extension*) and chemical mapping with DMS (*dimethyl sulfate*) were used. Modification sites were identified by primer extension reaction. There were used 6-FAM labeled primers (seven primers for segment7 vRNA and six primers for segment 8 vRNA). The data from both mapping were introduced to RNAstructure 5.4 software to generate secondary structure of segment 7 and 8 vRNA.

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Effect of chemical modification on G-quadruplex structure composed of two trinucleotide CGG repeats

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Our studies focus on the conformational properties of RNA fragments containing two trinucleotide CGG repeats. The CGG triplet repeat found within the 5′UTR of the FMR1 gene is involved in the pathogenesis of both fragile X syndrome and fragile X-associated tremor/ataxia syndrome (FXTAS). The repeats can form both hairpins and quadruplexes but the structure of CGG RNA repeats have not been well defined. In solution, the $r(CGG)_{17}$ molecule adopts a hairpin structure with the stem formed by alternating C:G, G:C and the non-canonical G:G base pairs (Sobczak et al., 2003). On the other hand, an evidence was presented that premutation – range $r(CGG)_{30\text{-}99}$ tracts can form stable intramolecular quadruplex (Ofer et al., 2009).

In the presence of sodium ions G-CGGCGG-C molecule exists in conformational equilibrium between duplex and quadruplex. Higher concentrations of G-CGGCGG-C drive equilibrium strongly towards tetramolecular quadruplex form. Analysis of NMR spectra recorded for 1.65 mM RNA concentrations revealed the presence of cross-peaks characteristic of the parallel quadruplex structure with external loops. This quadruplex folds into a dimeric structure containing four G:G:G:G tetrads and two G:C:G:C mixed tetrads (Fig. 1A). Changing the *anti/syn* conformational property of one of guanosine residues it is possible to invert orientation of the strands in this tetramolecular structure. The substitution of one of guanosine residues to 8-bromoguanosine or 8-methylguanosine (G-C^{Br}GGCGG-C, G-C^{Me}GGCGG-C), changes the topology of quadruplexes, from parallel into antiparallel arrangement. Both quadruplexes are formed in solution by association of two identical duplexes through their major grooves. The resulting antiparallel quadruplex is unusual because it is composed of two isolated G:$^{Br/Me}$G:G:$^{Br/Me}$G tetrads separated by two mixed G:C:G:C tetrads (Fig. 1B).

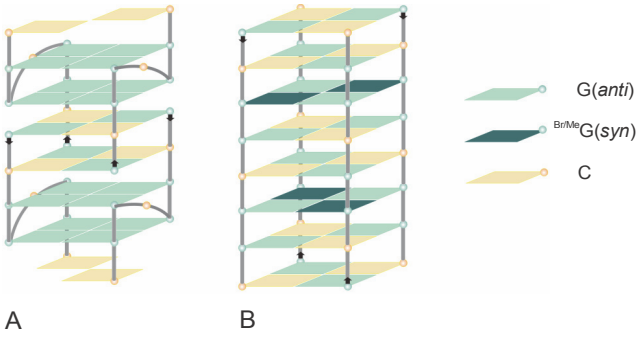

Fig. 1. Schematic structure of quadruplex formed by RNA sequence: A) GCGGCGGC, B) GC$^{Br/Me}$GGCGGC

Acknowledgements

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A novel *Medicago truncatula* ABC transporter from the G subfamily

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Legume plants have a unique capacity to interact symbiotically with nitrogen-fixing soil bacteria known as rhizobia. This intimate association results in the formation of root nodules that provide an environment suitable for atmospheric nitrogen conversion into a reduced form readily assimilable by a plant. A key event of the infection process required for nodule organogenesis is activation of the cytokinin signaling pathway in the root cortex, leading to the suppression of polar auxin transport and cortical cell division. However, elucidation of the mechanism that allows localized cytokinin transport/signaling in the inner root tissues remains elusive (Oldroyd et al., 2011). A major function fulfilled by ATP-binding cassette (ABC) proteins is transmembrane translocation of great variety of molecules. Mounting evidence suggests that full-size ABCG transporters could be responsible for transport of signaling molecules, crucial for successful symbiosis between legumes and rhizobia (Sugiyama et al., 2007).

Previously, we have identified and classified 19 full-size ABCG proteins from *Medicago truncatula* (Jasinski et al., 2009). Here we present a novel, root expressed full-size MtABCG transporter and we address a question about its putative role in the modulation of nitrogen-fixing symbiosis. The conducted qRT-PCR analysis revealed that the expression of this transporter is strongly up-regulated during inoculation with *Sinorhizobium meliloti* as well as cytokinin treatment. We hope that the presented data provide a foundation for further studies on the role of *Medicago* ABCG transporters in symbiotic interactions.

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New methods for the determination of RNA secondary structure – preliminary results

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Secondary structure of ribonucleic acids (RNAs) consists of double helices, pseudoknots and single stranded fragments, which can be divided into: internal loop, hairpins, bulges and junctions. Secondary structure of RNAs has direct influence on its biological function in *in vivo systems*, therefore its prediction is important for understanding molecular mechanism of RNAs action and origins of pathogenic processes. There are numerous methods for the determination of RNAs secondary structure including enzymatic, chemical, crystallographic or computational. Although all these methods provide useful information about secondary RNAs structure, they are time-consuming and laborious. Taking under consideration the rate of discovery of new RNAs and limitation of tools mentioned, the urgent need of development of novel, universal method for the determination of RNAs secondary structure appears (Kierzek et al., 2008).

Developed method is based on differences in chemical stabilities of 2′-5′ phosphordiester bonds in double and single stranded regions of RNA. Previous experiments conducted by Usher et al. revealed that 2′-5′ phosphodiester bond located in double stranded region of RNA undergoes hydrolysis at physiological or mild alkaline conditions. When 2′-5′ internucleotide bond is situated within the loop, its hydrolysis rate is similar to 3′-5′ phosphodiester bond hydrolysis rate (Usher et al., 1976). Although 2′-5′ phosphodiester bonds occur in nature during RNA processing, there is a lack of enzyme which could introduce this linkage to RNA molecules. This problem can be overcome by acid hydrolysis of 3′-5′ internucleotide bonds and their isomerization to 2′-5′ bonds (Mikkola et al., 2002).

In this work, RNA hairpins models with 2′-5′ phosphodiester bond placed in double (stem) or single (loop) stranded regions and 3′-5′ hairpins were folded and treated with 50 mM ethylene diamine hydrochloride solution at room temperature. Oligonucleotides fragments were separated by polyacrylamide gel electrophoresis. The method of introducing 2′-5′ internucleotide bonds into RNA was also carried out.

First experimental data confirmed observation made by Usher et al. Hairpins with 2′-5′ phosphodiester bonds localized in double strand region were more labile in ethylene diamine hydrochloride solution at room temperature than 2′-5′ single stranded hairpins and natural hairpins. The results presented show that the method allows reliable determination of single and double stranded region of RNAs.

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Changes in epigenetic marks level in response to herbicide stress in *Zea mays*

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The term "epigenetics" defines heritable states of gene activity level not encoded in the DNA sequence. In recent years, it has become clear that dynamic changes in chromatin properties like DNA methylation and histone modifications, also contribute to transcriptional and post-transcriptional regulation of gene expression, important for stress responses. Epigenetic regulation of gene expression is dynamically developing field of research, especially in plant biology.

The domestication of maize started 7500 years ago and it is widely cultivated till today. Among rice and wheat it is one of the most spread cultivated cereals in the world which cultivation develops dynamically (in 2012 in Poland corn area was approx. 1 mln has). The maize is one of the most important fodder crops cultivated in our climatic zone; with great economic consideration and wide industrial application. In cultivation of the corn widely applied is Roundup, a foliar herbicide of the systemic activity. It is absorbed by green parts of plants, and then translocated through the whole plant causing their necrobiosis.

In research carried out in our laboratory we observed various reaction of different strains of maize to herbicide. For our experiments we selected two strains displaying the natural tolerance or sensitivity to glyphosate. The aim of the work is examining the molecular mechanisms which determine this feature.

The use of the microarray technology with probes specific for *Zea mays*, allowed us to infer about changes in the genes expression level in the corn under stress conditions caused by herbicide Roundup, depending on the time (days 0 and 7 after treatment). Bioinformatic analyses of results were carried out to select genes involved in the plant response to the abiotic stress.

To investigate the effect of stress conditions on m^5C level in plants that could reveal difference in stress resistance, we applied two-dimensional TLC separation method of radioactive labelled nucleotides from enzymatic hydrolysate of total DNA. Subsequently we examined level of C methylation in sequence of genes. We successfully used bisulfite and ChIP methods to estimate level of m^5C and H3 modifications in sequence of genes which indicated significant changes in expression level and could be involved in molecular mechanisms of tolerance feature.

In our observations we imply that there is statistically significant difference in epigenetic marks level in plants sensitive and tolerant for herbicide stress and its correlation with changes in gene expression level.

Aryl nucleoside phosphoramidates as new anti-HIV prodrugs

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Nucleoside analogues (*e.g.* AZT) are important class of drugs used in AIDS therapy. Antiviral activity of nucleoside analogues strongly depends on their bioactivation by cellular kinases. The mechanism of action of these compounds is well recognized and proceeds *via* their enzymatic conversion to the corresponding mono, di- and finally triphosphates (HIV-RT inhibitors). Unfortunately, this strong dependence on enzyme-mediated activation sometimes may lead to loss of antiviral potency (in TK^- cells). This limitation in applications of nucleoside analogues, prompted to search of new type of antiviral drugs and as a result the idea pro-nucleotides was born.

The finding that nucleoside amino acid derived phosphoramidate monoesters are exceptionally highly active against HIV (Wagner et al., 1995) suggest that the nucleoside (*N*-aryl)phosphoramidates may constitute a unique group of nucleotide derivatives with high antiviral potency. In our laboratory we designed and synthesized nucleoside (*N*-aryl)-phosphoramidates of type **3** (Fig. 1).

For the preparation of these compounds we decided to use *H*-phosphonate chemistry. *N*-arylamines reacted smoothly with nucleoside *H*-phosphonate **1** in the presence of diphenylchlorophosphate (DPCP) as condensing agent, producing rapidly and nearly quantitatively nucleoside (*N*-aryl)-*H*-phosphonamidates **2**. These were oxidized and treated with an iodine-water system, yielding the respective (*N*-aryl)phosphoramidates **3** quantitatively.

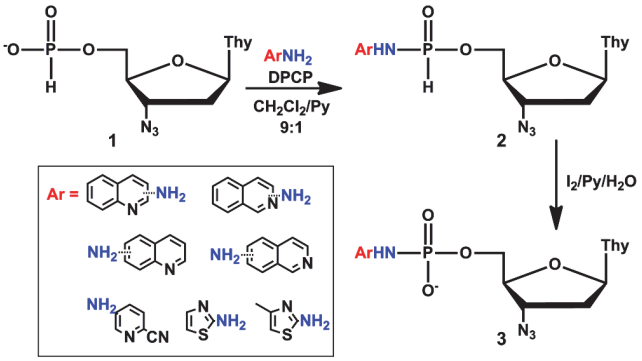

Fig. 1. Synthesis of nucleoside (*N*-aryl)phosphoramidates **3**

We examined stability ($t_{1/2,}$ HPLC) and decomposition pathways in cell culture media [RPMI, RPMI/FBS 9:1 (v/v)]. All investigated compounds were converted into biologically active form with optimal kinetics.

