40th Anniversary

Institute of Biotechnology
Vilnius University

MoBiLi

SEVENTH FRAMEWORK PROGRAMME

Vilnius University
Institute of Biotechnology
Biennial Report
2013–2014

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Vilnius University
Institute of Biotechnology

Report 2013–2014

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Director's note

2015 is the year of the 40-th anniversary of the Institute of Biotechnology. Founded in 1975 as an independent research institution (All-union Research Institute of Applied Enzymology), now part of Vilnius University, the Institute of Biotechnology strives to maintain the high standards of excellence in scientific endeavour, research training and technological advance with its main focus in a broadly defined field of molecular biotechnology including nucleic acid and protein technologies, bioinformatics, immunodiagnostics, drug design, microfluidics, next generation epigenomics and gene editing technologies. During this time, the Institute has become a recognized leader in life sciences both nationally and internationally. The Institute provides an interface between advanced education, basic research and technological development for the economic and social benefit of Lithuania and the European Union. The Institute is internationally acclaimed for its multidisciplinary research of structure and function of restriction-modification enzymes, CRISPR-Cas systems, modification of macromolecules, development of biomedical recombinant proteins, small molecule inhibitors and bioinformatics. Together with private capital the Institute has recently contributed to the establishment of the Sector of Microtechnologies. Such partnership is still unique in the Lithuanian life sciences sector. The highest level of research performed at the Institute in all these areas is attested by successful participation in the EU FP and other competitive programmes, scientific publications in top-tier journals, and the highest citation figures among the Lithuanian research institutions. In addition to fundamental research, IBT has a strong applied research component documented by the European/US patents and successful spin-off companies. During 2013-2014 researchers of the Institute have filled in 10 international patent applications, some of them were successfully licensed totally for about 1 million euro. We are sure, that the most important landmark in the history of the Institute of Biotechnology is the creation of the Lithuanian modern biotechnology industry, which is competitive on the world market. We take exceptional pride of our internationally recognized spin-offs UAB Fermentas (currently ThermoFisher Scientific Baltics), UAB Sicor-Biotech (currently Teva), UAB Biocentras, UAB Biok, and the new UAB Profarma (2007), UAB Nomads (2010), UAB Baltymas (2011), UAB IMD technologies (2012), UAB ThermoPharma Baltic (2014).

The future strategy of the Institute is focused on three major points: i) strengthening and consolidating on-going basic and applied biomedical research; ii) expanding research into new areas/cutting edge technologies, iii) making the research sustainable by attracting young researchers as group leaders. To achieve these long term goals a number of specific actions are envisioned: i) building of closer links between research and industry; ii) pro-active participation in the international research programmes and a deeper integration into ERA; iii) joining EMBC/EMBO and EMBL to get access to large international research infrastructures and cutting-edge technologies at pan-European research centres; iv) strengthening international co-operation, v) developing programmes to foster young talents.

In summer of 2015 the Institute will become part of the Life Sciences Centre of Vilnius University that will consolidate facilities and personnel of currently three separate entities of Vilnius University, namely, the Faculty of Natural Sciences, the Institute of Biochemistry and the Institute of Biotechnology. As such, the Life Sciences Centre will serve as a platform for strengthening biomedical research and molecular biotechnology. We hope that the convergence between science and education will benefit both science and studies.

Prof. Kęstutis Sasnauskas

Vilnius University Institute of Biotechnology:

Just the Facts and Numbers

Scientific staff of the Institute at the Annual meeting in Kernavės bajorynė on January 29, 2015

- The Institute of Biotechnology was established in 1990 after restructuring of the All Union Research Institute of Applied Enzimology. Since October 1, 2010 it has become an internal unit of Vilnius University. In 2015 the Institute will become part of the Life Sciences Centre of Vilnius University that will unite three separate entities of Vilnius University, namely, the Faculty of Natural Sciences, the Institute of Biochemistry and the Institute of Biotechnology.
- Located at V.A. Graičiūno 8, Vilnius.
- Total staff number is 135; research staff number is 100, it includes 59 researchers (PhD).
- The youngest Lithuanian research institute - average age – 38.
- Allocation of state budget (2014) comprises 18 % of income; other 82 % comes from outside sources (grants, programmes, contracts).
- High level scientific research in step with applied research. Thirteen scientists of the Institute were awarded the Lithuanian Science Prize:

  prof. V. Butkus and prof. A. Janulaitis (1994),

  prof. S. Klimašauskas and prof. V. Šikšnys (2001),

  dr. A. Ražanskienė, dr. A. Gedvilaitė and prof. K. Sasnauskas (2003),

  dr. Č. Venclovas (2010),

  dr. D. Matulis (2012),

  dr. A. Žvirblienė (2013),

  dr. G. Sasnauskas, dr. G. Tamulaitis and dr. M. Zaremba (2014).
- Top level 30-35 scientific papers in peer reviewed high impact journals each year; coming patent applications; 10 patent applications filled in and 4 patents published in 2013-2014.
- Successful participation in EC (FW5, FW6, FW7) and other competitive international programmes (HHMI, NIH, EEA).
- Selected as the Centre of Excellence in 2003 – EC FW5 tender – 600.000 Euros.
- A winner of the EC FW7 – Regional Research Potential: Coordination and Support action (FW7-REGPOT-2009—1) directed to the integration of European research entities into the European scientific research area - MoBiLi project – 1.600.000 Euros.
- Successful participation in projects of the European Social Fund under the Global Grant Measure – seven projects – 2.501.200 Euros for 2011-2015.
- Since 2000 after long term abroad 30 researchers have returned to the Institute and were involved in the establishment of new laboratories.
- Involved in education of students at Vilnius University, Gediminas Technical University, Kaunas University of Technology. A lot of the Institute lecturers are members of Committees on preparing Study Programmes.
- 25—30 students accomplish Bachelor or Master theses at the Institute each year.
- Forty four students are currently involved in Biochemistry or Chemical Engineering PhD studies at the Institute; all in all, three PhD theses were defended in 2013-2014.
- Famous Lithuanian Biotech spin-off companies emerged from the Institute (UAB Fermentas (presently Thermo Fisher Scientific Baltics) - 1995, UAB Sicor - Biotech (presently TEVA) - 1995, UAB Biocentras - 1991, UAB Biok – 1991, UAB Profarma – 2007, UAB Nomads – 2010, UAB Baltymas – 2011, UAB IMD technologies – 2012, UAB ThermoPharma Baltic – 2014).
- Skillful personnel for the Lithuanian Biotech are trained at the Institute; close connections with the Lithuanian Biotech industry are supported.
- Industrial Biotechnology Programme was initiated by the Institute.
- National Integrated Programme of Biotechnology & Biopharmacy was initiated by the Institute.

Doctoral theses

	Name	*Title*	*Supervisor*
2013	M. Tomkuvienė	Methyltransferases as Tools for Sequence-Specific Labeling of RNA and DNA	prof. S. Klimašauskas
	L. Baranauskienė	Analysis of Ligand Binding to Recombinant Human Carbonic Anhydrases I, II, VII, IX and XIII	dr. D. Matulis
2014	D. Kazlauskas	Computational analysis of DNA replication proteins in double-stranded DNA viruses	dr. Č. Venclovas

Dr. Miglė Tomkuvienė and Prof. Saulius Klimašauskas at the Best PhD Thesis 2013 awards in the Lithuanian Presidency

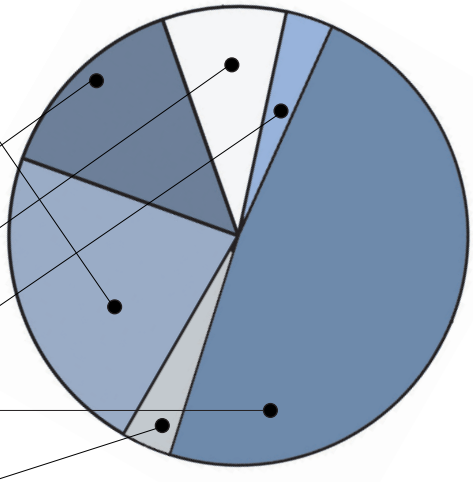
Just "released" Dr. Lina Baranauskienė together with her supervisor Prof. Daumantas Matulis

"New born" doctor Darius Kazlauskas with his supervisor Dr. Česlovas Venclovas

Financing Sources 2013 – 2014

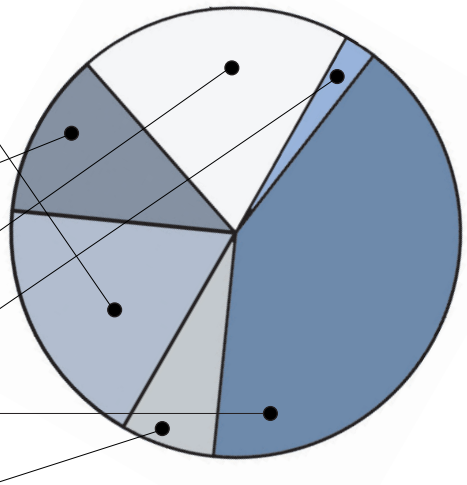
Funding 2013 5,21 MEuros

	Thous €	%
State Subsidy	1155,2	22 %
Research Council of Lithuania	737,6	14 %
Foreign Grants and Contracts	456	9 %
Agency for Science, Innovation and Technology	172,5	3 %
EU Structural Funds	2508,5	48 %
Other	181,2	4 %



Funding 2014 6,44 MEuros

	Thous €	%
State Subsidy	1174,3	18 %
Research Council of Lithuania	770,1	12 %
Foreign Grants and Contracts	1266,5	20 %
Agency for Science, Innovation and Technology	148,6	2 %
EU Structural Funds	2643,6	41 %
Other	432,9	7 %



National and International Grants

EUROPEAN COMMUNITY GRANTS
Framework 7 programme

Title	*Head of the project*	*Financing EUR thousand*	*Duration*
Strengthening and sustaining the European perspectives of molecular biotechnology in Lithuania (MoBiLi)	L. Pašakarnis	1603.9	2009-2013
Metastatic tumours facilitated by hypoxic tumour micro-environments (METOXIA)	dr. A. Kanopka	373.5	2009-2013
Pan-European network for the study and clinical management of drug resistant tuberculosis (TB PAN-NET)	dr. P. Stakėnas	104.4	2008-2013
Development of novel antiviral drugs against Influenza (FLUCURE)	dr. G. Žvirblis	256.0	2010-2014
Integrated Microfluidic System for Long Term Cell Cultivation, Monitoring and Analysis (BioCellChip)	dr. L. Mažutis	239.0	2012-2015
Towards construction of a comprehensive map of amyloid-ligand interactions: (-)-Epigallocatechin 3-Gallate and insulin amyloid (EGCG+INSULIN=)	dr. V. Smirnovas	100.0	2011-2015

OTHER INTERNATIONAL GRANTS
National Institutes of Health (USA)

Title	*Head of the project*	*Financing EUR thousand*	*Duration*
Approaches for genomic mapping of 5-hydroxymethylcytosine a novel epigenetic mark in mammalian DNA	prof. S. Klimašauskas	223.6	2010-2013
Direct single nucleotide mapping of genomic CpG marks	prof. S. Klimašauskas	223.4	2013-2015

EU FUNDS
National Integrated Programme

Title	*Head of the project*	*Financing EUR thousand*	*Duration*
Biotechnology and Biopharmacy: fundamental and applied research	prof. K. Sasnauskas	1437.7	2012-2015

JOINT RESEARCH PROGRAMME

Title	*Head of the project*	*Financing EUR thousand*	*Duration*
Development of new generation means for virus diagnostics and prophylaxis and application in veterinary medicine	dr. A. Gedvilaitė (together with the Lithuanian University of Health Sciences)	435.9	2013-2015

EUROPEAN SOCIAL FUND
Under the Global Grant Measure

Title	*Head of the project*	*Financing EUR thousand*	*Duration*
Molecular tools for epigenomics and RNomics	prof. S. Klimašauskas	456.4	2011-2015
Design of selective carbonic anhydrase, Hsp90, and Hsp70 inhibitors and investigation of their anticancer properties	dr. D. Matulis	405.5	2012-2015
The use of genome-wide analysis for engineering of new yeast strains with improved heterologous expression	dr. R. Slibinskas	379.2	2012-2015
Exploring flavones as universal inhibitors of amyloid-like fibril formation	dr. V. Smirnovas	401.9	2012-2015
Structure and molecular mechanisms of bacterial antivirus defence systems	prof. dr. V. Šikšnys	458.4	2011-2015
Making use of large-scale biological data for the development of a new method to assess protein models and for studying DNA replication and repair systems in bacteria and viruses	dr. Č.Venclovas	286.3	2013-2015
Novel chimeric proteins with antiviral activity	dr. A. Žvirblienė	399.8	2012-2015

NATIONAL GRANTS
Research Council of Lithuania

National Research Programme: Chronic Non-infectious Diseases

Title	*Head of the project*	*Financing EUR thousand*	*Duration*
Splicing factors and their regulated miRNA as cancer biomarkers for gastrointestinal system	dr. A. Kanopka	217.0	2012-2014
Carbonic anhidrase hCA XII as a potential marker for cancer cells	dr. D. Matulis	171.0	2012-2014
Studies on genetic and environmental allergy risk factors in the Lithuanian birth cohort	dr. A. Žvirblienė (partner of Vilnius University Faculty of Medicine)	200.4	2012-2014

NATIONAL RESEARCH PROGRAMME: Healthy and Safe Food

Title	*Head of the project*	*Financing EUR thousand*	*Duration*
Expression analysis of anthocyanin biosynthesis genes in horticultural plants	dr. V. Kazanavičiūtė (partner of the Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry)	197.1	2012-2015
Interspecific hybrids of orchard plant - a novel source of anthocyanins	dr. R. Ražanskas (partner of the Institute of Horticulture, Lithuanian Research centre for Agriculture and Forestry)	376.3	2011-2014

LITHUANIAN-SWISS COOPERATION PROGRAMME

Title	*Head of the project*	*Financing EUR thousand*	*Duration*
Directed evolution of computer engineered enzymes using droplet based microfluidics	prof. A. Janulaitis	703.9	2012-2016
Signalling control of pathogen induced plant immunity	prof. I. Meškienė	605.0	2013-2016

RESEARCH TEAM PROJECTS

Title	*Head of the project*	*Financing EUR thousand*	*Duration*
The role of Cas1 and Cas2 proteins in adaptation mechanism of CRISPR-Cas systems	dr. G. Gasiūnas	89.9	2014-2016
Expansion of the Crystallography Open Database (COD) and statistical analysis of crystal structures	dr. S. Gražulis	91.9	2013-2015
Genomic mapping of covalently tagged CpG sites	dr. E. Kriukienė	101.3	2013-2015
Bayesian nonparametrics for detection of distant protein homology	dr. M. Margelevičius	99.6	2013-2015
High-throughput screening of antibody-secreting cells using droplet-based microfluidics	dr. L. Mažutis	98.4	2012-2014
Protein ligand binding volume and its application in drug design	dr. V. Petrauskas	84.0	2014-2016
Studies on the mechanism of the cytolytic activity of the bacterial toxin vaginolysin	dr. M. Plečkaitytė	88.8	2012-2014

A universal method for recombinant synthesis of selenoproteins	dr. R. Rakauskaitė	85.8	2012-2014
Structure and function of 5-methyl and 5-hydroxymethylcytosine-directed restriction endonucleases	dr. G. Sasnauskas	100.5	2012-2014
Synthesis of Schmallenberg virus proteins and their application for diagnostic means	prof. K. Sasnauskas	99.3	2013-2015
Signalling components in stem cells	dr. A. Schweighofer	86.9	2014-2016
Looking for the origins of mammalian prion 'strains'	dr. V. Smirnovas	88.6	2012-2014
Structural and functional studies of restriction enzyme family	dr. G. Tamulaitienė	100.9	2013-2015
Investigation of RNA interference in bacteria	dr. G. Tamulaitis	101.1	2013-2015
Application of interatomic contacts for the assessment of three-dimensional RNA structural models	dr. Č. Venclovas	12.6	2012-2013
Studies on the biogenesis molecular mechanism of non coding RNAs in plants	dr. G. Vilkaitis	101.4	2012-2014
Function of a molecular motor in atypical restriction-modification system	dr. M. Zaremba	99.9	2012-2014
Development of recombinant antibodies against carbonic anhydrase	dr. A. Žvirblienė	101.1	2012-2014

AGENCY FOR SCIENCE, INNOVATION AND TECHNOLOGY (MITA) High-Technology Development Programme 2011-2013

Title	*Head of the project*	*Financing EUR thousand*	*Duration*
Development of microfluidics technology for monodisperse vesicles production and improved drug delivery	dr. L. Mažutis	69.4	2013

INDUSTRIAL BIOTECHNOLOGY PROGRAMME 2011-2013

Title	*Head of the project*	*Financing EUR thousand*	*Duration*
Development of innovative biocatalytic stain remover	dr. I. Matijošytė	58.0	2013
Development of innovative biotechnology for oil base lubricant production	dr. I. Matijošytė	4.89	2013

Crystallography Open Database (COD)

The COD project (abbrev. from "Crystallography Open Database", http://www.crystallography.net/) aims at collecting in a single open access database all organic, inorganic and metal organic structures [1] (except for the structures of biological macromolecules that are available at the PDB [2]).

The database was founded by Armel Le Bail, Lachlan Cranswick, Michael Berndt, Luca Lutterotti and Robert T. Downs in February 2003 as a response to Michael Berndt's letter published in the Structure Determination by Powder Diffractometry (SDPD) mailing list [3]. Since December 2007 the main database server is maintained and new software is developed at the Institute of Biotechnology, Vilnius University by Saulius Gražulis and Andrius Merkys. In 2014 data and software from CrystalEye project, managed by Peter Murray-Rust, were integrated into COD [4]. Currently, COD curates over 300 thousand records describing structures published in major crystallographic and chemical peer-reviewed journals [5].

The COD project aims not only at preserving data, but also at becoming a collaboration platform for scientists. The database presents itself on the Internet as a website (Fig. 1a) with basic data entry, search and retrieval capabilities. Registered researchers can upload their structures to COD using automatic data deposition system, and curate the existing COD data. The uploaded data may come directly from experiments (so called prepublication data), from previous publications or as personal communications using the deposition website. The deposition software performs rigorous checks of syntax and semantics, thus ensuring high quality of records deposited in COD. Searches can be carried out by entering basic crystallographic and chemical parameters or by providing a structural formula. The retrieved COD records can be viewed online (Fig. 1b) or downloaded for further processing. Data search and retrieval does not require any registration, nor is limited in any other way. The addition of functionality of public discussions and structure annotation is planned in the near future.

A great interest is also shown in relating COD crystal structures to their chemical information. In the beginning of 2014 Open Babel toolbox was used to create 35 thousand reciprocal links between the records of COD and ChemSpider [7], an open database of chemical structures; in the following year, an algorithm to restore stoichiometric molecular composition of a crystal [8]

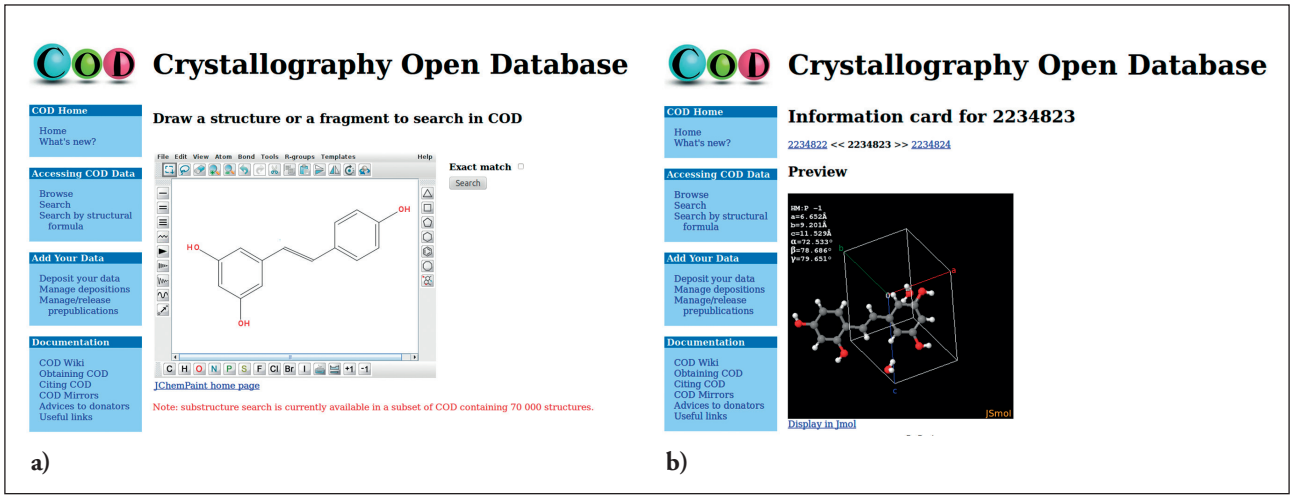


Fig. 1 a) Website and search interface of the Crystallography Open Database (COD) permits searches of crystallographic data by a range of crystallographic and chemical parameters, bibliography, as well as by structural formula. b) Data can be viewed on-line in the J(s)Mol applet or downloaded for further processing, either record-wise or in bulk.

was created and implemented. By utilizing the before mentioned algorithm and Open Babel toolbox, it is expected to automate link building between COD and PubChem, an open database of biological activities of small molecules.

The open nature of the COD permitted numerous mirrors around the globe [9-12] and specifically tailored COD database variants [13]. For massive data mining, COD permits downloads and updates of the whole database using Subversion, Rsync or HTTP protocols. The ease of access to COD data has spurred the use of this resource for software testing [14], teaching [13], and research [15].

COD software codebase also facilitated the creation of two additional open access databases PCOD (abbrev. from "Predicted COD", http://www.crystallography.net/pcod) and TCOD (abbrev. from "Theoretical COD", http://www.crystallography.net/tcod). PCOD, founded in 2008, now stores over a million records of first-principles-predicted crystal structures; TCOD, launched 5 years later in 2013, now stores a few hundred theoretically calculated or refined crystal structures. Even though TCOD is quite small at the moment fast database growth is expected in collaboration with various partners such as the work group of AiiDA [16] framework for atomistic simulations led by Nicola Marzari or the OQMD database [17] of DFT models of quantum materials supervised by Chris Wolverton.

Over the last 12 years COD has earned the recognition of the scientific community. After the integration of CrystalEye into COD in 2014, Saulius Gražulis was presented with the Blue Obelisk [18] award for achievements in promoting open data, open source and open standards. During the same year COD was added to the list of recommended data repositories [19] by the "Scientific Data" journal published by the Nature Publishing Group and was ranked 5th in accordance to the number of records by the analysis of Thomson Reuters data citation index [20]. At present, COD is the most comprehensive open resource for small molecule structures, freely available to all scientists in Lithuania and worldwide.

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Research overview

Bacterial viruses (bacteriophages) provide a ubiquitous and deadly threat to bacterial populations. To survive in hostile environments, bacteria have developed a multitude of antiviral defense systems. The overall research theme in our department is the **structural and functional characterization of enzymes and enzyme assemblies that contribute to the bacteria defense systems that target invading nucleic acids.** In particularly, we are interested in the molecular machinery involved in the CRISPR function and structural and molecular mechanisms of restriction enzymes. We are using X-ray crystallography, mutagenesis, and functional biochemical and biophysical assays to gain information on these systems.

Structure and molecular mechanisms of CRISPR-Cas systems

CRISPR-Cas systems in bacteria and archea provide acquired immunity against viruses and plasmids. CRISPR (clustered regularly interspaced short palindromic repeats) locus usually consists of short and highly conserved DNA repeats that are interspaced by variable sequences, called spacers (Figure 1). CRISPR arrays are typically located in the direct vicinity of *cas* (CRISPR-associated) genes. In response to phage infection, some bacteria integrate new spacers that are derived from phage genomic sequences, which contribute to the CRISPR-mediated phage resistance in the subsequent rounds of infection. Many mechanistic steps involved in invasive element recognition, novel repeat manufacturing, and spacer selection and integration into the CRISPR locus remain uncharacterized (see below for the details).

Streptococcus thermophilus DGCC7710 contains four distinct systems: CRISPR1, CRISPR2, CRISPR3 and CRISPR4, which belong to the three distinct Types (Figure 2). We aim to establish molecular mechanisms of CRISPR-immunity provided by the CRISPR systems of *S. thermophilus.*

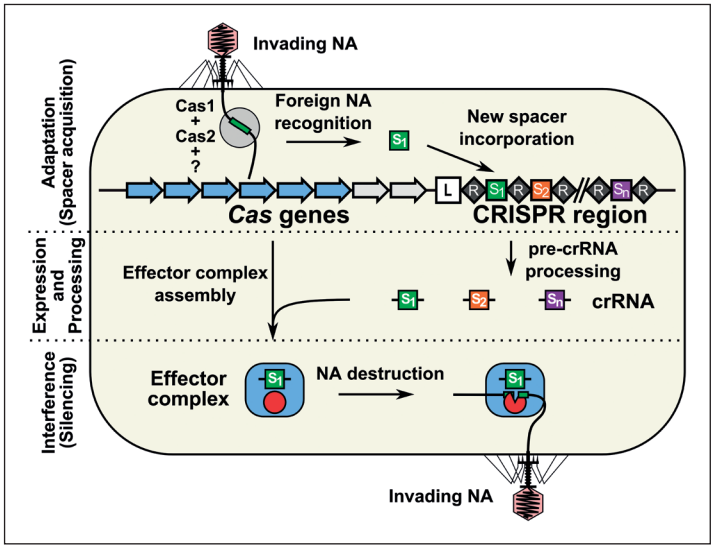

Figure 1. ***Schematic representation of CRISPR-Cas mechanism. The CRISPR–Cas mechanism is arbitrarily divided into three main stages: (1) adaptation or spacer acquisition, (2) expression and processing (crRNA generation), and (3) interference or silencing. During adaptation, Cas proteins recognize invasive nucleic acid (NA) and integrate short pieces of foreign DNA into the CRISPR region as new spacers (S1). Spacers are inserted at the leader (L) proximal end followed by duplication of the repeat (R). In the expression and processing stage, the CRISPR repeat-spacer array is transcribed into a long primary RNA transcript (pre-crRNA) that is further processed into a set of small crRNAs, containing a conserved repeat fragment and a variable spacer sequence (guide) complementary to the invading nucleic acid. crRNAs further combine with Cas proteins into an effector complex. In the interference or silencing stage, the effector complex recognizes the target sequence in the invasive nucleic acid by base pairing and induces sequence-specific cleavage, thereby preventing proliferation and propagation of foreign genetic elements.***

Cas9-dual RNA complex provides DNA silencing in the type II system

Type II CRISPR-Cas systems typically consist of only four Cas genes. We have shown that in the CRISPR3 system of *Streptococcus thermophilus* Cas9 protein associates with a dual crRNA:tracrRNA molecule to form an effector complex which specifically cleaves matching target dsDNA. The ternary Cas9-crRNA-tracrRNA complex of the type II CRISPR-Cas system functions as an Mg^{2+}-dependent RNA-directed DNA endonuclease that locates its DNA target guided by the crRNA and introduces a double-strand break at a specific site in DNA (Figure 2). The simple modular organization of Cas9, where specificity for the DNA target is encoded by a small crRNA and the cleavage reaction is executed by the Cas9 endonuclease, provides a versatile platform for the engineering of universal RNA-directed

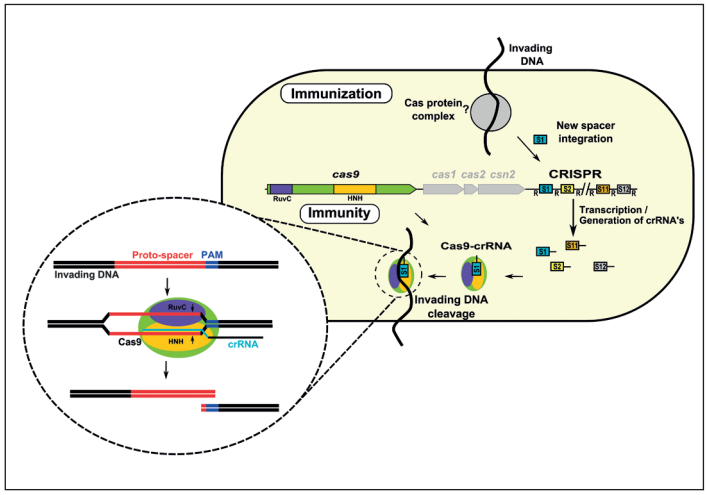

Figure 2. ***The Cas9-crRNA complex functions as an RNA-guided DNA endonuclease. Guided by the crRNA it finds a specific sequence in the target DNA and binds to it forming an R-loop. In the presence of Mg^{2+} ions, the Cas9 protein nicks each DNA strand 3 nt -upstream of the PAM sequence to generate blunt DNA ends, through RuvC- and HNH-like active sites that act on separate DNA strands.***

DNA endonucleases. Using *Streptococcus thermophilus* Cas9 as a model system, we demonstrated the feasibility of Cas9 as a programmable molecular tool for *in vitro* and *in vivo* DNA manipulations.

Cas9 for genome editing applications

Cas9 of *Streptococcus pyogenes* is currently used as a model system for genome editing applications. Typically, the DNA expression cassettes encoding nucleus-targeted codon-optimized Cas9 protein and sgRNAs are transfected into the cells. The efficiency of DNA cleavage by plasmid-delivered Cas9 in eukaryotic cells depends on multiple factors, including expression vector design, transfection efficiency, cell type, recovery yield of functional Cas9 complex, and usually requires optimization of a set of experimental conditions. Cas9 delivery by plasmid transfection is still difficult to achieve for some hard-to-transform cell lines including human primary cells and pluripotent stem cells. Moreover, plasmid transfection occasionally results in undesirable integration of vector plasmid into the genome and is often inefficient and stressful to cells. We developed an alternative way for the Cas9-mediated genome modification in eukaryotic cells by chemical transfection of *in vitro* reconstituted functionally active Cas9-crRNA-tracrRNA complex of *Streptococcus thermophilus* CRISPR3-Cas system.

Molecular basis for CRISPR immunity in type I systems

CRISPR-encoded immunity in type I systems relies on the Cascade ribonucleoprotein complex, which triggers foreign DNA degradation by an accessory Cas3 protein. To establish the mechanism for adaptive immunity provided by the *Streptococcus thermophilus* CRISPR4-Cas system (St-CRISPR4-Cas), we isolated an effector complex (St-Cascade) containing 61-nucleotide CRISPR RNA (crRNA). We show that St-Cascade, guided by crRNA, binds *in vitro* to a matching proto-spacer if a proto-spacer adjacent motif (PAM) is present. Surprisingly, the PAM sequence determined from binding analysis is promiscuous and limited to a single nucleotide (A or T) immediately upstream (-1 position) of the proto-spacer. In the presence of a correct PAM, St-Cascade binding to the target DNA generates an R-loop which serves as a landing site for the Cas3 ATPase/nuclease. We show that Cas3 binding to the displaced strand in the R-loop triggers DNA cleavage, and

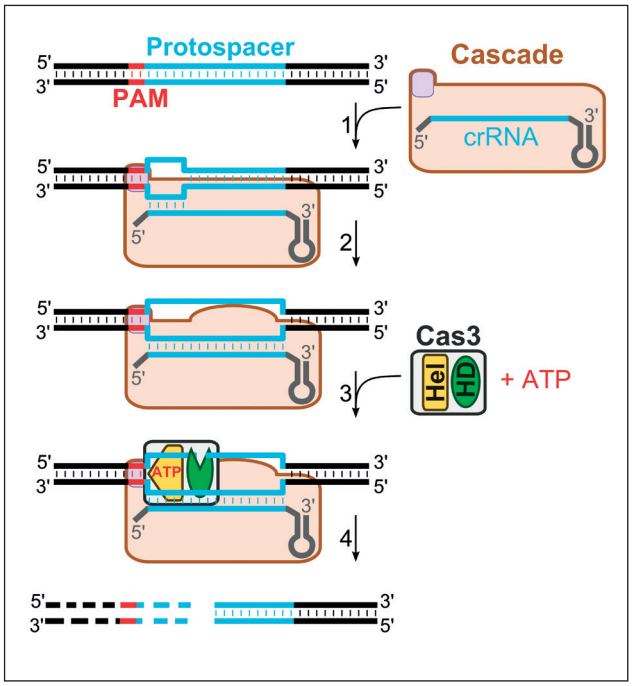

Figure 3. ***DNA-interference in the type I-E CRISPR-Cas systems. Cascade scans DNA for a protospacer sequence and PAM. Once the correct PAM and a short primary hybridization sequence ("seed") is identified (1), the guide of crRNA basepairs with a complementary DNA strand forming an R-loop, which is stabilized (locked) if a PAM-distal end of the protospacer is hybridized with the guide (2). Displaced DNA strand of the R-loop serves as a landing site for the Cas3 (3). Cas3 translocates in a 3'→5' direction powered by a helicase domain (Hel), whereas the HD domain degrades DNA in a unidirectional manner (4).***

if ATP is present, Cas3 further degrades DNA in a unidirectional manner. These findings establish a molecular basis for CRISPR immunity in St-CRISPR4-Cas and other Type I systems (Figure 3).

Single molecule observation of R-loop intermediate in the Cas9 and Cascade effector complex

Central to the defense against invading foreign DNA provided by the CRISPR-Cas systems is a ribonucleoprotein complex that produces RNA-guided cleavage of foreign nucleic acids. In DNA-targeting CRISPR-Cas systems, the RNA component of the complex encodes target recognition by forming a site-specific hybrid (R-loop) with its complement (protospacer) on an invading DNA while displacing the non-complementary strand. Subsequently, the R-loop structure triggers DNA degradation. Although these reactions have been reconstituted, the exact mechanism of R-loop formation has not been fully resolved. We used single-molecule DNA supercoiling to directly observe and quantify the dynamics of torque-dependent R-loop formation and dissociation for both Cascade- and Cas9-based CRISPR-Cas systems. We found that the protospacer adjacent motif (PAM) affects primarily the R-loop association rates, whereas protospacer elements distal to the PAM affect primarily R-loop stability. Furthermore, Cascade has higher torque stability than Cas9 by using a conformational locking step. Our data provide direct evidence for directional R-loop formation, starting from PAM recognition and expanding toward the distal protospacer end.