Antiviral potency and cytotoxicity of our nucleoside (*N*-aryl)phosphoramidates were examined in cooperating laboratory (dr A. Piasek, Warszawa). All investigated nucleoside (*N*-aryl)phosphoramidates were non-toxic ($CC_{50} < 200$) and highly anti-HIV potent ($EC_{50} < 0.0007$).

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Deep sequencing characterization of *Sus scrofa* piRNA fraction shared between female and male gonads

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Small RNAs are essential for proper germ cell development and analysis of mechanisms involving these RNAs in germ cell regulation is big challenge of molecular genetics. To address this challenge we characterized three families of small RNAs (piRNA, miRNA and ftRNA) present in *Sus scrofa* gonads using Illumina® approach. Particular attention was paid for RNA fraction shared between male and female gonads. In the case of piRNA, we demonstrate that despite of similar number of reads for the gonad's piRNAs, number of original piRNA sequences in ovaries is almost 10 fold lower. In addition 2.5% of piRNA and 10% of miRNA occurring in testis are also presented in ovaries. Shared piRNA fraction match in prevalence part to introns of ribosomal RNA and tRNA sequences similarly as ovarian piRNA and their biogenesis is proceeded from overlapping sequences. It should be noted that the shared miRNA family is conservative, among the most abundant miRNAs majority is identical with miRNA found in other organisms. In addition in ovaries highly abundant ftRNA sequences were found. We also suggest that the mechanism of *Sus scrofa* piRNA biogenesis is different than the ping-pong mechanism postulated for *Drosophila* piRNA biogenesis. Only piRNA population isolated from porcine testes contains 1U bias, however, lacks of 10A bias. While piRNA population isolated from ovaries does not include 1U bias.

Secondary metabolism of model Brassicaceae species responding to *Plectosphaerella cucumerina* infection

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Secondary metabolites constitute a highly diversified group of compounds, which for decades was considered to be not essential for growth and reproduction of flowering plants. However, some of them appeared to be critical for plant responses to the environmental stresses, including pathogen attack. Model plant *Arabidopsis thaliana* synthesizes and accumulates constitutively tryptophan-derived β-thioglucosides known as indole glucosinolate (IG). Recent studies revealed a pathway for IG metabolism that is triggered by a number of fungal and oomycete pathogens, and is essential for the pre-invasive defence of *A. thaliana* (Bednarek et al., 2009; Bednarek et al., 2011). In addition, infection of this model species leads to biosynthesis of other Trp-derived secondary metabolites with function in immunity. In our study we further analyzed conservation of Trp-metabolism between *A. thaliana* and its relatives – *Capsella rubella, Cardamine hirsuta* and *Arabis alpina.* For this purpose we performed LC/UV/MS metabolite profiling of leaf extracts from plants inoculated with *A. thaliana* adapted or non-adapted isolates of ascomycete pathogen *Plectosphaerella cucumerina* (Fig. 1). We also carried out bioinformatic analysis of accessible genomes of *A. thaliana* relatives, which included identification of possible ortholog genes encoding enzymes involved in Trp metabolism. Furthermore, we corroborated the above mentioned results with transcriptome analysis. To this end, we performed RT-PCR reactions for selected ortholog genes encoding enzymes involved in Trp metabolism and corresponding transcriptional factors found in *C. rubella* genome. Our studies indicated partial conservation of Trp-metabolism between *A. thaliana* and its tested relatives, which suggest an ancient and important role of this pathogen-triggered metabolic pathway in defence responses of Brassicaceae plants. However, we also identified some species-specific genes and metabolites.

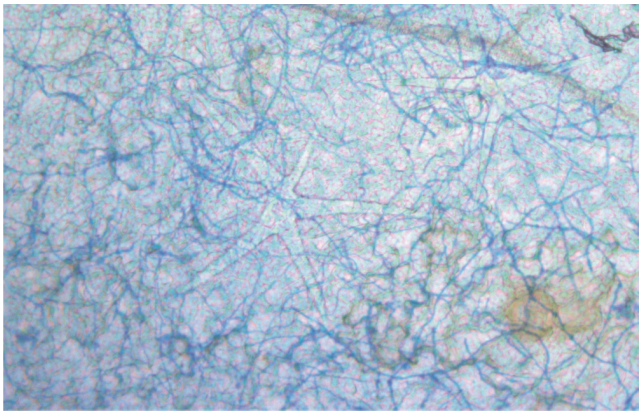

Fig. 1. Micrograph of *C. rubella* leaf inoculated with *P. cucumerina* isolate adapted on *A. thaliana* 72 hours post-infection

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Secondary structure and alternative splicing regulation of pre-mRNA MAPT gene

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Product of MAPT gene protein tau belongs to microtubule associated protein family. During alternative splicing from pre-mRNA MAPT gene are created six isoforms of tau. In adult human brain protein tau occurs in axonal part of neurons. The main function of this protein is polymerization and microtubule stabilization. In pathological state protein tau is presented in all parts of the nervous cell, moreover it can form into paired helical filaments and neurofibrillary tangles resulting the diseases known as tauopathies. In this group of diseases we can distinguish diseases linked to Alzheimer Disease (AD) and Frontotemporal Dementias (FTDs). Recently, diseases connected to dementias that were linked to chromosome 17 were named Frontotemporal Dementia and Parkinsonism Linked to Chromosome 17 (FTDP-17). In 1998 was found the first mutation in MAPT gene, nowadays were uncovered over 40. MAPT gene mutation can be divide to two types. The first type cause the change in biochemical properties of protein tau. The second, studied in our laboratory, disrupts alternative splicing exon 10. MAPT gene consist of 15 exons, furthermore alternative splicing exons 2, 3, 10 causes formation six protein isoforms. Alternative splicing of exon 10 produces form 3R (without exon 10) and 4R (with exon 10) protein isoforms. In healthy brain the ratio of form 3R and 4R is approximately 1. Our research focuses on the end of exon 10 and beginning of intron 10-11 interestingly this fragment forms hairpin structure. Destabilization of this hairpin are induced by mutation cause splicing reduction of exon 10, what follows change in ratio 3R/4R tau isoforms. The effect of disorder in quantity of 3R and 4R isoforms is pathological state moreover FTDP-17 disease. It is supposed that relaxed form of the hairpin is better available to interact with U1snRNP. Thermodynamic studies were performed using the 11 RNA molecules 25/26 nucleotides length with fragment with regulatory hairpin: wild type RNA-WT, six mutants naturally occurring in humans and destabilizing hairpin – DD-PAC, 11C, 12U, 13G, 16U, 19G, two molecules with DD-PAC mutation and with stabilizing mutations – DDI-17T, DD-10C, two molecules with stabilizing mutations – WTI-17T, WT-10C. UV melting experiments enabled to obtain the thermodynamic parameters all mutants, besides was done additional UV melting experiments with mutants and stabilizing hairpin molecules: neomycin, kanamycin, tobramycin, mitoxantrone. Fragments pre-mRNA MAPT gene 195/196 nucleotides length were used to predict secondary structure 11 RNAs the same mutation as used in UV melting experiments. RNA molecules consists of 39 nucleotides of intron 9-10, 93 nucleotides of exon 10 and 63/64 nucleotides of intron 10-11. To determine the secondary structures pre-mRNA MAPT gene were used chemical mapping DMS and SHAPE besides isoenergetic RNA microarray. Data from this experiment were used in predict 2D structure using programs: RNAStructure and vsfold5, in addition was proposed 3D structure using RNA Composer program.

Modified antisense oligonucleotides as allele-specific agents

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Antisense oligonucleotides have been studied for many years and are well known as tools for gene silencing. However, it's difficult to find a good antisense molecule, especially in case where a point mutation occurs and is the only factor differentiating wild type of gene from mutant. Based on thermodynamics of nucleic acid duplexes, we propose a strategy for allele-selective silencing of mutated RNA by RNase H activation mechanism, considering two approaches. The first one involves the use a pair of oligonucleotides, of which one as fully-complementary to mutant RNA, and capable to activate RNase H (so-called active one), should promote its hydrolysis, while the second, as fully-complementary to wild RNA (so-called passive), should protect it from RNase H cleavage. Essential for obtaining selectivity are modified nucleotides occuring in particular parts of antisense oligomers. Another proposed method to obtain allele-selectivity between wild type and mutant, is to use one antisense oligonucleotide, which introduce in the area of a formed duplex, different structural motifs, such as the one-side bulges and double mismatches. We assume that structure of formed duplex could affect RNase H binding and cleavage as well, and the motifs are the factors differentiating wild type RNA-ASO duplex from mutant-ASO one. Presence of mismatches and modified nucleotides in particular parts of formed duplex, should force hybridization of antisense oligonucleotide to proper RNA, providing alelle-selectivity of action.

G-quadruplex structures of AGG, CGG and UGG RNA repeats

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The tandem repeats of trinucleotide sequences (TNRs), also known as microsatellites, are present in many eucariotic and procariotic genomes and transcriptomes. The TNRs demonstrate extreme instability and are often polymorphic in length. Abnormal expansion of certain TNR sequences in some specific genes underlies a number of severe neuromuscular and neurodegenerative disorders. Bioinformatic studies have shown that some of repeats are widespread, while others are underrepresented in the human genome. Analysis of the human exonic sequences showed that among all 1030 TNRs tracts consisting of six or more consecutive trinucleotide units the most common are CGG (365 instances), CAG (301 instances) and the AGG repeats (169 instances). By contrast, these long UGG repeats are also found in human transcripts, but relatively rarely (only 6 cases) (Kozlowski et al., 2010).

The detailed knowledge of spatial structure of TNRs may be crucial for understanding the pathogenesis of many human diseases associated with their expansion and the structure of these molecules is believed to play a significant role (Krzyzosiak et al., 2012). Biochemical and biophysical studies of 20 different RNA triplet motifs (repeated 17 or 20 times) have shown that TNRs can be assigned to one of four structural classes: stable hairpins, semi-stable hairpins, G-quadruplexes and not forming any higher order structures (Sobczak et al., 2010).