Type III-A CRISPR2 system of Streptococcus thermophilus targets RNA

Immunity against viruses and plasmids provided by CRISPR-Cas systems relies on a ribonucleoprotein effector complex that triggers the degradation of invasive nucleic acids (NA). Effector complexes of type I (Cascade) and II (Cas9-dual RNA) target foreign DNA. Intriguingly, the genetic evidence suggests that the type III-A Csm complex targets DNA, whereas biochemical data show that the type III-B Cmr complex cleaves RNA. We aimed to investigate NA specificity

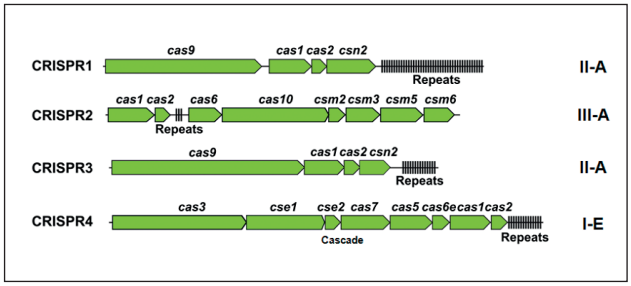

***Figure 4.** CRISPR-Cas systems of S. thermophilus DGCC7710. CRISPR1 and CRISPR3 systems belong to the type II, CRISPR2 to the type III whilst CRISPR4 belongs to the type I (E. coli subtype).*

and mechanism of CRISPR interference for the *Streptococcus thermophilus* Csm (III-A) complex (StCsm). When expressed in *Escherichia coli*, two complexes of different stoichiometry copurified with 40 and 72 nt crRNA species, respectively. Both complexes targeted RNA and generated multiple cuts at 6 nt intervals. The Csm3 protein, present in multiple copies in both Csm complexes, acts as endoribonuclease. In the heterologous *E. coli* host, StCsm restricts MS2 RNA phage in a Csm3 nuclease-dependent manner. Thus, our results demonstrate that the type III-A StCsm complex guided by crRNA targets RNA.

Structure and function of restriction endonucleases

Restriction-modification (RM) systems commonly act as sentries that guard bacterial cells against invasion by bacteriophage. RM systems typically consist of two complementary enzymatic activities, namely restriction endonuclease (REase) and methyltransferase (MTase). In typical RM systems REase cuts foreign DNA but does not act on the host genome because target sites for REase are methylated by accompanying MTase. REases from 4000 bacteria species with nearly 350 distinct specificities have been characterised. REases have now gained widespread application as indispensable tools for the *in vitro* manipulation and cloning of DNA. However, much less is known about how they achieve their function.
In the Department of Protein-Nucleic acids Interactions we focus on the structural and molecular mechanisms of restriction enzymes. Among the questions being asked are: How do the restriction enzymes recognize the particular DNA sequence? What common structural principles exist among restriction enzymes that recognize related nucleotide sequenc-

es? How do the sequence recognition and catalysis are coupled in the function of restriction enzymes? Answers to these questions are being sought using X-ray crystal structure determination of restriction enzyme-DNA complexes, site-directed mutageneses and biochemical studies to relate structure to function (see below for the details).

NTP-dependent restriction enzymes

The stress-sensitive RM system CglI from *Corynebacterium glutamicum* and the homologous NgoAVII RM system from *Neisseria gonorrhoeae* FA1090 are composed of three genes: a DNA methyltransferase (M.CglI and M.NgoAVII), a putative restriction endonuclease (R.CglI and R.NgoAVII, or R-proteins) and a predicted DEAD-family helicase/ATPase (H.CglI and H.NgoAVII or H-proteins). Size-exclusion chromatography and SAXS experiments revealed that the isolated R.CglI, R.NgoAVII and H.CglI proteins form homodimers, while H.NgoAVII is a monomer in solution. Moreover, the R.CglI and H.CglI proteins assembled in a complex with R2H2 stoichiometry. Next, we showed that H-proteins have ATPase activity that is dependent on double-stranded DNA and is stimulated by the R-proteins. Functional ATPase activity and extensive ATP hydrolysis (~170 ATP/s/monomer) are required for site-specific DNA cleavage by R-proteins. We further showed that ATP-dependent DNA cleavage by R-proteins occurs at fixed positions (6-7 nucleotides) downstream of the asymmetric recognition sequence 5'-GCCGC-3'. Despite similarities to both Type I and II restriction endonucleases, the CglI and NgoAVII enzymes may employ a unique catalytic mechanism for DNA cleavage.

To establish the molecular mechanism of the sequence recognition by NTP-dependent restriction enzymes we have solved crystal structures of the R.NgoAVII apo-protein and the R.NgoAVII C-terminal domain bound to a specific DNA (Figure 5). R.NgoAVII is composed of two domains: an N-terminal nucleolytic PLD domain; and a C-terminal B3-like DNA-binding domain identified previously in BfiI and EcoRII REases, and in plant transcription factors.

Structural comparison of the B3-like domains of R.NgoAVII, EcoRII, BfiI and the plant transcription factors revealed a conserved DNA-binding surface comprised of N- and C-arms that together grip the DNA. The C-arms of R.NgoAVII, EcoRII, BfiI and plant B3 domains are similar in size, but

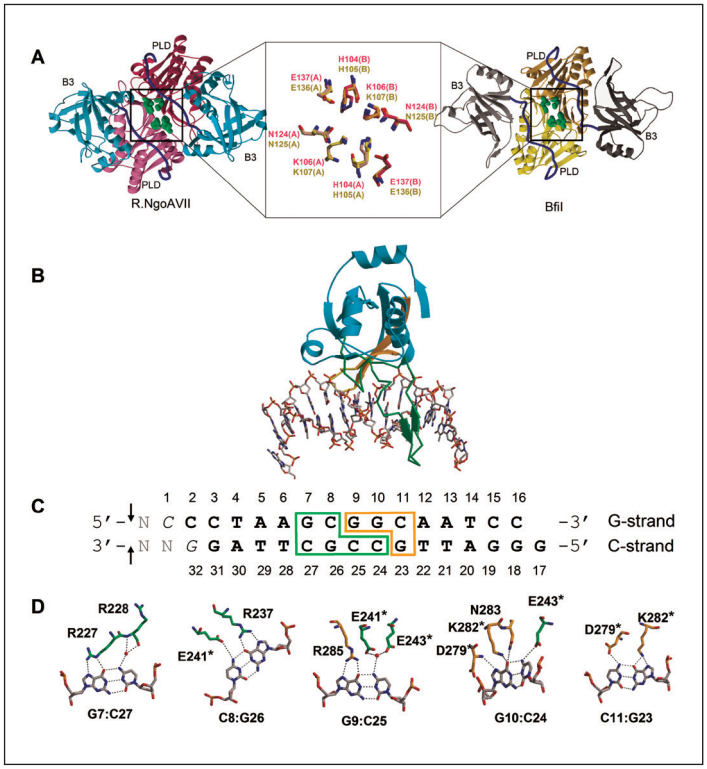

Figure 5. Crystal structure and DNA recognition by R.NgoAVII-B3. (A) Structure of R.NgoAVII. The R.NgoAVII N-terminal PLD domains (pink) form a dimer with a single active site (active site residues H104 and K106 are depicted in green). The C-terminal B3-like domains (cyan) are connected to the catalytic domains by long linkers (blue) and are positioned on both sides of a dimeric core. The R.NgoAVII dimer is similar to BfiI (PDB ID 2C1L). (B) Overall structure of the B3-like domain in DNA-bound form. The N-arm is colored green and the C-arm is colored orange. (C) The cognate oligoduplex used in co-crystallization. (D) Recognition of the individual base pairs by R.NgoAVII-B3. Residues from the N-arm are colored green and residues from the C-arm are colored orange. Residues involved in the recognition of more than one base pair are marked by asterisk.

the R.NgoAVII N-arm which makes the majority of the contacts to the target site is much longer. The overall structures of R.NgoAVII and BfiI are similar; however, whilst BfiI has stand-alone catalytic activity, R.NgoAVII requires an auxiliary cognate N.NgoAVII protein and ATP hydrolysis in order to cleave DNA at the target site. The structures will help formulate future experiments to explore the molecular mechanisms of intersubunit crosstalk that control DNA cleavage by R.NgoAVII and related endonucleases.

B3-like DNA binding domain of BfiI restriction enzyme

The B3 DNA-binding domains (DBDs) of plant transcription factors (TF) and DBDs of EcoRII and BfiI restriction endonucleases (EcoRII-N and BfiI-C) share a common structural fold, classified as the DNA-binding pseudobarrel. The B3 DBDs in the plant TFs recognize a diverse set of target sequences. The only available co-crystal structure of the B3-like DBD is that of EcoRII-N (recognition sequence 5'-CCTGG-3'). In order to understand the structural and molecular mechanisms of specificity of B3 DBDs, we have solved the crystal structure of BfiI-C (recognition sequence 5'-ACTGGG-3') complexed with 12-bp cognate oligoduplex (Figure 6). Structural comparison of BfiI-C-DNA and EcoRII-N-DNA complexes reveals a conserved DNA-binding mode and a conserved pattern of interactions with the phosphodiester backbone. The determinants of the target specificity are located in the loops that emanate from the conserved structural core. The BfiI-C-DNA structure presented here expands a range of templates for modeling of the DNA-bound complexes of the B3 family of plant TFs.

Structure and mechanism of modification-dependent restriction enzymes

Unlike conventional restriction endonucleases (REases) that recognize and cleave unmodified DNA sequences, cytosine modification-dependent REases recognize DNA sites containing 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC) or glucosylated cytosine. To date, several families of cytosine modification-dependent restriction endonucleases (REases) were identified, and some of them were employed as molecular tools for the epigenetic profiling of cytosine modifications in eukaryotic DNA. The MspJI family of modification-dependent REases recognize 5mC and 5hmC in various sequence contexts, and cleave DNA at a fixed distance from the modified cytosine. We aim to understand the structural and molecular mechanisms of MspJI family REases.

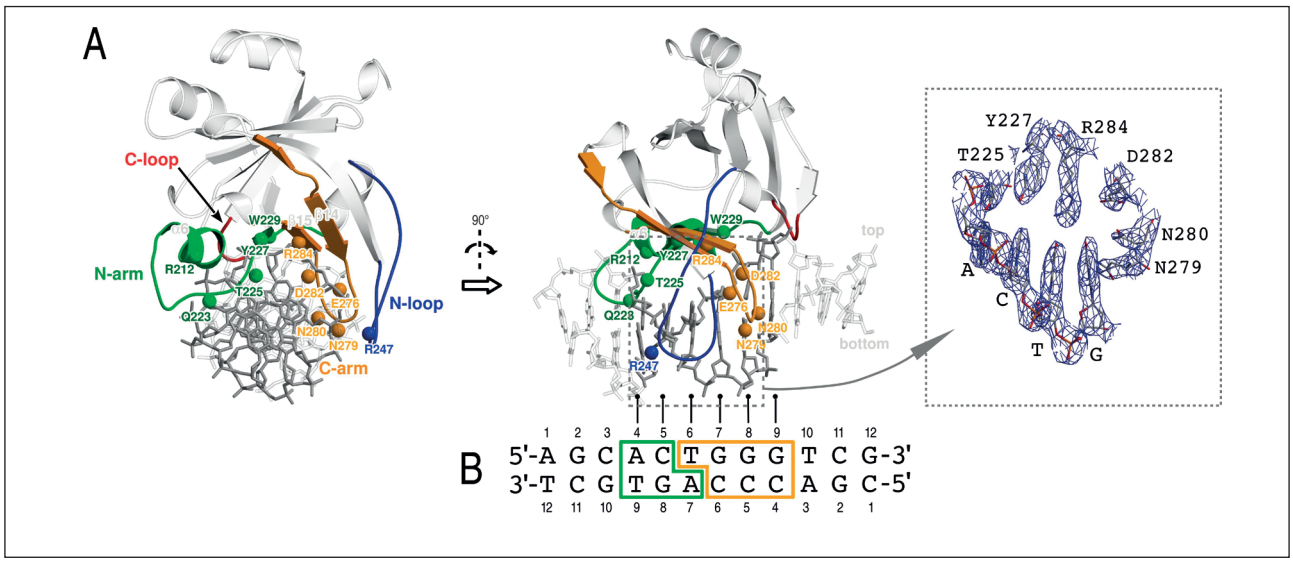

Figure 6. DNA recognition by BfiI-C. (A) The view of the BfiI-C–DNA complex along the long DNA axis (left) and the side view (right). The DNA-recognition site is colored dark grey. Spheres represent the Cα atoms of the DNA-recognition residues from the N- and C-arms. The N-loop is colored blue and the C-loop is red. A region of the top DNA strand (nucleotides A4-G7) and adjacent recognition residues are shown against their mFO-DFC SIGMAA-weighted-electron density contoured at 2.0 σ level. (B) The sequence and numbering of the cognate 12/12 oligoduplex used in this study. DNA bases that interact with the N- and C-arms are boxed in green and orange, respectively.

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Publications 2013-2014

1. **Tamulaitis G., Kazlauskiene M., Manakova E., Venclovas Č.**, Nwokeoji A.O, Dickman M.J., Horvath P. and **Siksnys V.** Programmable RNA shredding by the Type III-A CRIPSR-Cas system of Streptococcus thermophilus. Mol Cell 2014, 56(4):506-17.

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5. **Tamulaitiene G., Silanskas A., Grazulis S., Zaremba M., Siksnys V.** Crystal structure of the R-protein of the multisubunit ATP-dependent restriction endonuclease NgoAVII. Nucleic Acids Res 2014, 42(22):14022-14030.

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PATENT APPLICATIONS

1. **Siksnys V., Gasiunas G., Karvelis T.,** Lubys A., Zaliauskiene L., Glemzaite M., Smith A. RNA-directed DNA cleavage by the Cas9-crRNA complex from CRISPR3/Cas immune system of Streptococcus thermophilus. WO/2013/142578, PCT/US2013/033106. 2013/03/20

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3. **Siksnys V., Kazlauskiene M., Tamulaitis G.** Programmable RNA shredding by the Type III-A CRSIPR-Cas system of Streptococcus thermophilus. U.S. Patent Application Serial No. 62/046, 384.

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AdoMet-dependent methyltransferases (MTases), which represent more than 3% of the proteins in the cell, catalyze the transfer of the methyl group from S-adenosyl-L-methionine (SAM or AdoMet) to N-, C-, O- or S-nucleophiles in DNA, RNA, proteins or small biomolecules. In DNA of mammals, cytosines are often methylated at the 5-position of the pyrimidine ring to give 5-methylcytosine (5mC). DNA methylation profiles are highly variable across different genetic loci, cells and organisms, and are dependent on tissue, age, sex, diet, and disease. Besides 5mC, certain genomic DNAs have been shown to contain substantial amounts of 5-hydroxymethyl-cytosine (hmC). It was demonstrated that hmC is predominantly produced via oxidation of 5mC residues by TET oxygenases and that it can be further oxidized to form 5-formylcytosine (fC) and 5-carboxylcytosine (caC) (Fig. 1). Current evidence suggests that although hmC, fC and caC are intermediates on the pathway of active DNA demethylation, and the multiplicity of epigenetic states may also play independent roles in embryonic development, brain function and cancer progression. Therefore, a full appreciation of the biological significance of epigenetic regulation in mammals will require the development of novel tools that allow hmC, 5mC and C to be distinguished unequivocally.

Novel cofactor-independent reactions of DNA methyltransferases

S-Adenosylmethionine-dependent DNA methyltransferases (MTases) perform direct methylation of cytosine to yield 5-methylcytosine (5mC), which serves as part of the epigenetic regulation mechanism in vertebrates. Previously we found that (i) DNA C5-MTases catalyze covalent addition of exogenous aliphatic aldehydes to their target residues in DNA, yielding corresponding 5-α-hydroxyalkylcytosines and (ii) can promote the reverse reaction – the removal of formaldehyde from hmC in DNA (Liutkevičiūtė et al., Nat. Chem. Biol., 2009, 5: 400–402). We have also discovered that bacterial C5-MTases can catalyze in vitro condensation of aliphatic thiols and selenols to 5-hydroxymethyl-cytosine in DNA yielding 5-chalcogenomethyl derivatives (Liutkevičiūtė et al., Angew. Chem. Int. Ed., 2011, 50: 2090–2093). Most recently, we have demonstrated that both bacterial and mammalian C5-MTases can catalyze the direct decarboxylation of caC yielding unmodified cytosine in DNA in vitro but are inert toward fC. The observed atypical enzymatic C–C bond cleavage reaction provides a

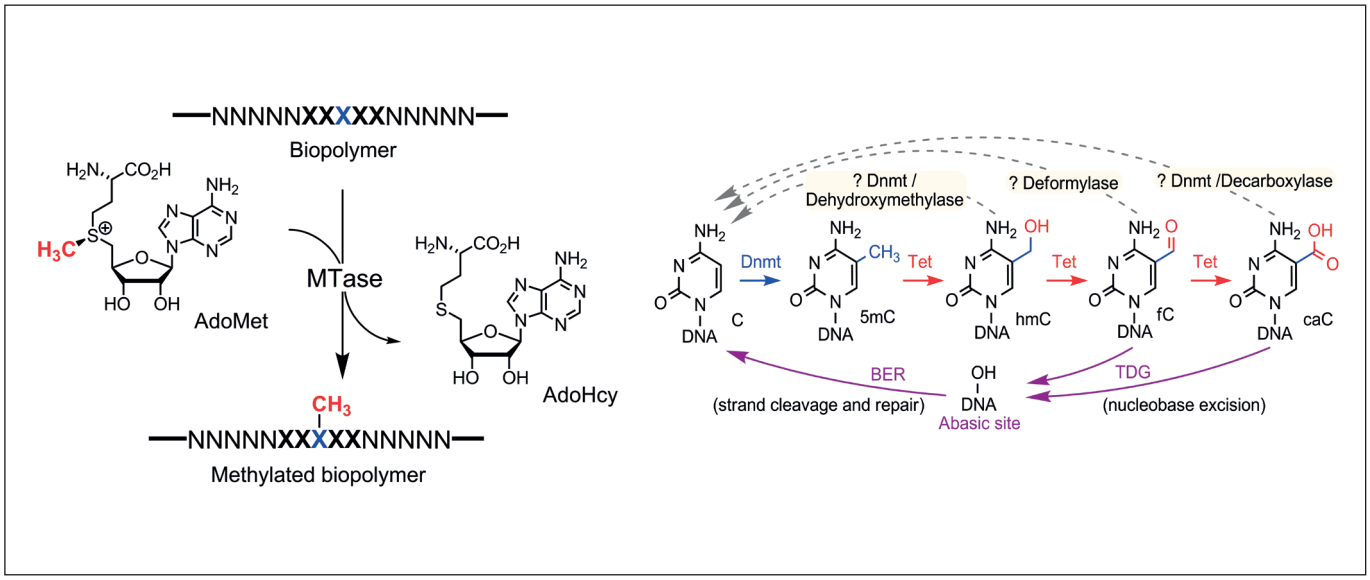

Figure 1. Biological Methylation and Demethylation. Left, targeted transfer of the methyl group (red) from the S-adenosyl-methionine cofactor (AdoMet) to a specific position of a biopoplymer by a methyltransferase (MTase) releasing S-adenosyl-homocysteine (AdoHcy) coproduct. Right, Biological DNA methylation and demethylation in vertebrates. Cytosine (C) is converted to 5-methylcytosine (5mC) by endogenous C5-MTases of the Dnmt1 and Dnmt3 families (blue); 5mC can be consecutively converted to 5-hydroxymethylcytosine (hmC), 5-formylcytosine (fC), and 5-carboxylcytosine (caC) by the TET oxygenases (red). Known reverse pathways (magenta) involve base excision repair (BER) of fC and caC by thymine DNA glycosylase (TDG), leading to transient formation of abasic sites in DNA. Dashed arrows denote the newly discovered 5-dehydroxymethylation and 5-decarboxylation reactions performed by C5-MTases and which may be carried out in vivo by Dnmts or other putative enzymes.