Studies of $(AGG)_{17}$ and $(UGG)_{17}$ transcripts revealed that only these molecules are able to easily fold into G-quadruplexes. Although structure probing and nondenaturating gel electrophoresis indicated that $(CGG)_{17}$ molecule can adopt stable hairpin form, however, CD spectra provided ambiguous results (Sobczak et al., 2010). We have found, that under specific conditions also molecules built from CGG repeats can fold into quadruplex. Our studies focus on the structure determination of RNA molecules composed of two AGG, CGG and UGG repeats. We propose structural motifs, which we believe, are unique to each type of TNRs. NMR, UV and CD spectroscopies demonstrate that 5′ AGGAGGA 3′ molecule exists in dimeric form with characteristic G:A:G:G:A:G hexad, 5′ GCGGCGGC 3′ molecule can also fold into dimeric structure containing characteristic C:G:C:G tetrad and finally, 5′ pUGGUGGU 3′ molecule adopts a very stable structure composed of G:G:G:G and U:U:U:U tetrads.

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Stereochemistry of phosphorylating agents comprising nucleophilic catalytic groups

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Synthetic nucleotide analogues comprising chiral internucleotide bond with defined configuration are interesting compounds that could improve antisense therapy (Eckstein, 2000).

Preparation of such analogues is a great synthetic challenge. One of solutions is the concept of intramolecular catalysis, in which nucleophilic protecting groups are covalently connected to the phosphorus atom as stabilizer of configuration (Almer et al., 2004).

In our investigations we are going to elaborate methods for the synthesis of stereochemically pure chiral phosphorylating agents containing nucleophilic catalytic groups. These compounds might serve as universal agents that introduce chiral phosphorus groups into any compound, e.g. nucleosides, amines, thiols (Fig. 1). This aim requires elucidation of reaction mechanisms in the first place.

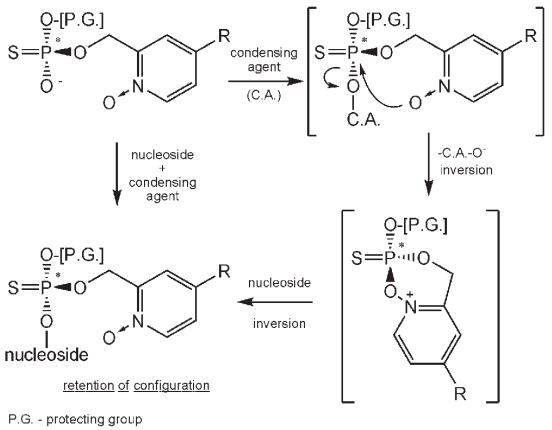

Fig. 1. Postulated mechanism of action for chiral phosphorylating agent comprising catalytic group

Preliminary experiments on achiral *H*-phosphonate monoesters comprising structures of pyridine N-oxide as catalytic groups are aimed to gain the knowledge about the mechanism and to optimize reaction conditions.

Condensation of *H*-phosphonates carrying catalytic groups revealed complexity of studied processes because, apart from the desired *H*-phosphonate diester, phosphate diesters and pyrophosphates were also formed due to competitive reactions. A noticeable relationship between donor-acceptor nature of substituent in the aromatic ring and catalytic as well as oxidative activity of N–O group was observed.

We found a number of factors influencing the course of reaction, e.g. type of condensing agent and solvent, presence of base and its pK_a, reaction time and molar ratio of reagents. Optimization of reaction conditions resulted in significant elimination of undesired by-products.

Selection of the most effective nucleophilic catalyst group built in the phosphorylating agents is a subject of current study.

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Structural dynamics of Dicer protein

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Dicer-like proteins (DCL) belong to the RNaseIII enzyme family and are involved in the RNAi pathway, which leads to sequence-specific translational repression of genes. The Dicer from parasite *Giardia intestinalis* (GiDicer) is a minimal functional enzyme and its crystal structure is available (MacRae et al., 2006). However little is known about dynamics of interaction between Dicer and its substrate: double-stranded RNA. Since flexibility of proteins plays a critical role in molecular recognition, present study was performed to predict dynamics of Dicer using computational methods. Comparative analysis of the dynamic properties of Dicer was carried out with ProDy (Bakan et al., 2011), which is a free and open-source Python package, designed for structure-based analysis of protein dynamics. Dominant patterns in structural variability were extracted by principal component analysis (PCA) of the four chains available in crystal structure of GiDicer and compared with theoretically predicted conformational dynamics. There were two ways to obtain theoretical data for protein dynamics: (i) prediction using normal mode analysis (NMA) based on elastic network models (ENMs): ANM and GNM, (ii) extraction from molecular dynamics (MD) trajectories using essential dynamics analysis (EDA).

The results obtained from all methods reveal mutual correlation. Theoretical predictions pick up the most flexible regions of GiDicer, in agreement with crystallographic β-factors. Three slowest modes of motions calculated by each of the applied analysis show similar directions of structural changes. Some results from the analysis of GiDicer flexibility are shown in Figure 1: A) network model, B) PCA vs ANM modes of motion for Dicer, C) X-ray β-factors vs calculated mean-square fluctuations (GNM).

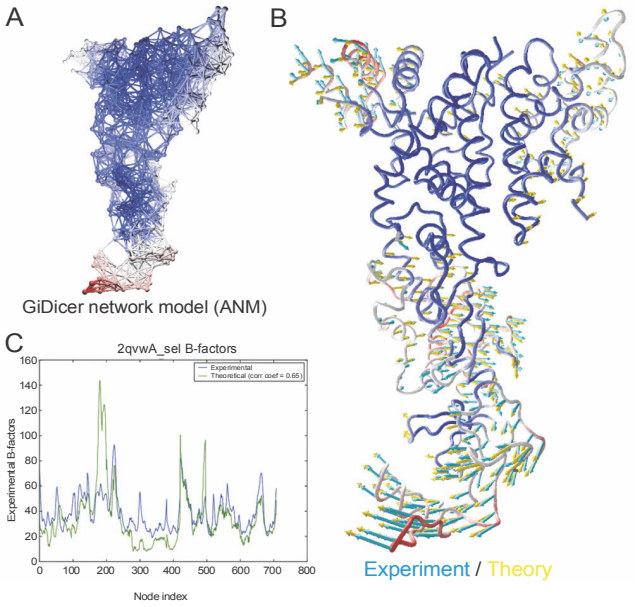

Fig. 1. Comparative analysis of GiDicer dynamics from experiments (PCA) and theory (ENM, MD) performed with ProDy

Computational methods allow us to generate an assemble of alternative conformations that can be used for docking proced ures of dsRNA or inhibitors.

Acknowledgements

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Novel structure of the primosomal protein N from bacteria solved ab initio

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Primosomal protein N (PriB) is one of the essential proteins for the bacterial primosome, a protein complex that plays an important role in DNA replication. At sites of DNA damage, the primosomal protein PriB is one of the components which catalyze the reactivation of stalled replication forks. The N-terminal domain of the thermostable PriB protein from the thermophilic bacterium *Thermoanaerobacter tengcongensis* (*Tte* PriB) was expressed, purified and its crystal structure was solved at the atomic resolution of 1.09 Å by direct methods. The protein chain, which encompasses the first 104 residues of the full 220-residue protein, adopts the characteristic oligonucleotide/oligosaccharide-binding (OB) structure consisting of a five-stranded β-barrel filled with hydrophobic residues and equipped with four loops extending from the barrel. In the crystal two protomers dimerize, forming a six stranded antiparallel β-sheet. The structure of the N-terminal OB domain of *T. tengcongensis* shows significant differences compared with mesophile PriBs. While in all other known structures of PriB a dimer is formed by two identical OB domains in separate chains, *Tte* PriB contains two consecutive OB domains in one chain. However, sequence comparison of both the N-terminal and the C-terminal domains of *Tte* PriB suggests that they have analogous structures and that the natural protein possesses a structure similar to a dimer of two N-terminal domains.

Small in size but large in function: RNA chaperone activity of HIV-2 NC protein

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RNA chaperones are non-specific RNA binding proteins that support RNA folding by resolving misfolded structures or preventing their formation. Proteins with RNA chaperone activity are very divers in structure and function. HIV-1 and HIV-2 nucleocapsid proteins (NC) are involved in key stages of HIV replication: dimerization, encapsidation and reverse transcription. Released from Gag, during viron maturation, nucleocapsid proteins (NC) are small basic proteins that possess two copies of highly conserved CCHC zinc fingers. The NC functions in HIV replication are correlated with their ability to act as nucleic acids chaperones (NAC). The multitude of performed functions makes them excellent targets for antiretroviral therapy.

NCp7 and NCp8 share only 67% sequence similarity and differences in packaging and dimerization have been reported for HIV-1 and HIV-2. In contrast to NCp7 protein (HIV-1), the NAC activity of NCp8 (HIV-2) is poorly recognized. This prompted us to examine the potential nucleic acid chaperone activities of recombinant NCp8 protein and chemically derived NCp8 peptide. We examined the ability of NCp8 to chaperone annealing of DNA with complementary strand as well as DNA and RNA strand exchange in duplex nucleic acids *in vitro*. Furthermore, we investigated whether NCp8 is capable of enhancing ribozyme-directed cleavage of an RNA substrate *in vitro*. We found that NCp8 possesses nucleic acids chaperone activity similar to but somewhat different than NCp7 protein.

Nucleocapsid proteins specific interaction with RNA by which the viral genome is selectively dimerized and packaged into the nascent virus particle are also presented. We recently proposed new sites of specific NCp8 binding within HIV-2 leader RNA (Purzycka et al., 2011). This region (560 nt) contains highly structured domains that play various regulatory roles in the viral replication. We showed that both SL-1 and TAR as isolated domains are bound by recombinant HIV-2 NC protein (NCp8) with high affinity, contrary to the hairpins downstream of SL1. The relatively tight binding of NCp8 to the TAR domain is especially interesting in the view of data indicating the involvement of the SL-1 and TAR hairpin III in the formation the HIV-2 leader RNA loose dimer (Purzycka et al., 2011).

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Experimental validation of novel microRNA candidates in *Pellia endiviifolia*

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MicroRNAs are short (18-24 nt) sequence-specific regulatory molecules of eukaryotic organisms. There are no data on any liverwort microtranscriptome. With the use of high-throughput sequencing techniques (SOLEXA, Illumina) we sequenced small RNAs from the liverwort *Pellia endiviifolia* species B. 270 conservative miRNA species belonging to 65 miRNA families that are identical to *Physcomitrella patens* or higher plants miRNAs were identified. The presence of selected conservative miRNAs identified in *P.endiviifolia* was confirmed by northern hybridization. Moreover, 108 miRNAs with one nucleotide substitution to known, conservative plant miRNAs and more than 534 with two nucleotide substitutions were found. With the use of bioinformatic approaches we study novel *Pellia endiviifolia* miRNA candidates which have not been previously described. A novel algorithm was implemented for the identification of new functional sRNAs. The annotation procedure involved clustering of sequence reads, size and expression profiling of cluster components as well as identification of functional sRNA features characteristic for already described sequences from other plants. Using northern hybridization and splinted ligation technique we evidenced the presence of forty one ~21-nt long stable, small RNAs, which can represent novel, unique liverwort miRNAs. Analysis of *P. endiviifolia* transcriptome revealed the presence of at least twenty miRNA putative precursors. Several of them were already verified using experimental approaches like RACE and genome walking. In the case of conservative miRNAs data obtained from these analyses show pre-miRNA structure conservation between liverworts and mosses however, the sequence conservation is restricted only to mature miRNA and miRNA* region. Using bioinformatics tools we selected putative targets for conservative and novel miRNAs. We are now validating selected targets using 5′RACE and modified by us splinted ligation assay.

The whole-genome identification and functional analyses of tRNA-derived small noncoding RNAs in *Arabidopsis thaliana*

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Small noncoding RNAs (18-35 nt sRNAs) have emerged as important factors in regulation of gene expression. They have been reported to play a significant role in regulation of plant growth and development, and are increasingly recognized as important players in responses to environmental stresses. The sRNAs act by silencing gene expression on post-transcriptonal and transcriptional level. Besides extensively studied microRNAs and siRNAs, other small functional RNAs have been identified in various organisms by using high-throughput sequencing techniques. Recent reports indicate that abundant, noncoding RNAs, like rRNA, tRNA and snoRNA may, aside of their primary function, be a source of small regulatory RNAs. We are interested in tRNA-derived small RNAs species. Initial screening of publicly available data from deep sequencing experiments allowed us to select four 20-22 nt long sRNAs derived from tRNAs. Their physical presence in the Arabidopsis transcriptome was experimentally verified by using Northern hybridization. Positive signals in the expected range of 20-30 nt may implicate a precise processing and stability of the fragments as well as indicate their potential regulatory capabilities. Moreover, we showed that precursor of one of the seven Gln-tRNA^{TTG} can, aside of being source of small RNA molecule, fold into an alternative secondary structure that is similar to and could function as a miRNA precursor. Northern hybridization technique revealed that the level of this tsRNA is SE, HYL1 and DCL1-dependant.

By using interdisciplinary, whole genome approach based on bioinformatics and molecular biology techniques, we plan to identify sRNAs derived from tRNAs, study molecular mechanisms involved in their biogenesis as well as determine their potential regulatory function in a model plant *Arabidopsis thaliana*. The initial phase of our research include identification of molecular mechanisms involved in biogenesis of tRNA-derived short RNA molecules. By using high-throughput sequencing techniques we plan to analyze changes in identified tRNA-derived sRNAs expression profiles in a set of representative *Arabidopsis* mutants selected based on the present knowledge about biogenesis and degradation of tRNA and microRNA/siRNA transcripts. We assume that sRNAs resulting from tRNA cleavages can represent a byproduct of tRNA maturation and/or degradation machinery or alternatively, can be processed by factors involving in siRNA/microRNA biogenesis.

Effect of glyphosate on the growth of *Pseudomonas putida* in soil

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Glyphosate is one of the most commonly used herbicides worldwide. The use of plant protection products on the interference has effect of the ecosystem (Liphadzi et al. 2005; Hart et al., 2009). The rhizosphere is rich in organic substances and high microbial activity (Kennedy, 2005; Kremer et. al., 2009) and the fate of glyphosate entering this environment is difficult to predict. *Pseudomonas putida* is a omnipresent anaerobic, Gram-negative bacterium present in soil, water, and rhizosphere. It performs a very important role in the breakdown of toxins (such as atrazine) and herbicides, to carbon dioxide and water. *P. putida* is an organism that may have far-reaching positive effects in cleansing the world of toxins that human produces through the use of chemicals in agriculture and other industrial processes.

The survey was conducted in 2013 at the University of Warsaw. The aim was to investigate the effects of glyphosate on the growth and respiration activity of *Pseudomonas putida* in the soil. Bacterial culture of *Pseudomonas putida* was isolated and subsequently characterized by sequencing of 16S rRNA genes and biochemical tests (Department of Geology, University of Warsaw). The resulting bacteria was subcultured in the medium Nutrient Yeast Broth (NYB). Amount of the final concentration of glyphosate in the substrate was established: 1 mg/ml, 5 mg/ml, 10 mg/ml. The microbial respiration activity was determined using a Micro-Oxymax respirometer (Columbus Instruments International Corp. Columbus) (Hollender et al., 2003). Measurement of carbon dioxide was carried out automatically for 135 hours (every 2.5 hours). The control was a culture without glyphosate. The experiment was performed in duplicate. The arithmetic mean and standard deviation were calculated. A cumulative production of carbon dioxide in 50 ml of culture was marked. The results show toxicity of glyphosate which concerns one of the most common soil bacteria.

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New type of anti-HIV phosphoramidate prodrugs

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Phosphoramidates derived from nucleoside analogues play an important role in antiviral therapy. These compounds have to fulfill several criteria of so-called pronucleotides: they should be a) uncharged, b) highly lipophilic, c) well soluble in water, d) stable in physiological media, e) should enter the target cells, and after this f) should be converted chemically and/or by enzymes into the respective nucleotide and subsequently into the active 5′-triphosphates. Recently, only a few types of compounds achieved the above criteria, *e.g.* amino acids or simple *N*-alkylamine derived phosphoramidates (Cahard et al., 2004) .

Here we present our studies on nucleoside (*N*-aryl)-phosphoramidates **1**, which have properties distinguishing them from other phosphoramidates and which can make them attractive anti-HIV agents. For our needs, a new synthetic protocols, based on *H*-phosphonate chemistry have been developed for preparation of this compounds.

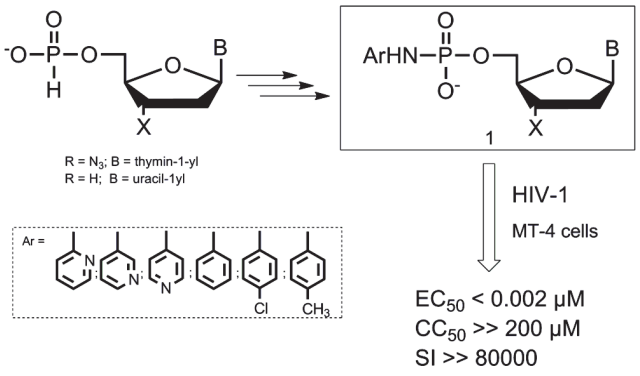

Fig. 1. A new phosphoramidate prodrugs

Stability and decomposition pathways ($t_{1/2}$, HPLC) of all phosphoramidates **1** were examined by incubation of the compounds in the cell culture media [RPMI/FBS 9:1 (v/v), 37°C]. Because phosphoramidates **1** decomposed very slowly (22 h to several days), it may suggest that they are able to enter the cell unchanged, where they are converted into biologically active nucleoside 5′-monophosphates.

Antiviral potency and cytotoxicity were examined in cooperating laboratories (dr A. Piasek). All the investigated derivatives of AZT showed high anti-HIV-1 potency ($EC_{50} < 0.002$ μM) and since activity was observed also for ddU-derived phosphoramidates, we can assume that these compounds act as pronucleotides. Finally, a very low cytotoxicity ($CC_{50} \gg 200$ μM), especially for aminopyridinyl derivatives, make phosphoramidates **1** attractive candidates for new anti-HIV prodrugs (Romanowska et al., 2011) .

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Dinucleoside cyclic phosphotriesters – a new type of anti-HIV prodrugs

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Antiviral nucleoside analogues require metabolic activation in infected cells by initial phosphorylation to the 5′-monophosphates by nucleoside kinases. To deliver these compounds into the cell, they have to be properly masked to cross cell membrane and then they are converted chemically and/or enzymatically into the respective nucleotides. Several pronucleotide approaches have been developed, for example aryl phosphoramidate diesters (McGuigan et al., 1993), diaryl phosphotriesters (Romanowska et al., 2009), cycloSal (Meier and Balzarini, 2006) or SATE (Lefebvre et al., 1995). Most of them contain protective groups which when free may be toxic for the cell. The main role of these masking groups is to provide proper physicochemical properties, required in pronucleotide approach.

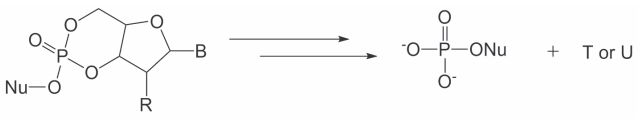

Fig. 1. A new type of pronucleotides

We designed a new type of anti-HIV prodrugs – dinucleoside 3′,5′-cyclic phosphotriesters, which contain, except of biologically active nucleoside, natural nucleoside units e.g. tymidine or uridine, exclusively (Fig. 1). These compounds may act as non-toxic, natural vehicles of anti-HIV nucleoside analogues, that release in the cell awaited 5′-monophosphates. Application of tymidine or uridine with AZT in the same molecule may triger additional effect – decreasing cytotoxicity of AZT.

We developed efficient methods for the synthesis of dinucleoside cyclic phosphotriesters based on *H*-phosphonate and P(V) chemistries. For proposed pronucleotides, both steps of their intracellular activation i.e., chemical hydrolysis towards nucleoside phosphodiester, and enzymatic hydrolysis to nucleoside 5′-phosphate, are equally important. Stability and decomposition pathways of all dinucleoside cyclic phosphotriesters and their intermediates (corresponding phosphodiesters) were examined (HPLC) in the cell culture media. Good solubility in water and very high anti-HIV activity together with very low cytotoxicity, make title phosphotriesters very promising candidates as a potential therapeutic agents against HIV.

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Crystal structure of aminotransferase from *Psychrobacter* sp. B6

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Aminotransferases are the enzymes commonly used in chemical and pharmaceutical industry for catalysis of the transamination reaction. The enzymes originating from psychrophilic organisms became the ones of interest, as their optimum reaction temperature is significantly lower than mesophilic enzymes. The most desired are enzymes, which exhibit activity of comparable level to their mesophilic homologues but in lower temperatures (Cavicchioli et al., 2002).

We present the structural studies of cold-adapted aminotransferase from *Psychrobacter* sp. B6 (*Psy*AT_B6), a gram negative soil strain from the Antarctic. The result of multiple sequence alignment in ClustalW has shown the two most similar proteins to the investigated one: PDB ID: 3FSL – tyrosine aminotransferase and 3QN6 – aspartate aminotransferase. The performed activity tests confirmed the enzymatic properties of *Psy*AT_B6 almost equal in respect to L-aspartate and L-tyrosine, making *Psy*AT_B6 a unique enzyme with both aromatic and acidic specificity. The performed crystallographic study may explain which structural futures are responsible for the broad substrate specificity.

Two native crystal structures of *Psy*AT_B6 were obtained. Both crystals grew in a monoclinic system ($P2_1$) in slightly different crystallization conditions; *Psy*AT_1 in the presence of magnesium nitrate and *Psy*AT_2 with magnesium chloride, which resulted in different crystal packing. The *Psy*AT_1 possess a dimer in the independent unit, while the *Psy*AT_2 consists of four dimers in the asymmetric unit (Fig. 1). The dimer is an active form of this enzyme with two active sites located on the dimer interface. The X-ray data for *Psy*AT_1 were collected at X13 DESY in Hamburg (2.19 Å), while the data for *Psy*AT_2 were collected at BL14.2 BESSY in Berlin (2.6 Å). For both crystals different cryoprotection was applied.