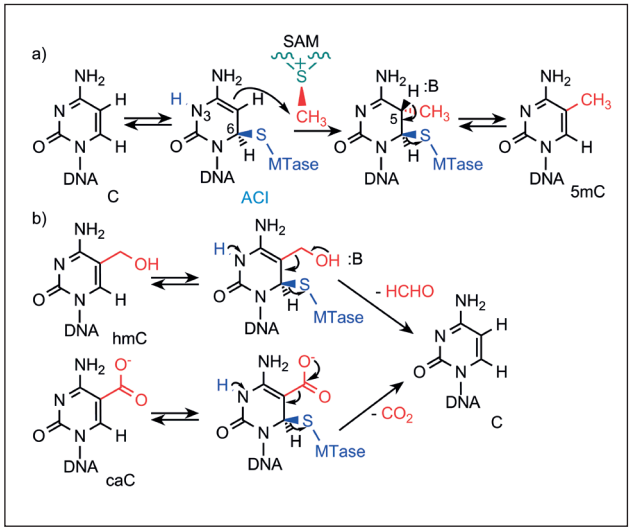

Figure 2. Mechanisms of covalent catalysis by DNA cytosine-5 MTases. A) Biological DNA methylation involves a covalent addition of a catalytic Cys residue at the C6 position of the cytosine, leading to an activated covalent intermediate (ACI) and sebsequnt tranfer of the activated methyl group from the SAM cofactor. B) Atypical C-C bond cleavage reactions catalyzed by MTases, dehydroxymethylation (upper) and decarboxylation (lower), also use the covalent activation mechanism.

plausible precedent for a direct reversal of caC to the unmodified state in DNA and offers a unique approach for sequence-specific analysis of genomic caC [5].

The MTase-directed decarboxylation reaction occurs under mild conditions and retains the cognate sequence- and nucleotide-specificity of the enzyme. The M.SssI MTase is also similarly active toward hmC, but not toward 5mC and fC. Based on these findings we developed a new method for CpG-specific analysis of genomic caC residues in which hmC sites are blocked by treatment of DNA with T4-β-glucosyltransferase (BGT), whereas C, 5mC, and fC sites are selectively eliminated by R.MspI cleavage and end-processing [5].

Engineering the catalytic reaction of methyltransferases for targeted covalent labeling of DNA

The ability of most MTases to catalyze highly specific covalent modifications of biopolymers makes them attractive molecular tools, provided that the transfer of larger chemical entities can be achieved. Our long standing effort is aimed at **redesigning the enzymatic methyltransferase reactions for targeted covalent deposition of desired functional or reporter groups** onto biopolymer molecules such as **DNA and RNA**. We had synthesized a series of model AdoMet analogs with sulfonium-bound extended side chains replacing the methyl group and showed that allylic and propargylic side chains can be efficiently transferred by DNA MTases with high sequence- and base-specificity (Dalhoff et al, Nature Chem. Biol., 2006, 2: 31–32; Klimašauskas and Weinhold, Trends Biotechnol., 2007, 25: 99–104) in collaboration with the group of Prof. Elmar Weinhold (RWTH Aachen, Germany). Using DNA MTases along with their novel cofactors that carry useful functional or reporter groups, we demonstrated that our new approach name mTAG (methyltransferase-directed Transfer of Activated Groups) can be used for sequence-specific functionalization and labeling of a wide variety of model and natural DNA substrates (see below). To further optimize the efficacy of the mTAG reactions we performed steric engineering of the cofactor pocket in the M.HhaI C5-MTase by systematic replacement of three non-essential positions (Lukinavičius et al., Nucleic Acids Res., 2012, 40, 11594–11602). Analogous replacements of two conserved residues in M.SssI C5-MTase also resulted in improved transalkylation activity [1] attesting a general applicability of the homology-guided engineering to the C5-MTase family and expanding the repertoire of sequence-specific tools for covalent in vitro and ex vivo labeling of DNA.

Improved synthetic AdoMet analogues for methyltransferase-directed labeling of DNA

Although clearly useful in certain applications, our previously described series of AdoMet analogues carrying sulfonium-bound 4-substituted but-2-ynyl side chains (Lukinavicius et al., J. Amer. Chem. Soc., 2007, 129: 2758), exhibited short lifetimes in physiological buffers. Examination of the reaction kinetics and products showed that their fast inactivation followed a differnt pathway than observed for AdoMet and rather involved a pH-dependent addition of a water molecule to the side chain. This side reaction was eradicated by synthesis of a series of cofactor analogues in which the separation between an electronegative group and the triple bond was increased from one to three carbon units. The designed hex-2-ynyl moiety-based cofactor analogues with terminal amino, azide, or alkyne groups showed a markedly improved enzymatic transalkylation

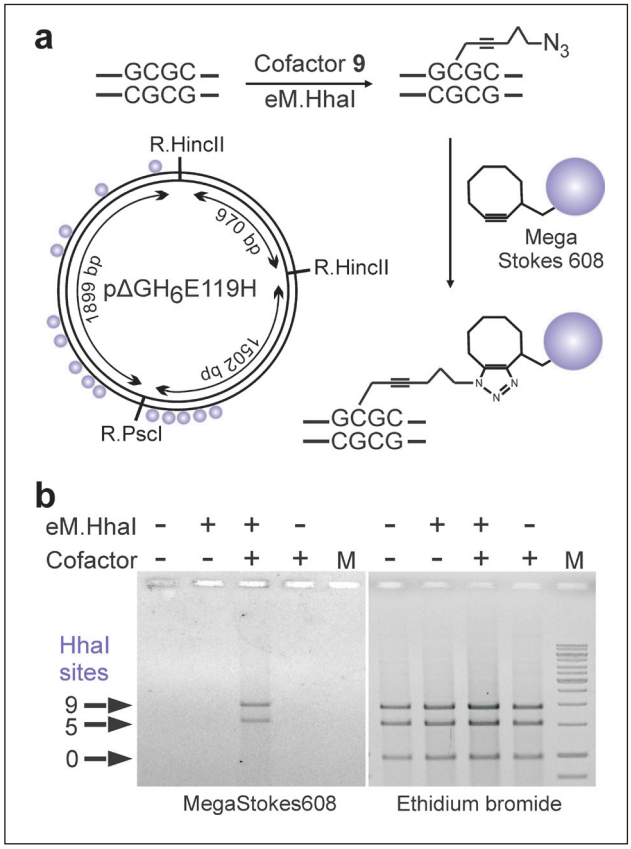

Figure 3. Sequence-specific two-step mTAG labeling of plasmid DNA using copper-free click reaction in crude cell extract. a) Escherichia coli ER2267 cells carrying the pΔGH6E119H plasmid were harvested, and crude lysate was treated with eM.HhaI (engineered HhaI MTase) and an azide cofactor analogue for 3 h at 37 °C. Alkyne MegaStokes 608 dye was added, and incubation continued for another 3 h. b) Image of agarose gel electrophoresis of the labeled plasmid fragmented with R.HincII and R.PscI endonucleases. Number of HhaI sites is indicated on the left of the fragment-pointing arrows. Control samples lacked M.HhaI or cofactor as indicated. M, DNA size marker.

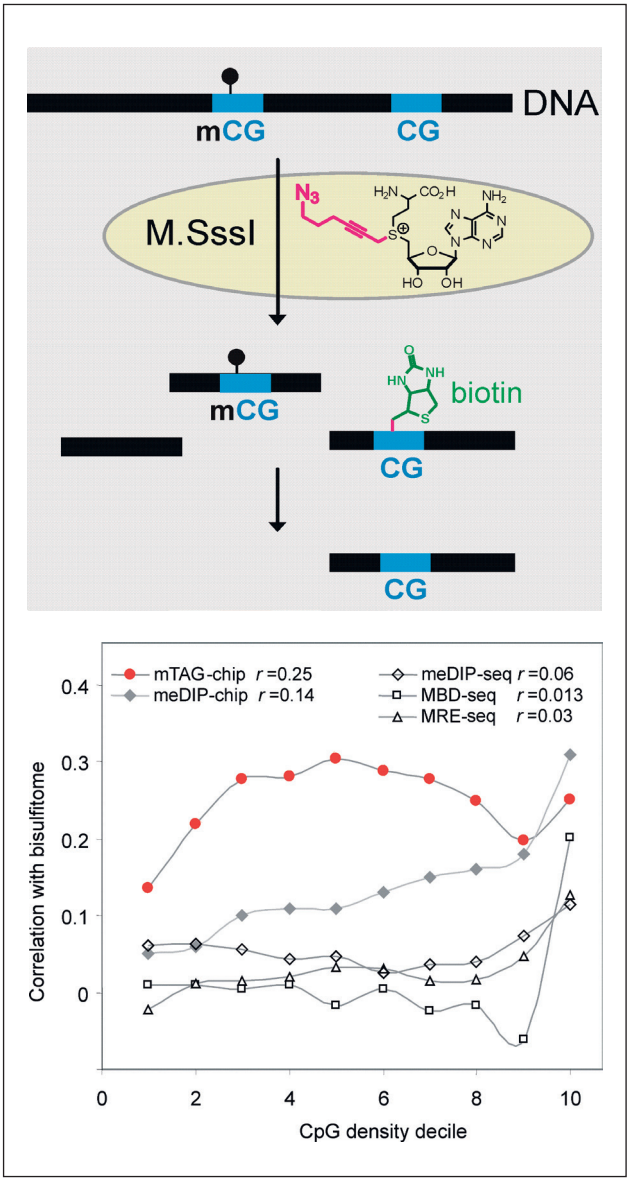

Figure 4. mTAG labeling-based enrichment and analysis of unmethylated CpG sites in the genome.. Left, (a) schematic outline of the analytical procedure. Right, Comparison of the mTAG approach with existing methylome profiling methods. Pearson correlations between experimental mTAG-chip, MeDIP-chip of IMR90 gDNA and published data of MeDIP-seq, MBD-seq, MRE-seq analysis of H1 gDNA were determined for 1 kb tiles on Chr4 against the corresponding MethylC-seq data and stratified according to local CpG density. Aggregate correlation numbers (r) obtained with each analytical procedure are shown above the plots.

activity and proved well suitable for methyltransferase-directed sequence-specific labeling of DNA in vitro and in bacterial cell lysates [2].

Molecular tools for genome analysis: epigenome profiling by covalent capture of unmodified CpG sites

Dynamic patterns of cytosine-5 methylation of CpG dinucleotide and its successive oxidated forms (see Figure 1) are part of epigenetic regulation in eukaryotes, including humans, which contributes to normal phenotypic variation and disease risk. However, no method alone can grasp the breath and chemical complexity of the mammalian epigenome. In collaboration with Prof. Art Petronis, CAMH, Canada, we went on to develop an approach for the mapping of unmodified regions of the genome, which we call the unmethylome [1]. Our technique exploits mTAG-based covalent biotin tagging of unmodified CpG sites using an engineered version of the M.SssI MTase followed by affinity enrichment and interrogation on tiling microarrays or next generation sequencing.

Control experiments and pilot studies of human genomic DNA from cultured cells and tissues demonstrate that, along with providing a unique cross-section through the chemical landscape of the epigenome, the mTAG-based approach offers high precision and robustness as compared with existing affinity-based techniques.

Covalent Labeling of miRNA and siRNA Duplexes Using HEN1 Methyltransferase

miRNAs are important regulators of mammalian gene expression. Individual miRNAs exhibit characteristic expression patterns that vary in normal and disease cells and therefore miRNA profiles provide valuable information for diagnosis and development of novel therapies. Current methods for small RNA discovery largely rely on size-dependent cloning of nucleic acids strands followed sequencing. However, such approaches lack the specificity required to discriminate against similarly sized other types of cellular RNA and DNA molecules and their degradation fragments.

We exploited the mTAG strategy and the specificity of the HEN1 methyltransferase in which miRNA duplexes are selectively targeted for labeling in mixtures of different types of RNA or DNA. We observe a highly efficient labeling of miRNA strands with biotin of fluorophores using a two-step procedure. The two-step approach offers the flexibility in selecting desired labeling chemistries and reporter groups by simply choosing different cofactors and matching chemical probes. We also demonstrate the first single-step labeling of RNA using a novel synthetically produced AdoMet analogue that carries a biotin reporter. The latter labeling modality may be more expensive but will be most useful when speed and simplicity is desired. Altogether, these features make our new molecular tools an extremely valuable addition to the existing analytical toolbox of small RNA paving the way to developing numerous novel analytical techniques. This is the first report of selective labeling of a whole class of cellular RNAs rather than defined nucleotides or sequences [6].

PhD student Milda Mickute caught in preparation for RNA methylation analysis

Roles of the plant 2'-O-methyltransferase HEN1 in microRNA biogenesis

Arabidopsis HEN1 belongs to a family of RNA 2'-O-methyltransferases widespread in both prokaryotes and eukaryotes that share conservative catalytic domain and transfer methyl group on 3'-terminal nucleotide of RNA molecules. The unique feature of the plant homologues is that they covalently modify double-stranded microRNAs or siRNAs. To achieve its biological function the plant HEN1 carries four additional domains preceding the catalytic methyltransferase domain. We experimentally showed that the two double-stranded RNA binding domains of HEN1 considerably but unequally contribute to the binding of mature miRNA/miRNA* duplex, and mapped residues in each domain responsible for this function. Detailed enzymatic and mutational analysis of the RNA interacting domains provided new important insights into the underlying mechanisms of double-stranded RNA specific recognition by 2'-O-methyltransferases [3, 7].

We also showed for the first time direct physical interactions of HEN1 with two proteins essential for plant microRNAs biogenesis, ribonuclease III-type enzyme DICER-LIKE 1 and double-stranded RNA binding protein HYL1, and the lack of direct interactions with SERRATE protein, a third component of the presumed plant miRNA processing complex. We further mapped a central domain of previously unknown function in HEN1 is a key factor for tight binding with HYL1 protein. Based on these findings, we propose model of plant microR-

NAs biogenesis wherein for the first time it was assigned a physical assembly for HEN1 methyltransferase with other proteins involved in maturation of microRNAs (Figure 5). Since HEN1 has been implicated and is the only invariable protein in biogenesis of all small RNA types in plants (miRNA, ta-siRNA, nat-siRNA, ls-siRNA, hc-siRNA and etc.), our studies thus pave the way to understanding the roles of RNA 2'-O-methylation in maturation of other classes of cellular small RNAs [7].

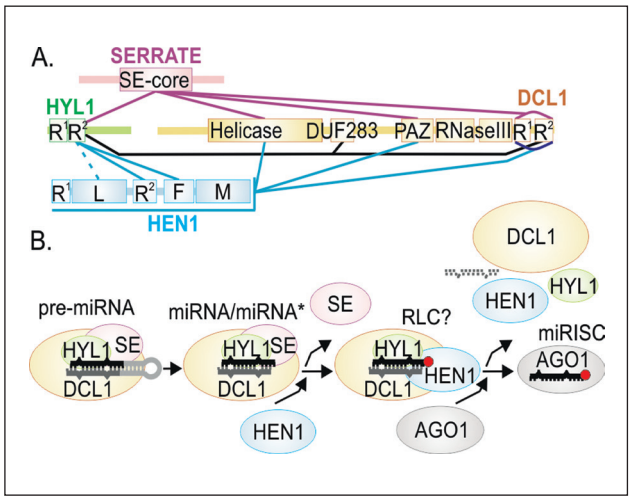

Figure 5. HEN1 interaction network and the proposed model of late stages of miRNA biogenesis. (A) Protein-protein interaction network involving SE, HYL1, DCL1 and HEN1. Cyan lines show interactions experimentally determined in this work, purple and black lines depict those reported previously. (B) Proposed model of miRNA biogenesis envisions that after the miRNR/miRNR duplex is cut out of its precursor, SE is expelled and HEN1 methyltransferase is bound in the microprocessor complex to form a HYL1-HEN1-DCL1 complex, which might represent the still unidentified plant RISC-loading complex (RLC). This complex directs HEN1 methylation (red circle) to the target miRNA strand (black) thus marking it for incorporation into AGO1 complex.*

Biosynthesis of selenoproteins with genetically-encoded photocaged selenocysteines

Engineering and in-cell production of recombinant proteins with desired catalytic capacity is widely exploited for structural and functional studies and for practical applications in medicine and industry. L-Selenocysteine (Sec), the 21st amino acid, endows engineered proteins with new valuable properties due to its enhanced chemical reactivity (higher nucleophilicity, lower pKa, and a lower redox potential) as

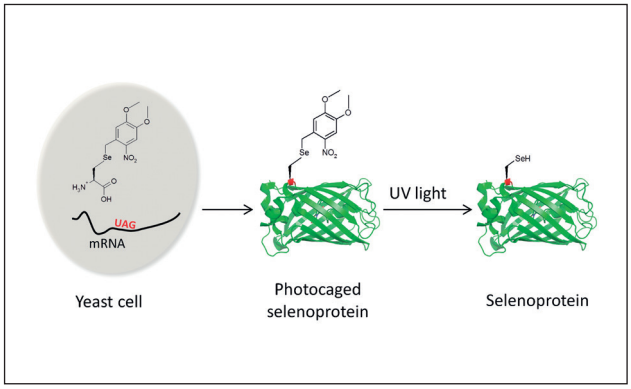

Figure 6. Strategy for incorporation of a photocaged selenocysteine into a genetically-encoded position of a recombinant protein in yeast cells followed by its photochemical decaging in vitro.

compared to cysteine. Despite its high technological potential, targeted incorporation of Sec into recombinant proteins is far from trivial. We developed the first general approach for efficient biosynthesis of selenoproteins containing photocaged selenocysteine residues at genetically predetermined positions. We explored a novel strategy based on a photolabile (4,5-dimethoxy-2-nitrobenzyl, DMNB) group to protect Sec in producing cells and during protein isolation using a yeast expression system originally designed to incorporate DMNB-Ser residues in proteins (Figure 6). We achieved efficient incorporation of DMNB-Sec in a model protein, EGFP, and also demonstrated an efficient photolytic removal of the protecting group from the Se atom, which has not been previously described for any protein or a synthetic peptide. Examples of light-controlled dimerization and site-specific labeling of such recombinant proteins further illustrate robustness and practical utility of the new technique. The generality of this approach is attested by our recent successful production of a HpaII DNA cytosine-5 methyltransferase fusion protein, in which an essential catalytic Cys is replaced with Sec. This paves the way to direct comparison of S- and Se-nucleophiles in the natural and atypical reactions (see above) potentially leading to design of improved molecular tools for genome studies [8].

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Thermo Fisher Scientific Baltics

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At present computational methods are playing an increasingly important role in biological research. Breakthroughs in technologies have resulted in a flood of various types of biological data such as genome sequences for different organisms, data on gene expression, protein-protein interactions, etc. Computational biology and bioinformatics are helping to make sense of all this vast biological data by providing tools for performing large-scale studies. In addition, computational biologists are utilizing available experimental data to improve various analytical and predictive methods that could help address specific biological problems.

Research carried out in our department covers a broad range of topics that can be collectively described as Computational Studies of Protein Structure, Function and Evolution. There are two main research directions:

- Development of methods for detecting protein homology (common evolutionary origin) from sequence data, comparative protein structure modeling, analysis and evaluation of protein 3D structure, analysis of protein-protein interactions in 3D.
- Application of computational methods for discovering general patterns in biological data, structural/functional characterization of proteins and their complexes; design of novel proteins and mutants with desired properties. We address a variety of challenging biological problems, yet our main focus is on proteins and protein complexes that perform work on nucleic acids.

Development of computational methods

Our major achievements in the area of methods development during the report period were in the evaluation and analysis of 3D structure of biological macromolecules and their complexes. We have also been actively developing an improved distant homology detection method and a framework for searching, analysis and modeling of protein-protein interactions in 3D.

CAD-score: contact area-based comparison of structures and interfaces of proteins, nucleic acids and their complexes

The evaluation of protein and RNA 3D structure is particularly important in computational modeling. Scoring models against the native structure is at the heart of development and benchmarking of protein and RNA structure prediction and refinement methods. It may seem that one-to-one correspondence between computational models and the native (reference) structure should make such evaluation trivial. Yet, contrary to this view, it is an open problem, because many aspects of the reference-based model evaluation still lack desired robustness. We have been actively researching how to improve reference-based model evaluation for proteins, nucleic acids and their complexes. As a result, we have developed CAD-score (Contact Area Difference score), a new score for measuring differences between physical contacts in two 3D structures of the same biological macromolecule or complex. CAD-score is both highly effective and universal as it can be applied to different types of biological macromolecules (proteins, RNA, DNA) and their complexes.

The new score uses the concept of residue-residue contact area difference (CAD) introduced by Abagyan & Totrov (J. Mol. Biol. 1997; 268:678–685). Contact areas, the underlying basis of the score, are derived using the Voronoi diagram of 3D balls that correspond to heavy atoms of van der Waals radii (Figure 1). The Voronoi diagram of balls is constructed using a new algorithm that is especially suited for processing macromolecular structures. We implemented the algorithm as open-source software, Voronota, which is available through our web site http://www.ibt.lt/bioinformatics/voronota/. The algorithm itself, the implementation details and the performance of Voronota are described in a paper in Journal of Computational Chemistry [5].

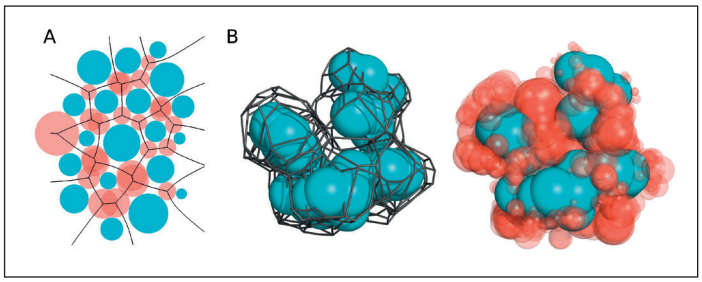

***Figure 1.** Voronoi diagram for two-dimensional (2D) and three-dimensional (3D) balls (a) Voronoi cells of 2D balls (blue) and the empty tangent spheres (red) corresponding to the Voronoi vertices. (b) Edges of the Voronoi cells of 3D balls (left) and the empty tangent spheres corresponding to the Voronoi vertices (right).*

The algorithm resolves residue–residue contacts at the level of atoms, making it possible to consider contacts not only between entire residues (amino acid or nucleotide) but also between subsets of residue atoms (main chain/backbone, side chain/base) (Figure 2).

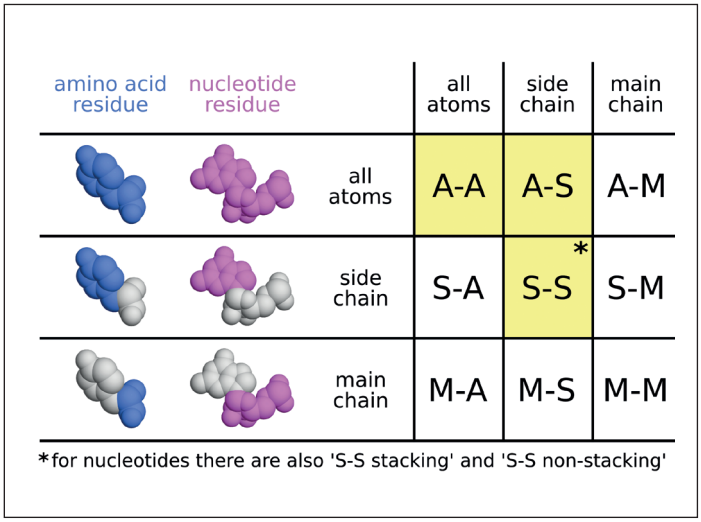

Figure 2. CAD-score variants based on standard subsets of residue (amino acid or nucleotide) atoms. 'A', 'S' and 'M' denote all atoms, side chain (base) and main chain (backbone), respectively. CAD-score variants highlighted in yellow are the most informative.

CAD-score has a number of attractive properties. It is based on physical contacts between residues, thereby directly reflecting interactions within the macromolecular structure. It is a continuous, threshold-free function that returns quantitative accuracy scores within the strictly defined boundaries. The definition of CAD-score does not contain any arbitrary parameters. In fact the only adjustable parameter used in CAD-score is van der Waals radii of atoms. Our new score provides a balanced assessment of domain rearrangement, removing the necessity for different treatment of single-domain, multi-domain and multi-subunit structures. Moreover, CAD-score makes it possible to directly assess differences between domain-domain and subunit-subunit interfaces. We believe that all these attractive properties will make CAD-score a valuable tool for both computational and experimental structural biologists at large. CAD-score has already been included into the standard set of reference-based evaluation scores in CASP experiments. These experiments aim for objective assessment of methods for protein structure prediction and are conducted by the Prediction Center (www.predictioncenter.org/).

To make CAD-score widely accessible we implemented it as a freely-accessible web server (http://www.ibt.lt/bioinformatics/cad-score/). The server provides a simple and intuitive interface for the use of the CAD-score method in the interactive manner. In particular, the server features highly interactive visualization options of local contact differences. An example of local differences mapped onto 3D structure is shown in Figure 3. The server is universal in several ways. It accepts both single-chain and multi-chain structures, works with all the major types of macromolecules (proteins, RNA, DNA and various complexes), allows flexible designation of substructures for the analysis and performs both global and local evaluation.

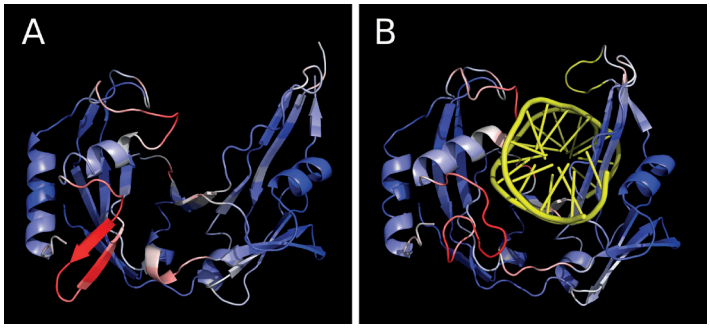

Figure 3. Color-coded local CAD-score differences between the structures of the restriction endonuclease BcnI crystallized (A) in the apo form (PDB id: 2odh) and (B) in complex with the cognate DNA (PDB id: 2q10).

The CAD-score method for proteins has been published in Proteins [1] and its adaptation for RNA in Nucleic Acids Research [6]. The description of CAD-score webserver has been published in Nucleic Acids Research web server issue [7] as NAR Breakthrough Article, putting it among the very best NAR papers.

PPI3D: a web server for searching, analyzing and modeling protein-protein interactions in the context of 3D structures

Most of biological processes are driven by protein-protein interactions. Therefore, the comprehensive understanding of molecular mechanisms of various biological pathways is impossible without the detailed knowledge of protein-protein interactions. With current technologies it is relatively easy to find out whether proteins interact. However, without knowing how they interact, these data alone have limited value. The details regarding protein interactions can be obtained from the three-dimensional (3D) structure of corresponding protein complexes. A large number of experimentally determined structures of protein complexes are already available at the Protein Data Bank (PDB). Even more protein complexes can be characterized computationally using the concept of homology, which implies that related protein complexes have similar sequences and 3D structures.

However, although available 3D structures of protein complexes in PDB offer numerous possibilities, there are several important problems that have to be solved. One of them is the highly redundant nature of PDB. Both the analysis and modeling of protein-protein interactions in 3D necessitates having regularly updated non-redundant set of protein-protein interactions. A related problem is how to define a protein-protein interaction interface and how to assess the interface similarity in constructing a non-redundant data set.

To enable researchers, in particular experimentalists, to make effective use of available PDB data for studies of protein-protein interactions, we have developed the PPI3D (Protein-Protein Interactions in 3D) web server. The web server provides a possibility to query experimentally determined 3D structures of protein complexes, to analyze the identified protein-protein interactions and to generate homology models of protein complexes. Structural data for experimentally determined protein-protein interactions are represented by PDB biological assemblies. All the protein-protein interactions accessible through PPI3D are clustered according to both sequence and interaction interface similarity. This removes the redundancy of structural data while preserving alternative protein binding modes. The server enables users to explore interactions for individual proteins or interactions between a pair of proteins (protein groups). In both modes, protein-protein interactions are detected using sequence search with BLAST or PSI-BLAST. The PPI3D output enables users to interactively explore both the overall results and every detected interaction. In addition, the server provides a possibility to construct a homology model for the protein complex. The workflow of the PPI3D server is provided in Figure 4. The server is freely accessible at http://www.ibt.lt/bioinformatics/ppi3d/. Manuscript describing PPI3D is in preparation.

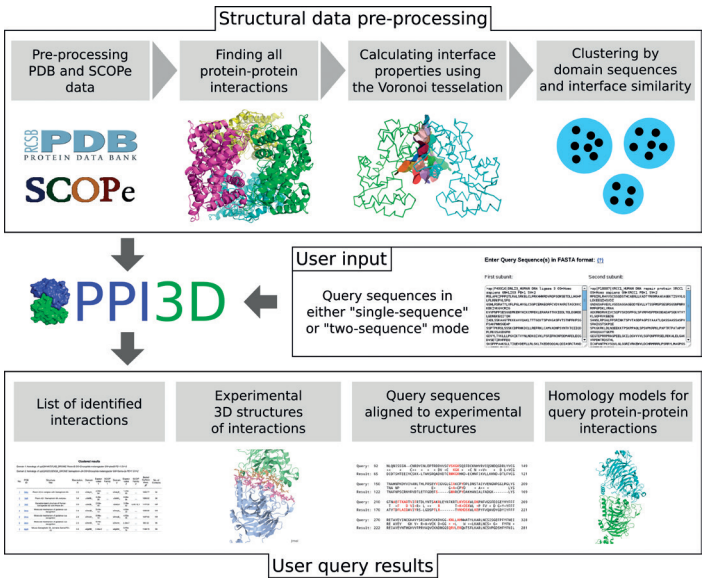

Figure 4. Schematic representation of data pre-processing, the user input and the results provided by the PPI3D server.

Application of computational methods to specific biological problems

An important component of our research is the application of computational methods either alone or in combination with experiments (through collaboration with experimental labs) for addressing specific biological questions. These biological questions are not restricted to some specific topic. Nevertheless, most research projects of this type involve proteins participating in nucleic acids binding or metabolism such as DNA replication and repair. Some of the projects accomplished during the reported period or those still ongoing are listed below.

- Computational analysis of structural and functional properties of DNA polymerases and their distribution in bacterial genomes
- Computational analysis of the nature and distribution of DNA replication proteins in genomes of double-stranded DNA viruses
- Exploring relationship between sequence, structure and dynamics of DNA polymerase processivity subunits from all kingdoms of life (collaboration with Prof. Penny Beuning from Northeastern University in Boston)
- Molecular mechanisms of DNA mutagenesis in *Mycobacterium tuberculosis*, causative agent of tuberculosis (collaboration with Prof. Valerie Mizrahi and Dr. Digby Warner from University of Cape Town)
- Experimental-computational studies of functional properties of human connexins, membrane proteins forming gap junction (GJ) channels that connect cytosols of neighbouring cells and thereby enable direct cell-to-cell communication (collaboration with Prof. Arvydas Skeberdis from the Institute of Cardiology, Lithuanian University of Health Sciences, Kaunas)
- Experimental-computational studies directed at understanding molecular mechanisms of CRISP-Cas systems that provide protection to bacterial cells against invading foreign nucleic acids (collaboration with Prof. Virgis Šikšnys at our Institute)

- Experimental-computational studies aimed at understanding of functions of individual domains in plant RNA methyltransferase HEN1 (collaboration with Dr. Giedrius Vilkaitis at our Institute)
- Experimental-computational studies of molecular mechanism of vaginolysin, a cholesterol-dependent cytolysin produced by pathogenic bacterium *Gardnerella vaginalis* (collaboration with Dr. Milda Plečkaitytė at our Institute)
- Experimental-computational studies of inhibitors of human carbonic anhydrase isoforms that are promising target candidates in various cancer types (collaboration with Prof. Daumantas Matulis at our Institute)

Two of these research projects are highlighted below.

Computational analysis of structural and functional properties of DNA polymerases and their distribution in bacterial genomes

DNA replication and repair are fundamental biological processes that involve multiple proteins, but the actual DNA synthesis is performed by DNA polymerases. The complete set of bacterial DNA polymerases has been extensively studied for only a few model organisms such as *Escherichia coli* that has five DNA polymerases belonging to four different families (A, B, C and Y). However, no systematic analysis of the composition and diversity of DNA polymerase sets encoded in bacterial genomes has ever been performed. As part of such global analysis we started with replicative DNA polymerases and their homologs that belong to C-family of DNA polymerases. We analyzed nearly 2000 bacterial genomes and found that they all without a single exception encode one or more C-family DNA polymerases (PolIII α-subunits). They come in two major forms, PolC and DnaE, related by ancient duplication. While PolC represents an evolutionary compact group, DnaE can be further subdivided into at least three groups (DnaE1-3). We performed an extensive analysis of various sequence, structure and surface properties of all four polymerase groups. Our analysis suggested a specific evolutionary pathway leading to PolC and DnaE from the last common ancestor and revealed important differences between extant polymerase groups. Among them, DnaE1 and PolC show the highest conservation of the analyzed properties. DnaE3 polymerases apparently represent an 'impaired' version of DnaE1. Non-essential DnaE2 polymerases, typical for oxygen-using bacteria with large, GC-rich genomes, have a number of features in common with DnaE3 polymerases. The analysis of polymerase distribution in genomes revealed three major combinations: DnaE1 either alone or accompanied by one or more DnaE2s, PolC+DnaE3 and PolC+DnaE1. The first two combinations are present in *Escherichi coli* and *Bacillus subtilis* respectively. The third one (PolC+DnaE1), found in *Clostridia*, represents a novel, so far experimentally uncharacterized, set.