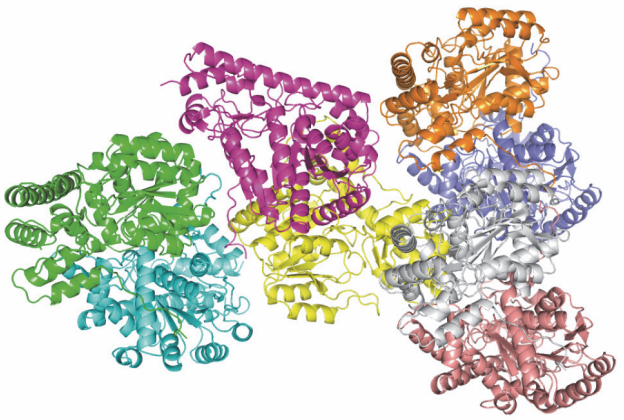

Fig. 1. The asymmetric unit of the *Psy*AT_2

Structural searching for similar protein in DALI server showed one more mammalian protein, besides the two previously found in ClustalW from mesophilic bacteria. The third one is an aspartate aminotransferase from *mus musculus*, PDB ID: 3PD6. The molecules of pyridoxal phosphate (PLP) associated with the protein were found in both presented structures, which proves that *Psy*AT_B6 belongs to the PLP-dependent enzyme family α (Woźniak and Koziołkiewicz, 2005).

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Modeling of biotin carboxylase from plants

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Plant acetyl coenzyme A carboxylase (ACCase) is a member of biotin dependent carboxylases – family of enzymes which share common catalytic mechanism involving fixation of CO_2 molecule on biotin covalently bound to biotin carboxyl carrier protein module (BCCP). This reaction is catalyzed by module of biotin carboxylase activity (BC) and depends on ATP. Third module of acetyl coenzyme A carboxylase is carboxyl transferase (CT) which transfer carboxyl group from carboxybiotin on acetyl coenzyme A (Fig. 1) (see Lombard et al., 2011, for BC evolution).

$$\text{BCCP-biotin} + HCO_3^- + \text{MgATP} \rightarrow \text{BCCP-biotin-}CO_2 + \text{MgADP} + P_i$$

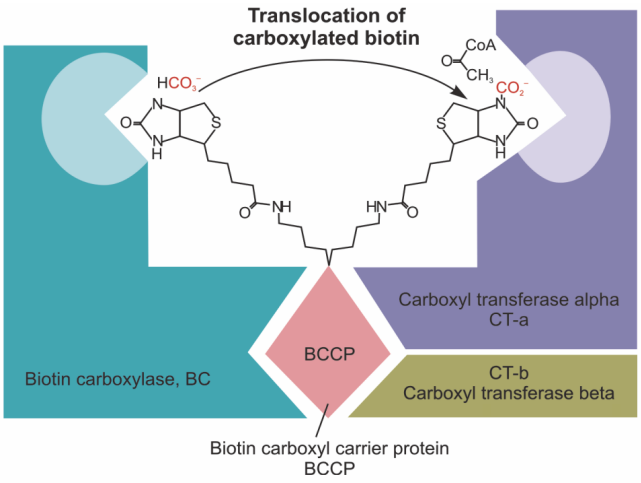

Fig. 1. Scheme – organization of BC, BCCP and CT complex

ACCase is an essential enzyme in all eukaryotes as its product – malonyl coenzyme A is the solely source of carbon for fatty acids synthesis. Due to the role of fatty acids in biology and importance of this compound for industry and food production, ACCases are objects of detailed studies using diverse approaches (Podkowinski et al., 2011).

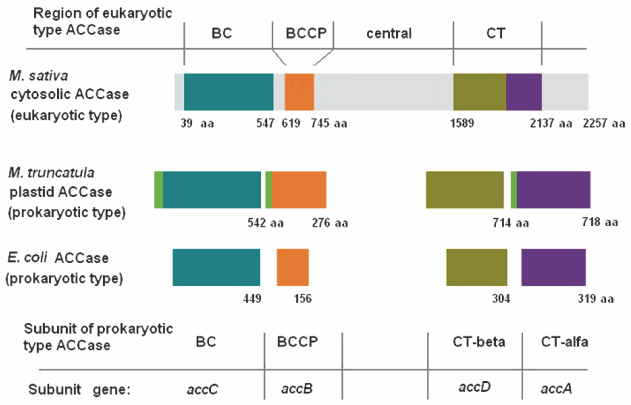

Fig. 2. Architecture of ACCases

All plants, excluding monocots (grass plants), possess plastid localized ACCase composed of four subunits carrying distinct enzymatic activity: BC, BCCP, CT-alpha and CT-beta (Fig. 2).

We decided to study the structure of biotin carboxylase subunit of plastid ACCase from model plant *M. truncatula* (MtBC) as this peptide is the entry point for all carbon atoms in fatty acids synthesis pathway and its structure is essential for understanding the reaction mechanism.

Modeling of MtBC structure was performed with homology or comparative modeling method – using a template structure of homologous protein resolved by experimental methods (NMR or X-ray diffraction).

Secondary and tertiary structure prediction was done by the GeneSilico MetaServer gateway (Kurowski et al., 2003) to obtain template proteins from top scored fold recognition alignments. Taking into consideration the fact that structure with ligand was needed, 3 structures were chosen based on the best score and identity to the target protein: 2VPQ (*S. aureus*), 2W70 (*E. coli*) and 3OUZ (*C. jejuni*) (chain A). For each template two

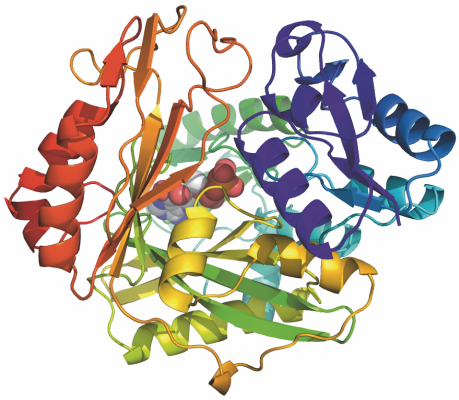

Fig. 3. Predicted 3D structure of *Medicago truncatula* biotin carboxylase with ATP docked into the active site (PyMol)

different alignments were prepared manually using DeepView (Swiss-PdbViewer) software. All six models were constructed in Modeller server (Sali et al., 1933) and then validated using two model quality assessment programs: Verify 3D (Eisenberg et al., 1997) and ProQ (Wallner et al., 2005). Scores reported indicated the best quality of the model built on 2W70 template which was chosen as a final prediction of MtBC tertiary structure. The final model was evaluated by the ProQ method as potentially "very good" (predicted LGscore: 5.713) or "good" (Predicted MaxSub : 0.556).

The model structure of MtBC has a similar tertiary structure as a bacterial BC. Superposition of MtBC model with 2VPQ and 2W70 (CA atoms) yields RMSD of 0.146 Å and 0.974 Å, respectively. Using structures of bacterial BC dimer as template, the MtBC homodimer model was generated and ATP was docked into the active site (Fig. 3). The model provides a framework for further analysis of the active site and the interactions at the interface of MtBC dimer.

This work was carried out in frame of project Sormisol supported by The National Center for Research and Development (NCBiR), grant nr. PBS1/8A/9/2012.

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Structural insights into the role of equine serum albumin in drug transport

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Serum albumin, the most abundant vertebrate transport protein, carries a large number of different molecules through the bloodstream. Among ligands of albumin, a large group of drugs can be found, including non-steroidal anti-inflammatory drugs, antibiotics, tranquillizers or anesthetics. In its helical structure, serum albumin possesses multiple binding sites, where drugs and other molecules are specifically bound (Ghuman et al., 2005).

Administration, distribution, metabolism and excretion (ADME) are important stages of drugs interacting with organism. There are multiple factors which are responsible for an effective drug dose reaching the target site of action (Balani et al., 2005). Since albumin can bind drugs, it is obvious that the protein is involved in the second step of ADME – distribution. Both too strong and too weak drug binding by albumin can lead to undesirable effects of absorbed drugs. On the one hand overly high affinity to albumin can cause a drug to be ineffective or increase its half-life time. On the other hand, if the drug is very poorly bound by plasma proteins, its distribution is disturbed and the threat of precipitation of the drug inside the circulatory system arises. Also the stoichiometry of the drug-albumin interactions and identifying the drug binding sites are crucial for drug transport by albumins.

Here we present the structural study of the equine serum albumin (ESA) in complexes with commonly used non-steroidal anti-inflammatory drugs (diclofenac, naproxen) and antibiotic (ampicillin). Human (HSA) and other mammalian serum albumins are structurally similar (Bujacz, 2012), however there is a little structural knowledge about ligand binding properties of non-human serum albumins and their comparison with HSA. Presented crystal structures of complexes with ligands provide valuable information about the binding sites responsible for drugs distribution in serum albumin.

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The role of the precursor structure in the biogenesis of microRNA in plants

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MicroRNAs (miRNAs) are small non-coding RNAs of about 21 nt in length, which take part in a wide variety of physiological and cellular processes. miRNAs act by regulating gene expression in a sequence-specific manner. The target mRNAs are first recognized by miRNAs and subsequently cleaved or blocked inhibiting translation. In plants, miRNAs are encoded mostly by independent transcriptional units and are transcribed into long non-coding primary transcripts (pri-miRNAs), containing miRNA sequences. They can be found mostly in exons, but there are examples where miRNA are located within introns.

We have already identified the structure of 44 *Arabidopsis MIR* genes. It has been also reported that 11 plant miRNAs are encoded within introns of protein-coding genes. Our further bioinformatic analyses revealed there the existence of additional 18 microRNAs within introns of functional genes. Expression of these miRNAs is probably regulated by their host-gene promoters.

Primary transcripts of miRNA genes are synthesized almost exclusively by RNA polymerase II and contain characteristic hairpin-like secondary structures, in which sequences of mature miRNAs are embedded. Pri-miRNAs are processed by DCL1 (DICER-LIKE 1) accompanied by CBC (a nuclear cap-binding protein complex, composed of two subunits, CBP20 and CBP80), HYL1 (HYPONASTIC LEAVES 1) and SE (SERRATE, zinc-finger protein) proteins into miRNA precursors (pre-miRNAs) which are further cleaved into mature miRNA/miRNA* duplexes. Interactions between these five proteins are necessary to improve the efficiency and precision of miRNA formation, however, apart from DCL1, the function of the rest of plant miRNA processing proteins is still unclear.