This study has been published as Survey and Summary in Nucleic Acids Research and has also been presented as a Highlight Paper at ECCB 2014, a key European Computational Biology conference. Our current research includes a similar analysis for all of the DNA polymerase families in bacteria. We hope that the results of this large-scale computational study will provide answers to a number of important questions. What is the minimal sufficient set of DNA polymerases in a bacterial cell? What functional properties are implied by different sets? Is there a dependence of the DNA polymerase set and the genome size, GC content of the genome, living environment, pathogenicity and other attributes?

Exploring relationship between sequence, structure and dynamics of DNA polymerase processivity subunits

Proteins typically function as specific 3D structures which, however, are not static. Instead, they display dynamic properties that are essential for biological function. In this collaborative

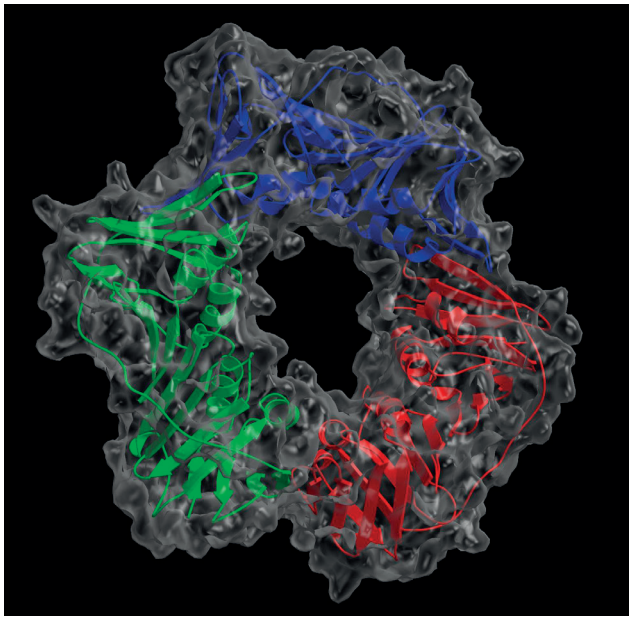

Figure 5. ***Crystal structure of yeast PCNA, a homotrimeric DNA sliding clamp.***

study (with Prof. Penny Beuning at Northeastern University in Boston) we combined experimental and computational approaches to explore whether protein structural similarity translates into similarity of their dynamics. We addressed this question using eight structurally conserved DNA polymerase processivity subunits representing all kingdoms of life. These proteins form doughnut-shaped clamps that can freely slide on the DNA (Figure 5). Therefore, their function is directly related with dynamic properties. Our collaborators in Boston have determined the dynamics of these sliding clamp proteins using the in-solution experimental approach of hydrogen-deuterium exchange mass spectrometry (HX MS). These experiments revealed that, despite similar 3D structures, clamp proteins display a wide range of dynamic behavior. Differences were apparent both for structurally similar domains within proteins and for corresponding domains of different proteins. Several of the clamps contained regions that underwent local unfolding with different half-lives. They also observed a conserved pattern of alternating dynamics of the α-helices lining the inner pore of the clamps. To understand the reason for that, here, at the Institute of Biotechnology, we performed molecular dynamics (MD) simulations. MD results showed that the alternating dynamics of these α-helices correlate with the number of salt bridges and hydrogen bonds. Thus the main conclusion of this study was that protein tertiary structure and dynamics are not directly correlated and that amino acid sequence plays an important role in dynamics. The paper reporting our results has been published in Structure as a featured article.

Collaborative interactions

In addition to extensive scientific interactions with our colleagues at the Institute of Biotechnology we are involved in a number of external collaborations:

Prof. Penny Beuning, Northeastern University, Boston, MA, USA
Prof. Maris Laan, Institute of Molecular and Cell Biology, University of Tartu, Estonia
Prof. Valerie Mizrahi & Dr. Digby Warner, University of Cape Town, South Africa
Prof. Arvydas Skeberdis, Institute of Cardiology, Lithuanian University of Health Sciences, Kaunas, Lithuania

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* Featured article

** NAR Breakthrough article

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Research overview

Department of Eukaryote Gene Engineering is focused on the research directions of recombinant viral and human protein analysis, expression and assembly of proteins into virus-like particles (VLPs) as well as mechanisms of plant signaling and biosynthesis. We use methods of gene cloning and expression in different host systems, electron microscopy, immunological and functional biochemical assays of proteins, biosynthesis and purification of essential amounts of target recombinant proteins with application area including diagnostics and therapeutics. Please find few examples of our research studies during 2013-2014.

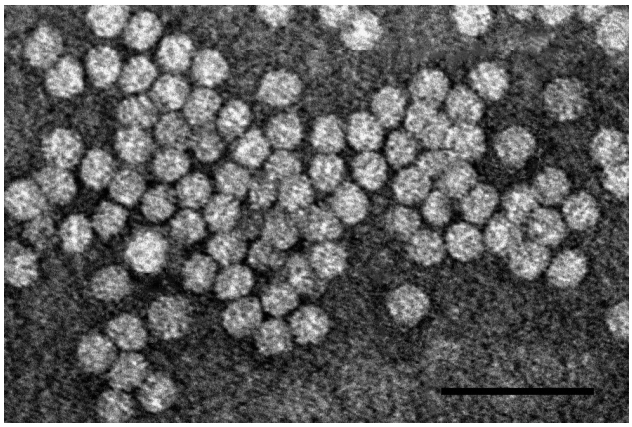

Figure 1. Electron microscopy pictures of VLPs formed by PCV2-Cap protein generated in yeast stained with 2% aqueous uranyl acetate solution and examined by Morgagni 268 electron microscope. Scale bar 100 nm.

Development of porcine circovirus type 2 diagnostics tools

Porcine circovirus type 2 (PCV2)-associated diseases are responsible for significant mortality among pigs and remains a serious economic problem to the swine industry worldwide leading to significant negative impacts on profitability of pork production. The two major groups designated as PCV2a, PCV2b are found worldwide and have been demonstrated to be virulent enough to trigger PCV2-associated diseases. We determined the genomes of PCV2 virus spread in Lithuania pig farms in the samples collected between 2009 and 2013 and confirmed that all isolated virus genomes belonged to the PCV2b genotipe. Three slightly different open reading frame 2 (ORF2) variants were amplified by PCR from native PCV2 genomes as well as ORF2 of one PCV2-Cap gene variant was codon-optimized by S. cerevisiae codon usage and used for the expression in yeast S. cerevisiae. Recombinant PCV2b capside protein generated in yeast assembled into particles (Cap VLPs) similar to native virions (Fig.1). Yeast-derived PCV2 Cap VLPs were capable to induce the generation of PCV2-specific MAbs that did not show any cross-reactivity with PCV1-infected cells. Moreover, yeast-derived recombinant PCV2 Cap VLPs were used as antigen in newly developed the indirect IgG PCV2 Cap VLP-based ELISA. The high sensitivity and specificity of newly developed the indirect IgG PCV2 Cap VLP-based ELISA clearly suggested that this assay is potentially useful diagnostic tool for screening PCV2-suspected samples [Nainys J et al, 2014]. This work was funded by European Social Fund, grant No VP1-3.1-ŠMM-10-V-02-017.

Production of recombinant human polyomavirus-like particles in yeast

Over recent years eleven new human polyomaviruses (HPyVs) have been identified. According to preliminary serological studies all this variety of HPyVs subclinically infects the general population at an early age. As many HPyVs cannot be easily cultured, major capsid protein VP1 is an ideal protein for generation of virus-like particles (VLPs) which resemble native virions they are derived from in structure, immunogenicity and tropism, but do not contain any viral genetic material. Recombinant VP1 VLPs originated from ten newly identified HPyVs were efficiently produced in yeast. Merkel cell polyomavirus (MCPyV) and trichodysplasia spinulosa-associated polyomavirus (TSPyV) derived VP1 self-assembled into homogeneous in size VLPs. Karolinska Institute polyomavirus (KIPyV), HPyV7, HPyV9, HPyV10 and St. Louis polyomavirus (STLPyV) VP1 proteins formed heterogeneous in size VLPs with diameters ranging from 20 to 60 nm. Fractions of smaller VLPs (25–35 nm in diameter) dominated in preparations of VP1 proteins originated from Washington University polyomavirus (WUPyV) and HPyV6. HPyV12 VP1 VLPs were generated from the second of two potential translation initiation sites in VP1-encoding open reading frame. Yeast-produced recombinant VP1 VLPs originated from different HPyV demonstrated distinct HA activity and could be useful in virus diagnostics and capsid structure studies or investigation of entry pathways and cell tropism of new HPyVs replacing corresponding viruses until cell culture systems for the new

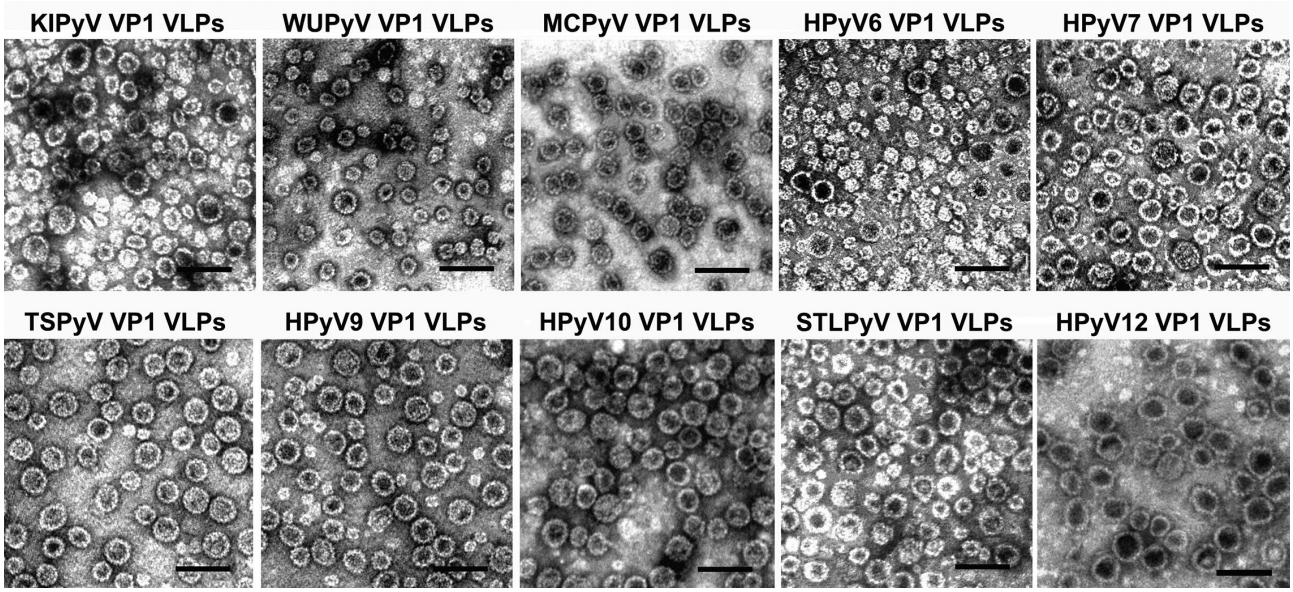

Figure 2. Electron microscopy pictures of VLPs formed by novel human polioma viruses-derived VP1 VLPs generated in yeast stained with 2% aqueous uranyl acetate solution and examined by Morgagni 268 electron microscope. Scal bar 100 nm.

HPyVs will be developed (Figure2). This work was funded by the European Social Fund under National Integrated Program Biotechnology & Biopharmacy, grant No. VP1-3.1-SMM- 08-K01-005.

Analysis of genomic DNA sequences of hamster polyomaviruse isolated from GASH:Sal Syrian golden hamsters suffering from lymphomas

Almost 16 % (90 males and 60 females) of the 975 GASH:Sal Syrian golden hamsters (Mesocricetus auratus) were affected during a 5-year period by the development of a progressing lymphoid tumour and exhibited similar clinical profiles, and a rapid disease progression resulting in mortality within 1 to 2 weeks. A TaqManprobe-based real-time PCR analysis of genomic DNA from different tissue samples of the affected animals revealed the presence of a DNA sequence encoding the hamster polyomavirus (HaPyV) VP1 capsid protein. Additionally, immunohistochemical analysis using HaPyV-VP1-specific monoclonal antibodies confirmed the presence of viral proteins in all hamster tumour tissues analysed within the colony. An indirect ELISA and western blot analysis confirmed the presence of antibodies against the VP1 capsid protein in sera, not only from affected and nonaffected GASH:Sal hamsters but also from control hamsters from the same breeding area. The HaPyV genome that accumulated in tumour tissues typically contained deletions affecting the noncoding regulatory region and adjacent sequences coding for the N-terminal part of the capsid protein VP2. The analysis of the non-deleted HaPyV genome derived from GASH-Sal hamster tumour tissues (JX416849) in comparison with the sequence of HaPyV isolated from Syrian hamsters in Berlin-Buch (JX036360) revealed 132 nucleotide exchanges (112 nucleotide exchanges were in the coding sequence, which resulted in a total of 23 amino acid exchanges in six viral proteins, suggesting that in the Salamanca new strain of HPyV was responsible for the infection which did not originate directly from the same source as Berlin-Buch HaPyV strain [Munoz LJ et al, 2013].

Functional analysis of recombinant lipolytic protein encoded in phytoplasma phage based genomic island

Wall-less bacteria known as phytoplasmas are obligate transkingdom parasites and pathogens of plants and insect vectors. These unusual bacteria possess some of the smallest genomes known among pathogenic bacteria, and have never been successfully isolated in artificial culture. Disease symptoms in-

Scientists: Alma Gedvilaitė, Monika Valavičiūtė, Evaldas Čiplys, Mindaugas Zaveckas. PhD students: Milda Norkienė, Justas Lazutka. Students: Juozas Nainys, Karolis Goda, Vaida Kurmauskaitė, Aliona Špakova, Karolis Simutis.

duced by phytoplasmas in infected plants include abnormal growth and often severe yellowing of leaves, but mechanisms involved in phytoplasma parasitism and pathogenicity are little understood. A phage based genomic island (sequence variable mosaic, SVM) in the genome of Malaysian periwinkle yellows (MPY) phytoplasma harbors a gene encoding membrane-targeted proteins, including a putative phospholipase (PL), potentially important in pathogen-host interactions. Since some phytoplasmal disease symptoms could possibly be accounted for, at least in part, by damage and/or degradation of host cell membranes, we hypothesize that the MPY phytoplasma putative PL is an active enzyme. The present study provided for the first time phytoplasma gene expression investigated and compared in two different protein expression systems: bacterial (E.coli) and yeast (S.cerevisiae). Despite the low level of expression of rPL in both hosts, a sufficient amount of the enzyme was purified for activity testing. The enzyme activity tests revealed that phytoplasmal rPL exhibited lipolytic activity on several different substrates. rPL from MPY phytoplasma demonstrated only moderate PLA1and low PLA2 enzyme activity on substrates incorporated in liposomes and may be called a phospholipase with low PLA activity. Interestingly, rPL expressed in yeasts exhibited slightly higher lipolytic activity in comparison with MPY rPL expressed in E.coli, as well as having a slightly different molecular mass, suggesting distinctive protein modifications which eventually affected enzymatic activity. In conclusion, the results obtained in the present study show the existence of an active lipolytic enzyme, encoded in the genomic SVM region of MPY phytoplasma [Gedvilaite A. et al., 2014].

Generation of recombinant Schmallenberg virus nucleocapsid protein and development of virus-specific monoclonal antibodies

Schmallenberg virus (SBV), discovered in continental Europe in late 2011, causes mild clinical signs in adult ruminants, including diarrhoea and reduced milk yield. However fetal infection can lead to severe malformation in newborn offspring. To develop improved reagents for SBV serology a high-level yeast expression system was employed to produce recombinant SBV nucleocapsid (N) protein. Recombinant SBV N protein was investigated as an antigen in SBV-specific IgG enzyme immunoassay and used for generation of monoclonal antibodies (MAbs). Yeast-expressed SBV N protein was reactive with anti-SBV IgG-positive cow serum specimens collected from different farms of Lithuania (Figure3). After immunization of mice with recombinant SBV N protein, four MAbs were generated. The MAbs raised against recombinant SBV N protein reacted with native viral nucleocapsids in SBV-infected BHK cells by immunofluorescence assay (Figure 4.). The reactivity of recombinant N protein with SBV-positive cow serum specimens and the ability of the MAbs to recognize virus-infected cells confirm the antigenic similarity between yeast-expressed SBV N pro-

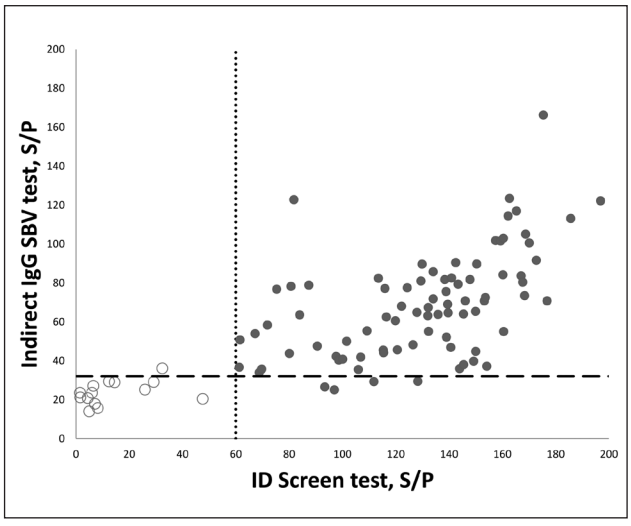

Figure 3. Antibody responses of individual cow serum specimens defined by the newly developed indirect IgG SBV ELISA based on yeast-expressed SBV N protein in comparison to the commercial ID screen test. The S/P ratios of reactivity were plotted. Grey markers represent positive and white negative serum samples obtained by commercial ID screen test. The dotted line represents the cut-off value of the newly developed indirect IgG SBV ELISA.

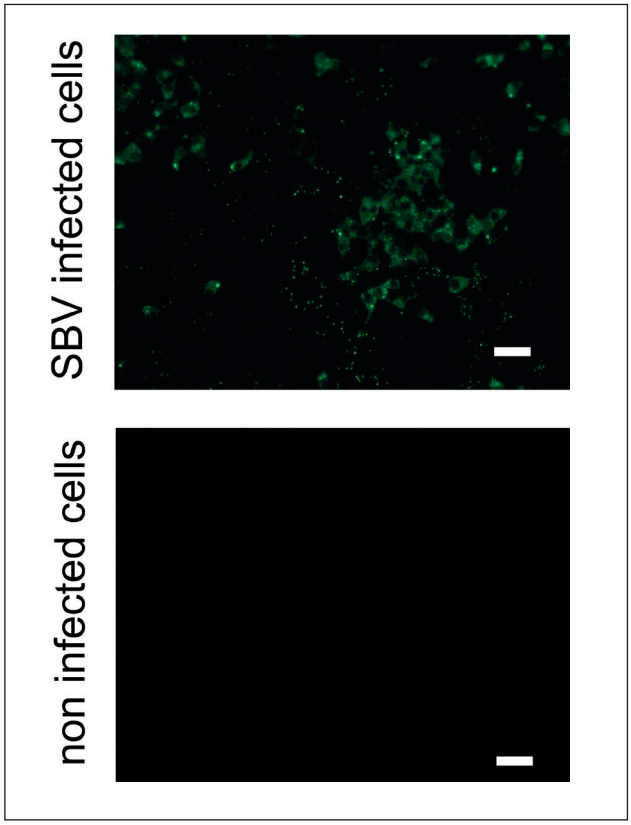

Figure 4. Fluorescence microphotographs showing the reactivity of the MAbs 4F3 with BHK cells infected with SBV BH80/11 strain (upper). Noninfected BHK cells were used as a negative control ((lower). Hybridoma culture supernatants were used at a dilution of 1:10. Scale bar: 100 µm.

tein and native viral nucleocapsids. In summary, yeast-expressed SBV N protein and newly developed SBV-reactive MAbs may provide useful reagents for diagnostics and seroprevalence studies of SBV infection. The study demonstrates that yeast expression system is suitable for high-level production of recombinant SBV N protein and provides the first evidence on the presence of SBV-positive antibodies in cow serum specimens collected in Lithuania [Lazutka J. et al, 2014]. This work is supported by Lithuanian Science Council grant MIP-044/2013.

Investigation of porcine parvovirus

Porcine parvovirus (PPV) is a widespread infectious virus that causes serious reproductive diseases of swine and death of piglets. The gene coding for the major capsid protein VP2 of PPV was amplified using viral nucleic acid extract from swine se-

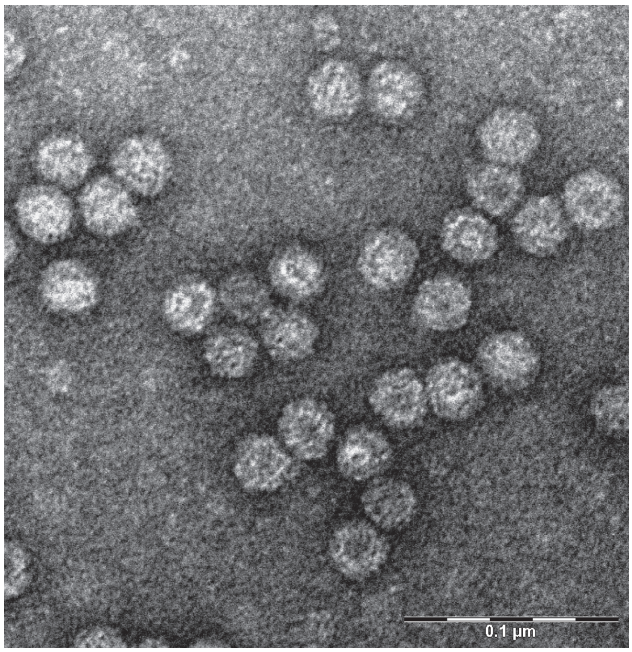

Figure 5. Electron micrograph of recombinant PPV VP2 VLPs generated in yeast. Scale bar 100 nm.

rum and inserted into yeast Sacharomyces cerevisiae expression plasmid. Recombinant PPV VP2 protein was efficiently expressed in yeast and purified using density gradient centrifugation. Electron microscopy analysis of purified PPV VP2 protein revealed the self-assembly of virus-like particles (VLPs) (Figure 5). Nine monoclonal antibodies (MAbs) against the recombinant PPV VP2 protein were generated. The specificity of the newly generated MAbs was proven by immunofluorescence analysis of PPV-infected cells. Indirect IgG ELISA based on the recombinant VLPs for detection of PPV-specific antibodies in swine sera was developed and evaluated. The sensitivity and specificity of the new assay were found to be 93.4 % and 97.4 %, respectively. Yeast S. cerevisiae represents a promising expression system for generating recombinant PPV VP2 protein VLPs of diagnostic relevance [Tamošiūnas P-L et al., 2013; Tamošiūnas P-L et al., 2014].

Study of human parainfluenza virus 2 nucleocapsid protein

Human parainfluenza virus 2 (HPIV2) causes respiratory tract infections. It belongs to the genus Rubulavirus of the family Paramyxoviridae. The research was aimed to produce HPIV2 nucleocapsid protein N in yeast S. cerevisiae. The gene encod-

Name	Introduced sequence change	Variant-specific aa
NR	NP_598401.1:p.K194_M195insR	R195
NV	NP_598401.1:p.D331V	V331
NP	NP_598401.1:p.Q358P	P358
NVR	NP_598401.1:p.[K194_M195insR; D331V]	R195, V332
NVRP	NP_598401.1:p.[K194_M195insR; D331V; Q358P]	R195, V332, P359

Table 1. HPIV2 N protein variants with mutations in respect to reference sequence and with resulting variant-specific amino acids listed.

ing amino acid sequence identical to RefSeq NP_598401.1 has been expressed but the protein did not form nucleocapsid-like particles (NLPs), which are characteristic to yeast-produced Paramyxoviridae N proteins. N protein sequences were obtained by conceptual translation of GenBank HPIV2 genomes and an alignment was generated. Three instances of sequence disagreement characteristic of NP_598401.1 exclusively were identified. PCR mutagenesis of N gene was used to introduce mutations resulting in amino acids characteristic to alignment consensus (Table 1).

No NLPs were present in the preparations of NR and NP. The samples of NV, NVR, and NVRP contained NLPs typical of Paramyxoviridae (Figure 6), implying substitution NP_598401.1:p.D331V concluded in NLP formation. N protein 3D-structure model was calculated by ModBase (http://salilab.org/modbase) using crystal structure of the Nipah virus RNA-free nucleoprotein-phosphoprotein complex 4CO6 as a template. The model suggested V332 was situated in the hydrophobic core of the C-terminal domain facing the inside of the domain. It is plausible that a negatively charged aspartic acid side chain in place of valine disrupts the packaging of α-helices, resulting in deformed monomers unable to multimerize. NVR was used to generate monoclonal antibodies which will be evaluated for virus detection.

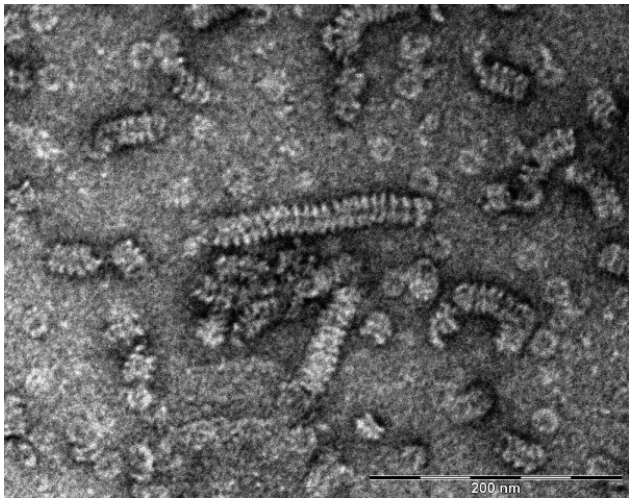

Figure 6. Electron micrograph of CsCl density gradient purified HPIV2 NVRP NLPs. Scale bar 200 nm.

Analysis of yeast expression mechanism

Yeast expression mechanism has been investigated by different techniques. Two-dimensional gel electrophoresis (2DE) is one of the most popular methods in proteomics. Research group of Dr. Slibinskas R. employed non-equilibrium pH gradient electrophoresis (NEPHGE)-based 2DE technique for studying stress responses in recombinant protein producing yeast. Comparison of broad range (pH 3–10) gradient-based 2DE methods suggested that NEPHGE-based method is preferable over commonly used immobilized pH gradient (IPG)-based 2DE method for the analysis of basic proteins (Figure 7), [Slibinskas R. et all, 2013].

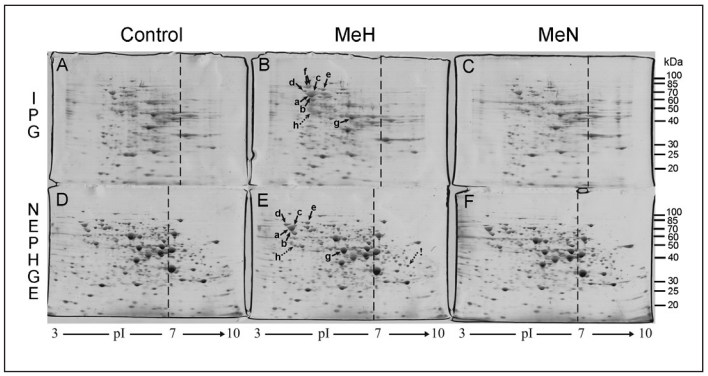

Figure 7. 2DE of yeast whole cell lysates using IPG (A-C) and NEPHGE (D-F) based methods at high protein load. [Proteome Sci. 2013, 11:36].

Plant anthocyanin research

Anthocyanins are essential contributors to the fruit coloration, an important quality feature and a breed determining trait of a sweet cherry fruit. It is well established that the biosynthesis of anthocyanins is regulated by the interplay of specific transcription factors belonging to MYB and bHLH families accompanied by the WD40 protein. In this study, we isolated and analysed

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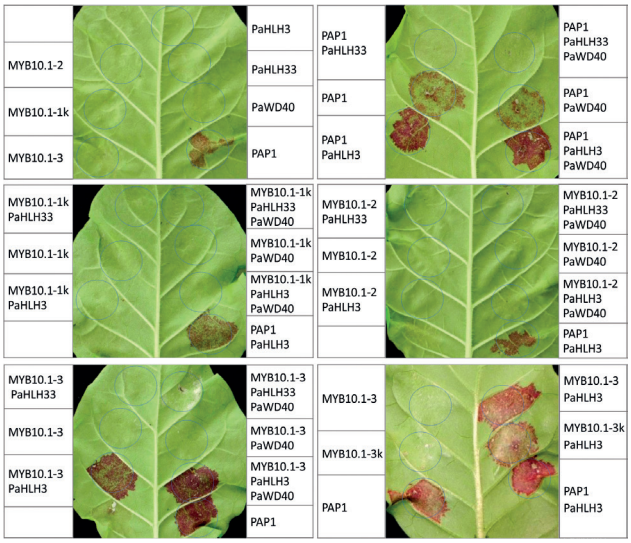

Figure 8. Transient expression of putative regulators of anthocyanin synthesis in leaves of Nicotiana tabacum.

WD40, bHLH3, bHLH33 and several closely related MYB10 gene variants from different cultivars of sweet cherry, analysed their expression in fruits with different anthocyanin levels at several developmental stages and determined their capabilities to modulate anthocyanin synthesis in leaves of two Nicotiana species. Our results indicate that transcription level of one MYB10 variant correlates with fruit coloration, but anthocyanin synthesis in Nicotiana was induced by another variant, moderately expressed in fruits. The analysis of two fruit-expressed bHLH proteins revealed that bHLH3 enhances MYB-induced anthocyanin synthesis, whereas bHLH33 has strong inhibitory properties (Figure8). This study is supported by National Science Program "Healthy and safe food" grant No. SVE-11008.

Infiltrated control cDNAs: PAP1, Arabidopsis thaliana PAP1 (MYB75); GFP, Aequorea victoria GFP. Tested Prunus avium genes isolated from following cultivars: PaWD40, PaHLH33, MYB10.1-1, MYB10.1-3 from 'Irema BS'; PaHLH3 from 'Kitayanka'; MYB10.1-3 from 'Regina'. MYB10.1-1k – cDNA from 'Kitayanka' fruits; MYB10.1-3k – cDNA expressed in N. benthamiana leaves from infiltrated 'Irema BS' gene

Studies of plant signaling mechanisms

During plant responses to stress or developmental cues signaling via mitogen-activated protein kinases (MAPKs) mediates fast, precise and specific responses in cells. The mechanism of this signaling in plants is similar as in other eukaryotes and is relatively well understood, whereas termination of this process by the MAPK phosphatases is less known. The study is aimed to understand the biological roles of PP2C-type MAPK phosphatases in the model plant Arabidopsis thaliana. We found that PP2C-type phosphatases regulate MAPKs. PP2C can influence cell fate decisions during stomata development. Stomata are cells essential in water/gas exchange between plant and environment and thus supporting our ecosystem. Gene expression analysis enabled identification of specific MAPKs and a PP2C phosphatase induced during stomata cell development [Fuchs, S et al., 2013]. This study is supported by Lithuanian Science Council grant No. MIP-003/2014.

PP2C-type phosphatases also control plant cell signaling pathways in reponse to pathogens. We have identified a novel PP2C function in signaling induced by bacteria. This study is supported by Lithuanian-Swiss Program grant No. CH-3-ŠMM-01/10.

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Publications 2013-2014

1. **Tamošiūnas P.L., Simutis K., Kodzė I.**, Firantienė R., Emužytė R., **Petraitytė-Burneikienė R., Zvirblienė A., Sasnauskas K.** Production of human Parvovirus 4 VP2 virus-like particles in yeast and their evaluation as an antigen for detection of virus-specific antibodies in human serum. Intervirology 2013, 56(5):271-7.

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Book Chapters

Schweighofer A., Shubchynskyy V., **Kazanaviciute V.**, Djamei A., **Meskiene I.** Bimolecular fluorescent complementation (BiFC) by MAP kinases and MAPK phosphatases // Methods in Molecular Biology. Editors: George Komis and Jozef Šamaj. Humana Press 2014, 1171:147-58.

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Department of Immunology and Cell Biology comprises of three research groups. In 2013-2014, the research was focussed to the following topics: immunogenicity studies of oligomeric antigens; development of monoclonal antibodies; studies on bacterial virulence factors (research group of dr. A. Žvirblienė), regulation of gene expression by alternative splicing (research group of dr. A. Kanopka), molecular epidemiology of tuberculosis (research group of dr. P. Stakėnas).

Reconstitution of *G.vaginalis* toxin vaginolysin into artificial membranes: implications for bioanalysis

Vaginolysin (VLY) is a cholesterol-dependent cytolysin, the main virulence factor of bacteria *Gardnerella vaginalis*. In the current study, we have investigated the functional reconstitution of VLY into artificial tethered bilayer membranes (tBLMs) composed of synthetic dioleoylphosphocholine with variable amounts of cholesterol. The reconstitution was followed in a real-time by the electrochemical impedance spectroscopy (EIS). Changes of the EIS parameters of tBLMs upon exposure to VLY solutions were consistent with the formation of water-filled pores in membranes. It was found that reconstitution of VLY is a strictly cholesterol dependent, irreversible process. In the absence of cholesterol no effects on membrane permeability or dielectric properties were detected, while increased effect was observed with increasing cholesterol content in tBLM. At constant cholesterol concentration, reconstitution of VLY occurred in a VLY concentration-dependent manner thus allowing monitoring protein concentration and activity in vitro and opening possibilities for tBLM utilization in bioanalysis. Inactivation of wild-type VLY by amino acid substitutions led to a noticeably lesser tBLM damage. Pre-incubation with the neutralizing monoclonal antibody inactivated the membrane damaging ability of VLY in a concentration-dependent manner, while non-neutralizing antibody exhibited no effect. Interestingly, contrasting earlier findings a membrane-damaging interaction between VLY and tBLM was observed in the absence of a human CD59 receptor, which is known to strongly facilitate hemolytic activity of VLY. Estimates allowed us to conclude that in the absence of the CD59 much smaller amount of pores are formed in cells so that hemolytic activity of VLY is almost suppresed. Taken together, our study demonstrates applicability of tBLMs as a bioanalytical platform for the detection of the activity of VLY, and possibly other cholesterol dependent cytolysins.