To outline their role in miRNA biogenesis we performed studies using our comprehensive platform, called mirEX, based on the high-throughput real-time PCR technique. It allows us to check the level of all known *A. thaliana* pri-miRNAs (289) at the same time. In our experiments we tested 14-day-old *se-1*, *cbc*, *hyl1-2* mutants and wild type plants. The expression level of over 50% precursors was changed in *se-1* and *cbc* mutants and about 40% in *hyl1-2* plants. We divided 73 miRNA genes whose structure is known into three categories: miRNAs encoded by the independent transcriptional units without introns and containing introns and miRNAs located within introns of a given host-gene. Next, we compared the influence of a given mutant on miRNA biogenesis. Among pri-miRNAs which are encoded by the independent transcription units (intron-less and intron-containing precursors), almost all were HYL1-, SERRATE- and CBC-dependent. In the case of miRNAs embedded in the introns of protein-coding genes, we observed disturbances in pri-miRNA expression level in all analyzed mutants in less than 50% cases. It clearly shows there are other players, apart from known miRNA biogenesis machinery proteins that influence the maturation process of miRNAs located in host-gene introns.

Natural inhibitors of aminoacyl-tRNA synthetases as antimicrobial agents

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The rising number of bacteria resistant to conventional antibiotics has made the search for new anti-infective drugs a priority task. Examples of bacteria that are already causing a major threat to public health are methicillin resistant *S. aureus* (MRSA), penicillin resistant *S. pneumoniae* or multi-drug resistant *M. tuberculosis* (MDMT). New drugs have to be vital for the cell function, very selective for the bacterial target, and difficult for the bacteria to develop resistance by mutations.

The aminoacyl-tRNA synthetases (aaRS) are enzymes found in all living organisms (Doolittle and Brown, 1994; Nagel and Doolittle, 1995). They catalyze the attachment of the correct amino acid to its cognate transfer RNA (tRNA), and thus play a fundamental role in translating the genetic code. The first stage of acylation reaction involves the formation of an enzyme bound aminoacyl-adenylate, which is then resolved into the 2′ or 3′-hydoxyl of the terminal ribose of the tRNA. Inhibition of one of these two enzymatic steps disrupts tRNA charging, which in turn, stalls elongation of growing polypeptide chains (Tao and Schimmel, 2000). AARSs, as the essential enzymes for protein synthesis, represent the promising targets for development of cures against pathogenic species. Additionally prokaryotic aaRSs are phylogenetically conserved and show significant structural difference from their eukaryotic counterparts (Pohlmann and Brotz-Oesterhelt, 2004).

The aim of our work was to find new inhibitors of aaRS that potentially can be used to develop new and effective antibacterial agents. Looking for effective inhibitors of bacterial growth showing minimal toxicity to human cells we turned to natural substances. Good candidates are flavonoids, a group of plant products with many biological and pharmacological activities; antibacterial, antiviral, antioxidant, and antimutagenic effects and inhibition of several enzymes have been demonstrated.

We show the effects of different flavonoids on activity of Tyr-RS from *E. coli* and *S. aureus*. Inhibitory activity of compounds was analyzed in *in vitro* aminoacylation reaction. The most potent inhibitors are acacetin, kaempferide, biochanin A (IC_{50} – 5.12, 25.14 and 25.8 mM, respectively). Structure – function relationship analysis shows that for Tyr-RS inhibition hydroxyl group at position 5 and 7 as well as methoxy group 4′ are necessary (Fig. 1).

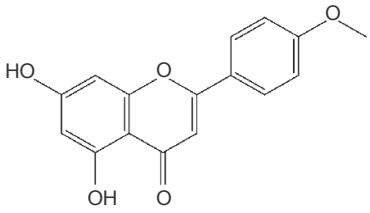

Fig. 1. Acacetin structure

Flavones showed slightly higher inhibitory activity than flavonols. Glycosylation of aglycone weakens the interaction with the enzyme.

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Secondary structure conserved motif of influenza virus mRNA

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Influenza A virus is real threat for human due to seasonal epidemics and occasional pandemics. This virus can generate new strains that are dangerous for humans (Korteweg, 2008). In this situation, it is important to create drug that will stop spreading of the virus. RNA that is essential in its life cycle appears to be an alternative target for stopping the virus life cycle.

Influenza virus contains eight single-stranded viral RNA segments of negative polarity. Each vRNA is associated with a viral polymerase complex and numerous copies of a nucleoprotein. Viral polymerase complex replicates the influenza's RNA genome and transcribes its mRNA. Replication begins with vRNA acting as a template to produce a (+)RNA intermediate which in turn becomes the template for producing more vRNA strands. The viral polymerase is also capable of using vRNA for producing mRNA (Olson, 2010).

For understanding role of RNA in virus's life cycle the information about RNA secondary structure is of critical importance. Unfortunately, this knowledge is largely undiscovered but more and more bioinformatics analysis are performed. In addition experimental data show that each vRNA segment is involved in a direct contact with at least one other vRNA (Fournier, 2012). Furthermore, some data suggest that structure of (+)RNA can have critical function in replication of the virus (Heldt, 2012). Besides, it was shown that adopting specific secondary structure can serve as mechanism of controlled splicing (Moss, 2012). Moreover, the secondary structure of influenza virus's RNA can be essential for segments ressortment (Priore, 2012).

Interestingly, many regions of influenza A (+)RNA are predicted to have unusual thermodynamic stability, conserved base pairing and also have suppressed third codon position variability. This data suggest that fragments of influenza virus RNA are likely to fold into functional structure (Moss, 2011).

Segment 5 of influenza A virus encodes nucleoprotein. The bioinformatics analysis of influenza RNA predicts existence of a structure motif in (+)RNA of segment 5. This RNA motif with average sequence of segment 5 (+)RNA was synthetised *in vitro*. Enzymatic, chemical structure probing and oligonucleotide binding mainly confirmed the prediction of secondary structure of this conserved RNA motif.

Conservation in secondary structure should have biological meaning. Described here conserved RNA structural motif could be target for new therapeutics.

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Structural investigation of avidin complexes with metallocene biotin derivatives

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Avidin is positively charged glycosylated protein and the object of various biochemical investigations due to its unique binding properties with vitamin H that is one of the strongest interaction in nature. Homotetramer of avidin binds up to four molecules of biotin in non-covalent interaction with high affinity ($K_d \approx 10^{-13}$-10^{-15} M). This interaction is several orders of magnitude higher than in the typical antigen-antibody complexes (Rosano et al., 1999). Nowadays, due to the high biotin affinity to avidin, such complex is commonly used in numerous laboratory techniques including: Western blotting, Cell-surface labeling, Affinity purification, Fluorescence-activated cell sorting (FACS), Electromobility shift assays (EMSA) and Enzyme linked immunosorbent assay (ELISA).

The aim of the study is to determine the crystal structures of avidin, the glycosylated protein from Hen egg white, in the form of complexes with the metallocene biotin derivatives (Plazuk et al., 2011). Metallocene and its derivatives are of increasing importance regarding their biological applications. Introducing metallocene moiety into molecules of biological relevance may reveal new useful features and properties of these compounds (Rudolf et al., 2010). These combinations exhibit therapeutically important properties that may open new possibilities in the treatment of diseases like malaria and cancer.

Structural analysis of avidin complexes with the metallocene biotin derivatives will provide information about the nature of protein-ligand interactions and the differences in ligands affinity to the protein. This might be important in the application of such complexes in the laboratory techniques. Crystal structure shows how the ligand binds to protein molecule and initiates protein conformational changes upon ligand binding. The specific properties of ligands and their interactions with protein may be useful in biochemical and medical applications.

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Functionalization of a *cis*-diol system of rybonucleosides

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Differentiation of *cis*-diol system in ribonucleosides is still a challenging topic. All methods require several steps to obtain $O^{2'}$,$O^{5'}$-diprotected nucleosides with an exception of partial acylation (Kamaike et al., 1987) or partial silylation (Hakimelahi et al., 1981). Recently N^4,$O^{2'}$,$O^{5'}$-tripivaloylcytidine was successfully obtained in crystalline form in good yields (Fogt et al., 2009). The free 3′-hydroxyl group was subjected to several modifications like deoxygenation, mesylation and further substitution with inversion of configuration. To obtain 3′-substituted derivatives with retention of configuration 3′-anhydrocytidine was synthesised with strong basis from 3′-mesylic derivative with yield of 24%. However, the obtained yield was lower, than reported (Mizuno and Sasaki, 1965) and therefore the interest went to uridine, which can be transformed to anhydro form much easier. However, in the case of uridine the partial acylation gave a mixture of two dipivaloic isomers with free 3′-hydroxyl group or 2′-hydroxyl in a ratio 4:1 respectively. Derivatization with Mitsunobu reaction (Mitsunobu, 1981) gave surprisingly single product 2,2′-anhydro-$O^{3'}$,$O^{5'}$-dipivaloyluridine (**1**). The probable mechanism of regioselective formation of **1** based on similar situation (Perali et al., 2011) is proposed. At the first stage the hydrogen from free hydroxyl group of sugar residue was cleaved by adduct of diethyl azodicarboxylate and triphenylphosphine generating two isomeric nucleosyl oxyanions 3′ and 2′. Oxyanions 3′ and 2′ are isomerising, while amount of 2′ is depleting because of formation of nucleosyl oxyphosphonium zwitterion. The observed product **1** is formed from nucleosyl oxyphosphonium zwitterion in intramolecular S_N2 substitution, where oxygen in the position 2 of nucleobase is an attacking nucleophile. This phenomenon could be caused by two factors: different steric hindrance and different electron density on oxygen atom O2′ and O3′.

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Identification of small RNAs involved in response of *Zea mays* to herbicidal stress

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Plants, exposed to different types of abiotic and biotic stress, developed several mechanisms allowing them to adapt to adverse environmental conditions, basing on a regulation of gene expression at transcription and translation levels. In past few years it was shown that small noncoding RNA are also involved in plant response to abiotic and biotic stress conditions.

Roundup and Titus (active ingredients: glyphosate and rimsulfuron, respectively) are the systemic herbicides widely used in crops. Different lines of maize (*Zea mays*) have proven to react differently to stress caused by using the herbicides in plant culturing – some are naturally resistant and some are sensitive. Understanding of molecular mechanism of plant response to this type of stress conditions is an important issue for agriculture.

We aim to identify small RNAs that can be involved in maize response to herbicide stress at the level of translation. To achieve this goal we applied two approaches. The *in vitro* selection (so-called SELEX) was used to obtain RNAs that bind with high affinity and specificity to ribosomes isolated from *Zea mays* and therefore might act through the protein biosynthesis regulation. The RNA molecules were selected against ribosomes isolated from three different maize lines treated with herbicides Roundup and Titus, displaying various reactions to stress conditions, and from control lines. After 8 rounds of selection the RNAs were cloned and sequenced. The bioinformatic analysis was carried out to determine sequence motives, as well as to confirm their presence in maize genome. Next 7 cycles of SELEX were carried out and obtained RNAs are to be analysed in the same way.

Basing on the recent reports on degradation of constitutive RNA molecules as a result of oxidative stress and the ability of glyphosate to cause oxidative stress in plant cell the second approach was designed. We try to find out if ribosomal RNAs can be the source of small RNAs with possible functions in herbicide stress response. In this project leaves of *Zea mays* (resistant and sensitive lines, both treated with Roundup and control plants) were harvested on the day of herbicide application and a week later. Ribosomal RNA was isolated from plant material and subsequently subjected to digestion with the usage of shortened variant of ribonuclease Dicer-like 1 (DCL1), which is involved in miRNA and siRNA biogenesis. The main cleavage products size ranges were 10-27 and 35-50 nt, what is in agreement with sizes reported for miRNAs and RNAs generated from constitutive RNAs, respectively.

RNAs obtained by both approaches will be seuquenced and subsequently tested to estimate their potential as regulators of translation activity in context of herbicide stress response.

Complexes of chagasin with proteolytic enzymes

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Chagas disease, caused by the protozoan pathogen *Trypanosoma cruzi*, harvests about 50 000 lives every year in the Americas. Near 18 million people in Central and South America are infected with the parasite. Approximately one-third of those develop the more severe, death-leading, chronic symptoms of the disease such as cardiac problems and enlargement of the esophagus or bowel.

The major protease expressed in *T. cruzi* is a potent papain-like (C1) cysteine protease, called cruzipain. Cruzipain is essential both for the life-cycle of the parasite and for its interaction with host cells. The enzyme is, therefore, a promising target for therapy. The endogenous natural inhibitor of cruzipain is called chagasin. Chagasin is a 110 residue-long protein. Chagasin takes part in the controlling of cruzipain activity. Thus the knowledge of the structure is very important to find the treatment of Chagas disease.

The structures of recombinant chagasin in free form and in complexes with a human cathepsins L (Ljunggren et al., 2007), B (Redzynia et al., 2008) and also with plant papain (Redzynia et al.,2009) reveal the novel fold of this protein and mode of enzyme inhibition generally similar to cystatin (natural mammalian inhibitors of lysosomal C1 cysteine proteases) but different in details. In terms of the folding pattern, chagasin defines a new structural family, with some resemblance to immunoglobulin domain, which can be described as a distorted jelly-roll barrel with an intrusion of an N-terminal β-hairpin in parallel orientation to the neighboring strands. This folding pattern has no analogy to any of the known classes of cysteine protease inhibitors, such as cystatins or staphopains.

Comparison with the structure of papain in complex with human cystatin B reveals that, despite entirely different folding, the two inhibitors utilize very similar atomic interactions, leading to essentially identical affinities for the enzyme. Comparisons of the chagasin–papain complex with high-resolution structures of chagasin in complexes with cathepsin L, cathepsin B and falcipain allowed the creation of a consensus map of the structural features that are important for efficient inhibition of papain-like enzymes. The comparisons also revealed a number of unique interactions that can be used to design enzyme-specific inhibitors. Such information, coupled with our identification of specificity – conferring interactions, should be important for the development of drugs for treatment of the devastating Chagas disease caused by this parasite.

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Drought-regulated microRNAs in barley (*Hordeum vulgare*)

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MicroRNAs (miRNAs) are small RNA molecules, usually of 21 nucleotides in length, which regulate gene expression through driving the target mRNA cleavage or repressing its translation. In plants miRNAs function as regulators in development, signal transduction, protein degradation, pathogen invasion and response to environmental stresses. However, the role of miRNAs in barley response to drought is poorly determined.

In this study, we used Illumina deep sequencing technology to analyze the global miRNA expression level in severe drought (20% SWC) treated barley plants. We identified 304 conserved miRNAs in both, control and drought stressed plants. The expression levels of 120 miRNAs were downregulated under drought conditions, 111 miRNAs were upregulated, and the level of 74 remained unchanged. From this miRNA data set, we chose eight miRNAs: miRNA156, miRNA159a/b, miRNA164a, miRNA168-5p, miRNA171, miRNA172, miRNA319b and miRNA5051, which are differently expressed in plants under stress conditions. Northern hybridization was used to confirm the deep sequencing results obtained. The changes of the level of these eight miRNAs were examined by northern not only in severe drought stress treated plants but also in plants under minor drought (30% SWC), as well as six hours after rehydratation. The expression level of all miRNAs studied, except miRNA172, decreased in drought treated plants, what is in agreement with our deep sequencing results. We also determined the pre-miRNA levels using northern, and the pri-miRNA levels by quantitative PCR. We conclude, that during drought stress the levels of barley miRNAs are regulated not only at the level of transcription, but also at the step of pri-miRNA processing (posttranscriptional regulation).

We provide new data describing changes in the expression profile of miRNAs, pre-miRNAs and pri-miRNAs in control and minor to severe drought conditions, as well as after rehydratation. Our next goal is to find targets of the drought-regulated miRNAs characterized.

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Searching protecting groups for 2′-hydroxyl function in RNA synthesis

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The research aiming to develop at creating an efficient chemical method of RNA synthesis began in the 1950s. Nowadays, the methods of chemical DNA synthesis are developed much better than methods of RNA synthesis. Finding new functions of RNA, and most importantly, discovering the phenomenon of RNA (RNAi) interference, has significantly increased the interest in synthetic RNA. New regulatory functions of RNA, the development of structural research of RNA and numerous reports in the literature presenting practical applications of these oligomers make the demand for synthetic RNA fragments higher. However, chemical RNA synthesis is very problematic and it is a challenge faced by many chemists all over the world. Numerous studies have been conducted in many laboratories in order to find new protecting groups for 2′-hydroxyl function, as well as efficient methods of entering them into nucleosides.

One of methods of protecting 2′-OH position in ribonucleosides is a method in which a suitably blocked nucleoside (**1**) component is converted into a more active nucleoside 2′-O-methylthiomethyl derivative (**2**). Then, the isolated derivative nr **2** is reacted with appropriate alcohol (to become a part of protecting group) in the presence of N-iodosuccinimide (NIS) and trifluoromethanesulfonic acid (TfOH) as activators.

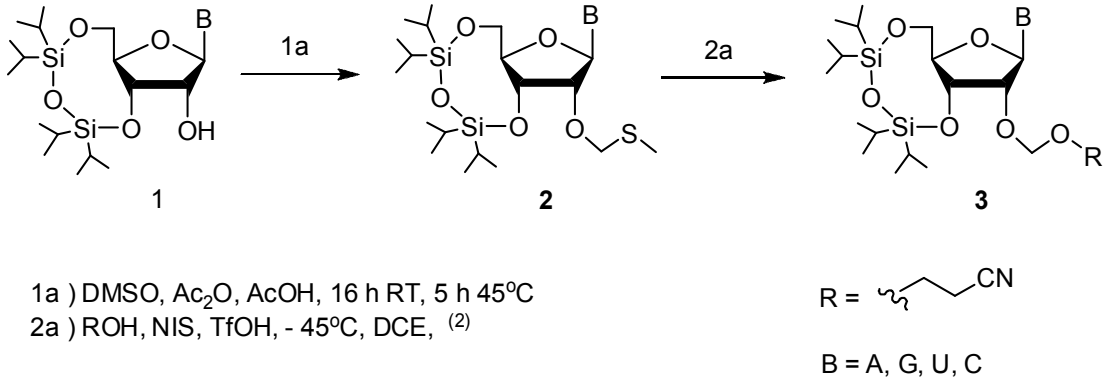

Fig. 1. Synthesis of 2′-O-cyanoethoxymethyl-3′,5′-O-tetraisopropyldisiloxane-1,3-diyl ribonucleoside

In the last few years, acetal derivatives of formaldehyde have been studied as protecting groups of 2′-OH function in RNA synthesis. Such groups have numerous advantages and the most significant one is a small spacial hindrance. However, entering acetal blocking groups to ribonucleoside requires developing more comfortable and efficient methods than those which have been described in the literature thus far.

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Small-Angle X-ray Scattering study of the effect of ionic strength and temperature on the conformation of plant Hsp90 protein

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Inside the cell newly translated polypeptide chains and disordered proteins constantly risk misfolding and aggregation that may have serious consequences on cell physiology and may lead to possibly fatal diseases like Alzheimer's disease. All organisms have specialized assemblies that protect proteins from misfolding. Molecular chaperones are class of assemblies that help proteins adopt proper fold and protect them from misfolding at stress conditions.

Heat Shock Protein 90kDa (Hsp90) is a molecular chaperone which assists in proper folding of proteins in response to heat stress but also plays role in maintaining metastable active/inactive conformation of many cellular factors at normal physiological conditions (Taipale et al., 2010). Hsp90 protein is involved in many processes like kinaseand nuclear receptors activation, protein degradation, immunity. Hsp90 protein is chaperone for many mutated and viral tyrosine kinases like v-Src or Bcr-Abl. In plants Hsp90 protein associates with cytosolic nucleotide-binding sites and leucine-rich repeats receptors that recognize pathogen effectors and trigger immune response.

Hsp90 protein binds to ATP during its chaperone cycle. Binding to the client proteins causes ATP hydrolysis and conformational changes that leads to client proteins stabilization and folding. Hsp90 protein interacts with many proteins, co-chaperones which modulate ATPase activity of Hsp90 protein. Other co-chaperones bind to other chaperones or to the client proteins.

Domain structure of Hsp90 protein consists of three domains: N-terminal ATPase domain (NTD), middle domain (MD) that is responsible for substrate binding and C-terminal dimerization domain (CTD). NTD and MD are connected by the charged linker, which has important role in Hsp90 protein function. From crystallographic, X-ray scattering and electron microscopy studies it is known that Hsp90 protein possesses different conformations (Krukenberg et al., 2008). In solution Hsp90 protein exists in equilibrium between open and compact states and the equilibrium constant is species dependent. In closed state N-terminal domains interacts which each other. Environmental factors like pH and osmolytes, changes conformational equilibrium.

In this study we investigated the effect of ionic strength on the conformation of *Triticum aestivum* Hsp90 protein in apo, ADP and AMPPNP bound states. We also examined effect of temperature on Hsp90 protein without bound nucleotides. In low ionic strength conditions Hsp90 protein exists in rather compact conformation characterized by the maximum particle size (D_{max}) equal to 22 nm in both nucleotide and apo states. Ionic strength cause shift to the more open, expanded conformation characterized by the D_{max} equal to 30 nm. Higher temperature also changes equilibrium to the more extended conformation. Detailed analysis revealed two, distinct open conformations.

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Crystal structures of M-PMV protease in dimeric form

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Mason-Pfizer Monkey Virus (M-PMV) is a D-type retrovirus that causes simian acquired immunodeficiency syndrome (SAIDS) in rhesus monkeys. Its protease (PR) processes the retroviral polyproteins to release the final protein products necessary for virion maturation. M-PMV PR is a member of retropepsins (pepsin-like aspartic proteases) and acts as a homodimer, but in absence of substrates or inhibitors the equilibrium is shifted toward the monomeric state.

In this work, we have crystallized M-PMV PR with two different active-site inhibitors to obtain the dimeric form of the protein. For the crystallization experiments, a C7A/D26N/C106A mutant of M-PMV PR was used. To prevent autodigestion the active site aspartate was mutated to the isosteric asparagine (D26N). The cysteine residues (C7 and C106) were replaced by alanine to remove the possibility of uncontrolled disulfide-driven aggregation. The peptidomimetic inhibitors have the sequences Pro-(O-Me)Tyr-Val-Pst-Ala-Met-Thr (INHB1) and Pro-Tyr-Val-Pst-Ala-Met-His (INHB2), where (O-Me)Tyr denotes Oη-methylated tyrosine and Pst denotes pepstatin, a standard residue used in inhibition of pepsin-like enzymes, which has a non-hydrolyzable surrogate of the peptide bond.

The protease crystallized in two polymorphic forms: triclinic ($P1$) and monoclinic ($P2_1$). The crystallization conditions for the $P1$ structures (PR+INHB1, PR+INHB2) were similar (citrate buffer pH 5.6, 2-propanol). For the $P2_1$ structure (PR+INHB1), crystallization was based on the condition reported for the monomeric form of M-PMV PR. Diffraction data extending to 2.0-2.4 Å resolution were collected using synchrotron X-ray radiation, and the structures were solved by molecular replacement and are being refined. The models have good geometry and the refinement has converged in all cases to R-factors of 18% or better.

All structures show a dimeric protease despite the fact that the crystallization conditions used for the $P2_1$ structure were very similar to those reported for the monomeric form. The dimers are cemented mainly by a four-stranded intermolecular β-sheet interface composed of the N- and C-termini of both subunits, in a way that is typical for homodimeric retroviral proteases. The inhibitor molecules located in the active site, are visible in electron density maps in one orientation. The mobile flap loops extending over the inhibitors locked in the active site are in most cases partially disordered, especially in the $P2_1$ structure.

Sucrose Phosphate Synthase – a key enzyme in sucrose metabolism applied in plant biotechnology

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Sucrose is essential compound in plant physiology due to its role in plant energy metabolism, long distance transport and sequestration of carbon fixed in the course of photosynthesis, as well as its role in signalization. Set of enzymes involving sucrose phosphate synthase (SPS; EC 2.4.1.14) is responsible for processing hexoses produced in the course of photoassimilation to sucrose (Fig. 1). SPS which converts Fru6P and UDPGlu to Suc6P is the limiting step of the pathway and regulates the pathway efficiency. SPS associations with sucrose content in plant, or fixed carbon sequestration in sink organs make this enzyme attractive target for traditional breeding and biotechnological projects including metabolic engineering.

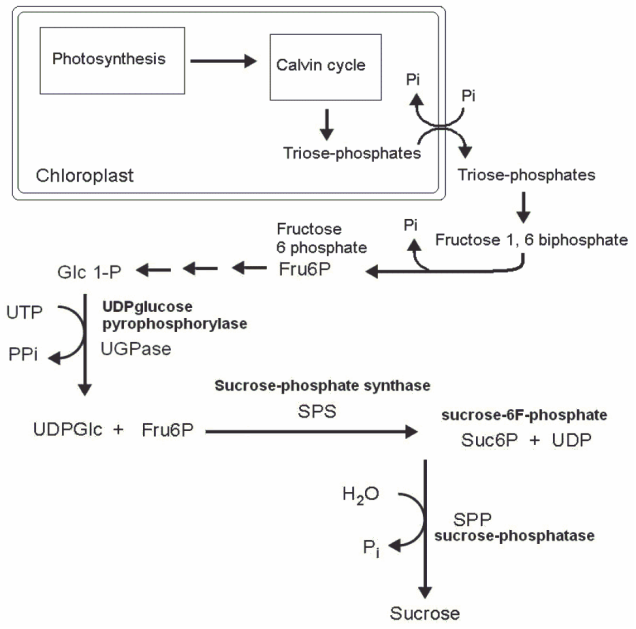

Fig. 1. SPS role in processing photoassimilates into sucrose

Current phylogenetic classification of SPS genes from cyanobacteria, monocotyledonous and dicotyledonous plants assumes the presence of five distinct SPS families (Lutfiyya et al., 2007). SPS sequences of monocots are split into three groups, with one group specific for monocotyledonous plants (group including grasses – *Poaceae*). The other two groups harbor SPS sequences from monocots and dicots. Based on the presence of five SPS forms described in sequenced plant genomes of maize and rice it is highly probable that all grass plants possess five SPS homologues of different functional specificity.

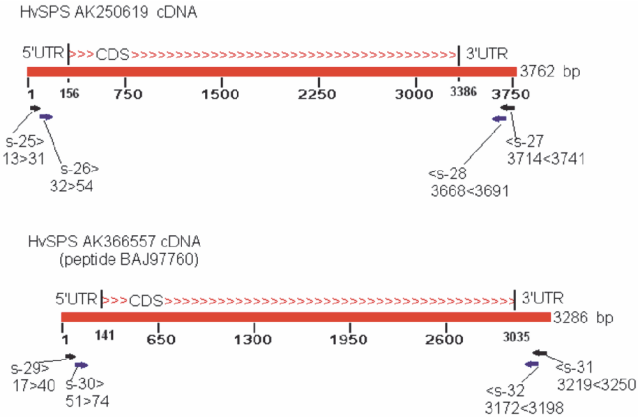

Fig. 2. Strategy for cloning two barley SPS

Four barley (*Horedeum vulgare*) SPS sequences were identified in amino acid databases searched with blastp program and rice SPS (GenBank accession no. A2WYE9) as a query. Two of the barley SPS homologues – GenBank accession no. AK250619 and BAJ97760 were applied for further studies – cDNA amplification and constructs assembly (Fig. 2). The AK250619 and BAJ97760 peptides differ from each other with respect of the presence of Pfam domains and regulatory phosphorylation sites: Ser-158 – light-dark regulation, Ser-229 – 14-3-3 protein binding and Ser-424 – osmotic stress activation (Fig. 3).

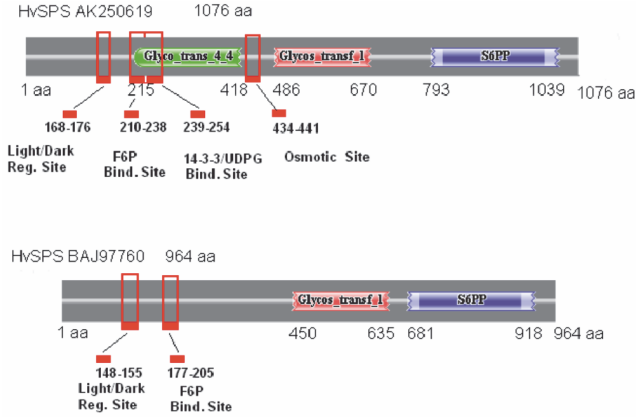

Fig. 3. SPS peptide architecture – functional domains and regulatory motifs

Our studies on SPS in grasses possess two goals: 1 – investigate role of SPS homologues in sucrose metabolism and 2 – increase sucrose synthesis efficiency in grasses. The studies are part of project Sormisol where various method including genetic engineering with SPS harboring constructs will be applied to increase sucrose, and decrease lignin content in *Sorghum sp.* and *Miscanthus sp.* – grass plants used as stock for bioethanol production.

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Serum albumin – a universal carrier

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Serum albumin, naturally abundant plasma macromolecule, is one of the most extensively investigated proteins. It is difficult to find another protein that accommodates such a diversity of small molecule ligands both endogenous and exogenous. Albumin is synthesized in the liver and exported as a single non-glycosylated polypeptide chain. This multidomain protein attracts great interest from the pharmaceutical industry since it shows an extraordinary ligand binding properties and can bind numerous physiological ligands such as: fatty acids, hormones and others. Binding of drugs to albumin enhances the solubility and the transport capacity of blood thus enabling for better distribution of ligands in the body. Toxic metabolites, such as bilirubin, are transported by albumin to their disposal sites (Curry, 2002). The complex mechanism of protein-ligand interactions among mammalian serum albumin analogues is an important structure-function correlation studied among monomeric proteins.

Albumin appears to be formed by three homologous helical domains without any β-sheet element, arranged in a globular heart-shaped conformation. Each domain is composed of two unique subdomains showing a certain degree of binding specificity. The structural organization of serum albumin provides a various distribution of ligand binding sites. According to Sudlow, bulky heterocyclic anions bind to Drug Site I, while aromatic carboxylates with an extended conformation bind to Drug Site II (Sudlow et al., 1975). Excessive small molecule binding by albumin causing attenuated efficacy of the drug has been the reason to start investigations of serum albumin complexes with a range of natural ligands and drugs. The topology of serum albumin binding pockets has been well characterized based on the human serum albumin (HSA) crystal structures and it has been explored to other species thanks to the research conducted in our group (Bujacz, 2012).

Bovine serum albumin (BSA) is used as a standard in drug affinity tests and binding kinetics of drugs among others, while ovine (OSA) and leporine (LSA) serum albumins constitute an interesting research goal since many of the pharmacological and physiological *in vivo* studies are performed on sheeps and rabbits. We have obtained the crystal structure of BSA in complex with 3,5-diiodosalicylic acid (DIU). This ligand is used as an intermediate for veterinary anthelmintic agents, a medication capable of causing the evacuation of parasitic intestinal worms. Additionally, it possesses the properties of salicylates. The structural investigations of serum albumins provide the information about their surface shape and potential that can be utilized as specific epitopes for antibodies.

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Nuclear import of N receptor in tobacco is regulated by posttranslational modification of SGT1 protein

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Resistance to Tobacco Mosaic Virus is mediated by the tobacco N protein, which belongs to the TIR-NB-LRR (Toll-Interleukin Receptor; Nucleotide-Binding; Leucine-Rich Repeat) class of plant resistance R proteins. NB-LRR receptors recognize in the cytoplasm proteins delivered by pathogens into plant cells, and this event initiates downstream signaling pathways. Subsequently, R proteins have to be transported to the nucleus where they play a key role in regulation of defense-related gene expression. However, little is known how nucleocytoplasmic transport of those receptors is regulated since they are large and usually do not contain a discernible nuclear localization signal within their sequences.

Most NB-LRR receptors, including N, require essential eukaryotic protein SGT1 (Suppressor of G2 allele of SKP1) to execute their function. In plants, SGT1, HSP90 (Heat Shock Protein) and RAR1 (Required for Mla Resistance 1) form a molecular chaperone complex, which maintains R proteins in an inactive but signal-competent state.

We show that forced nuclear import of SGT1 leads to increased nuclear accumulation of N protein. Co-localization studies of SGT1 with N derived domains revealed that SGT1 may regulate LRR nuclear import and export, whereas localization of TIR and NB domains was unaffected. This is consistent with reports that SGT1 interacts with NB-LRR receptors through their LRR domains. Based on our previous results showing that phosphorylation state of SGT1 affects its distribution within cell, we propose a model in which nucleocytoplasmic partitioning of N protein during TMV-triggered defense response is modulated by SGT1 phosphorylation.

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