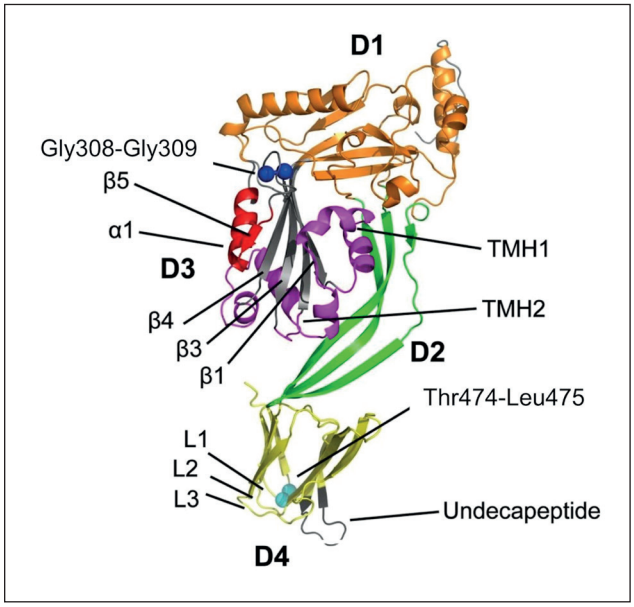

Figure 1. Molecular model of vaginolysin (created by dr. Č. Venclovas). Domains are designated as D1-D4; TMH1-TMH2 motifs—purple ribbons; the double Gly motif is shown as dark blue space-filled spheres; the Thr-Leu pair in D4 is shown as light blue spheres.

This study has been performed in collaboration with the Institute of Biochemistry of Vilnius University.

Reference:

Budvytyte et. al., Plos One, 2013; 8(12):e82536. doi: 10.1371.

Studies on the immunogenicity and cytotoxicity of amyloid beta oligomers

The central molecule in the pathogenesis of Alzheimer's disease (AD) is believed to be a small-sized polypeptide – beta amyloid (Aβ) which has an ability to assemble spontaneously into oligomers. Various studies concerning therapeutic and prophylactic approaches for AD are based on the immunotherapy using antibodies against Aβ. It has been suggested that either active immunization with Aβ or passive immunization with anti-Aβ antibodies might help to prevent or reduce the symptoms of the disease. However, knowledge on the mechanisms of Aβ-induced immune response is rather limited. Previous research on Aβ1-42 oligomers in rat brain

cultures showed that the neurotoxicity of these oligomers considerably depends on their size. In the current study, we evaluated the dependence of immunogenicity of Aβ1-42 oligomers on the size of oligomeric particles and identified the immunodominant epitopes of the oligomers. The analysis of serum antibodies in mice immunized with various Aβ oligomers revealed that small Aβ neurotoxic oligomers (1-2 nm in size) are highly immunogenic. In contrast, larger Aβ oligomers and monomers did not induce a detectable IgG response. Monoclonal antibodies against 1-2 nm Aβ oligomers were generated and used for the antigenic characterization of Aβ oligomers. Epitope mapping demonstrated that the main immunodominant region of the Aβ oligomers is located at its N terminus (aa 1-13) thus indicating its surface localization and accessibility to the B cells.

We have investigated whether monoclonal antibodies to Aβ oligomers would prevent their neurotoxicity in primary neuronal-glial cultures. However, surprisingly, the antibodies dramatically increased the neurotoxicity of Aβ oligomers. Moreover, antibodies to other oligomeric proteins (recombinant virus-like particles) strongly potentiated the neurotoxicity of their target antigens. The neurotoxicity of antibody-antigen complexes was abolished by removal of the Fc region from the antibodies or by removing microglia from cultures. This indicates that that immune complexes formed by Aβ oligomers or other oligomeric antigens and their specific antibodies can cause death and loss of neurons in primary neuronal-glial cultures via Fc-dependent microglial activation.

The results of the current study may be important for further development of Aβ-based vaccination and immunotherapy strategies.

This study has been performed in collaboration with the Institute of Biochemistry of Vilnius University and the Lithuanian University of Health Sciences (Kaunas).

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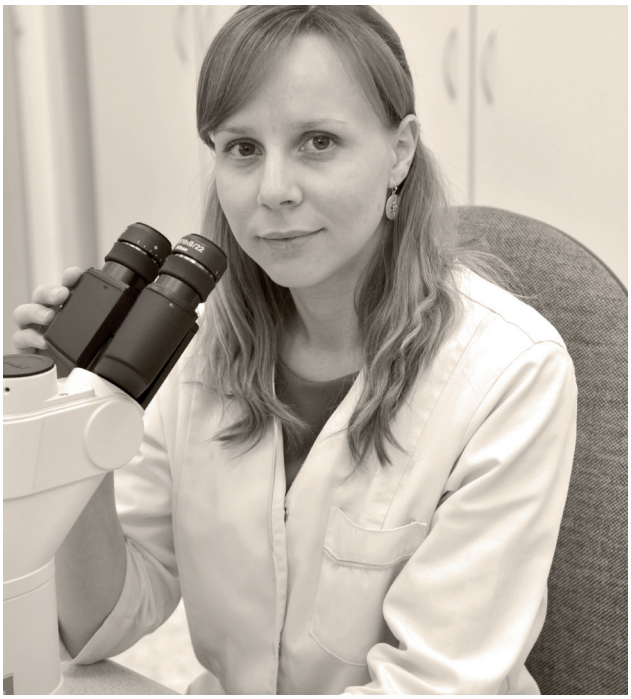
Development and characterization of monoclonal antibodies against cellular and viral antigens

The Department has long-term experience in development and characterization of monoclonal antibodies against various targets. During 2013-2014, a large collection of monoclonal antibodies against viral and cellular proteins has been generated.

Previous studies have shown that recombinant viral structural proteins with their intrinsic capacity to self–assemble to highly-organized structures - virus-like particles (VLPs) or nucleocapsid-like particles (NLPs) – are highly immunogenic and represent promising antigens for developing virus-specific antibodies. In collaboration with the Department of Eukaryote Gene Engineering, novel monoclonal antibodies against recombinant yeast-expressed viral antigens have been generated and employed for diagnostic assays. Those include antibodies against hamster polyomavirus VP1 protein (Munoz et al., Arch Virol. 2013), human parvovirus 4 capsid protein (Tamošiūnas et al., Intervirology 2013), porcine parvovirus capsid protein (Tamošiūnas et al., J Immunol Res 2014) , Schmallenberg virus nucleocapsid protein (Lazutka et al., J Immunol Res 2014) and hantavirus glycoprotein (Zvirbliene et al., Viruses 2014).



Junior scientist R. Lasickienė

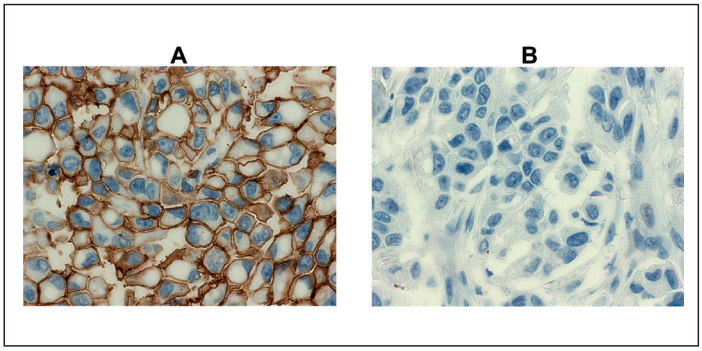


Figure 2. Immunohistochemistry staining of human invasive ductal carcinoma for CA XII expression using the CA XII-specific antibody 3D8 (A). An irrelevant antibody 1F8 is used as a negative control (B). CA XII in cell membrane is shown in brown, cell nucleus is shown in blue.

Recombinant antigens have been used to generate novel monoclonal antibodies against human carbonic anhydrase XII, a potential biomarker of tumor cells (Dekaminaviciute et al., J Enzyme Inhib Med Chem 2014, Dekaminaviciute et al., Biomed Res Int 2014). The diagnostic relevance of the newly developed antibodies has been confirmed using tumor cell lines and clinical specimens (Figure 2).

A large collection of neutralizing monoclonal antibodies against DNA polymerases has been developed (contract with Thermo Fisher Scientific, No AP5-560000-1032).

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Dekaminaviciute et al., Biomed Res Int 2014, 2014:309307. doi: 10.1155/2014/309307.

Regulation of hypoxia-dependent alternative pre-mRNA splicing

Hypoxia has been recognized as a common feature of solid tumours and a negative prognostic factor for response to treatment and survival of cancer patients. Biological responses to hypoxia involve induction of transcription of a network of target genes, a process which is co-ordinately regulated by three structurally related hypoxia-inducible transcription factors (HIFs): HIF-1, HIF-2 and HIF-3. HIFs recognize hypoxia response elements of target genes as heterodimeric complexes (HIF-1α, HIF-2α and HIF-3α) with the transcription factor Arnt.

Research group of dr. A. Kanopka: I. Pečiulienė, A. Mazėtytė, K. Vaidžiulytė, dr. A. Kanopka, E. Jakubauskienė, L. Vilys

A striking change has been observed in alternative splicing patterns of genes and alterations in splicing factor expression under pathologic conditions especially in human cancers. Cancer cells are often confronted with a significant reduction in oxygen availability. The splicing machinery heavily contributes to biological complexity especially to the ability of cells to adapt to different developmental stages and altered cellular conditions. The selection of alternative splice sites can be regulated in a different manner related to tissue specificity, developmental stage, physiological processes, sex determination and in response to various stress factors. A number of reports describe changes in alternative pre-mRNA splicing patterns induced by hypoxia. The mechanism underlying oxygen tension-dependent changes in splicing remains unknown.

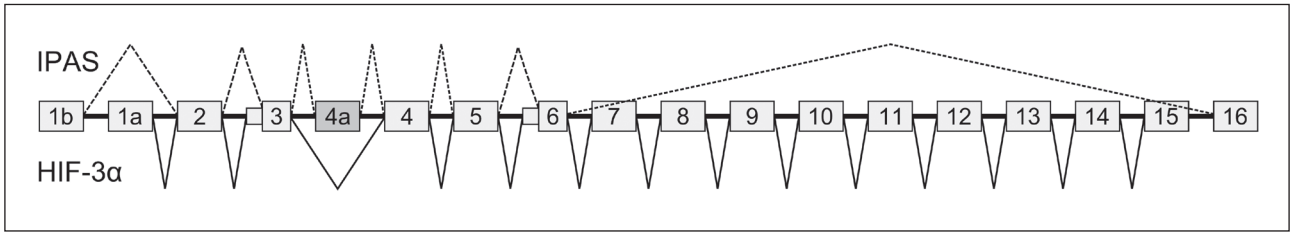

Figure 3. ***Alternative hypoxia-dependent HIF-3α pre-mRNA splicing in mice. HIF-3α mRNA is produced both in normoxic and hypoxic cells. IPAS mRNA is produced strictly in hypoxic cells.***

Our goal is to establish mechanism and factors involved in hypoxia-dependent splicing regulation. We established that hypoxia-inducible factor HIF-1 indirectly is involved in such regulation. Also we established that a change in activity of essential splicing factors determine oxygen-dependent pre-mRNA splicing.

Thus we identified one of hypoxia-dependent pre-mRNA splicing regulator which might re-program cellular events and could not only be useful for the potential therapeutic applications but also for their application as an analytic tool.

This work was supported by the EU Framework 7th Programme (project Metoxia).

Molecular epidemiology of *Mycobacterium tuberculosis*

Tuberculosis (TB) caused by *M. tuberculosis* complex bacteria remains a serious health problem in Lithuania. Incidence of TB and in particular multidrug-resistant (MDR TB) is one of the highest in the European Society. The aim of our study is to characterize population of *M. tuberculosis* strains that circulate in Lithuania including the genetic determinants of drug resistance. The research was carried out in collaboration with Infectious Diseases and Tuberculosis Hospital, Affiliate of Vilnius University Hospital Santariskiu Klinikos and other partners from the research of TB networks. Genotyping was performed by international standardized molecular methods (MIRU-VNTR typing, spoligotyping) and the polymorphisms of *M. tuberculosis* genome were identified by a direct sequencing. The data were submitted to the relevant multinational databases that facilitate understanding of the spread of TB and emergence of drug resistance. Analysis of *M. tuberculosis* genotypes indicated that many of the Lithuanian isolates are in the cross-borders clusters. Therefore, we started sub-typing of the strains by using an additional hypervariable MIRU-VNTR locus in order to improve a discrimination power of MIRU-VNTR typing. Also, we continued the identification of the mutations occurring in the well-known genomic regions of *M. tuberculosis* involved in drug resistance and search for polymorphisms in the putative targets for the first and second line anti-TB drugs. A large multicenter study on a deep characterization of polymorphisms in the pncA gene involved in the resistance to the key drug pyrazinamide was completed and published.

This work was supported by the EU Framework 7th Programme (project TB PAN-NET).

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Publications 2013-2014

1. Tamošiūnas P-L., **Petraityte-Burneikiene R., Lasickiene R.**, Akatov A., Kundrotas G., Sereika V., Lelešius R., Žvirbliene A., Sasnauskas K. Generation of recombinant porcine parvovirus pirus-like particles in Saccharomyces cerevisiae and development of virus-specific monoclonal antibodies. J Immunol Res 2014, 2014:573531.

2. Lazutka J., **Zvirbliene A., Dalgediene I.**, Petraityte-Burneikiene R., Spakova A., Sereika V., Lelesius R., Wernike K., Beer M.D and Sasnauskas K. Generation of recombinant Schmallenberg virus nucleocapsid protein in yeast and development of virus-specific monoclonal antibodies. J Immunol Res, 2014, 2014:160316.

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10. **Dalgediene I., Lasickiene R.**, Budvytyte R., Valincius G., Morkuniene R., Borutaite V., **Zvirbliene A.** Immunogenic properties of amyloid beta oligomers. J Biomed Sci 2013, 20:10. doi: 10.1186/1423-0127-20-10.

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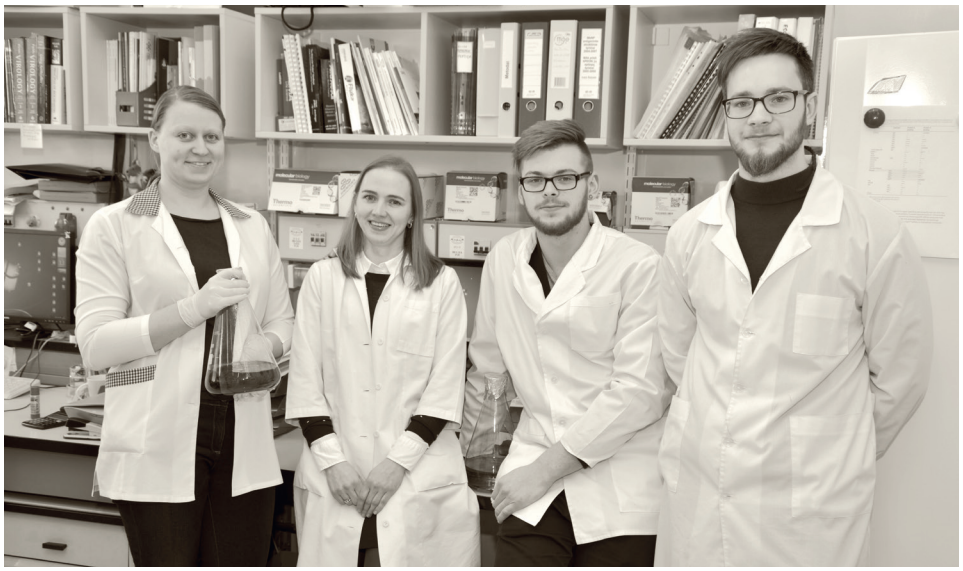

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Gerda Blantaitytė,
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Dovilė Janušaitė,
Ieva Kunigėlytė,
Fausta Labanauskaitė,
Arūnas Maisaitis,
Katažyna Milto,
Valdemaras Petrošius,
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Tomas Šneideris,
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Aušra Želvytė

The Department of Biothermodynamics and Drug Design (DBDD) was established in 2006 based on the former Laboratory of Recombinant Proteins. The DBDD designs novel chemical compounds for therapeutic purposes. The efficiency of both naturally occurring and synthetic compounds is evaluated by structural biothermodynamics and molecular modeling methods.

The laboratory's personnel consist of five teams according to their research goals and activities:

The team of Molecular and Cellular Biology gathered after the discussion at a group meeting

The Team of Molecular and Cell Biology, headed by Dr. Jurgita Matulienė (Ph. D. in cell biology from the University of Minnesota, USA, 2003), makes target proteins by gene cloning, expression in E.coli, insect, or mammalian cells, and chromatografic purification of large quantities of active proteins sufficient for biothermodynamic measurements of compound binding. Several projects involve the design of mutants and truncated protein domain constructs. Live human cancer cells are cultured for the evaluation of compound anticancer activity. Dr. Vilma Petrikaitė has a Ph. D. in pharmacy and performs compound testing in mice xenografts. The team collaborates with the Department of Immunology and Cell Biology in antibody design and diagnostic markers.

The Team of Organic Synthesis, headed by Dr. Virginija Dudutienė (Ph. D. in organic synthesis from the Vilnius University, 2005), synthesizes compounds that are designed to bind carbonic anhydrases and other drug target proteins. Compounds are designed by computer docking, molecular modeling, and comparison with naturally occurring or previously synthesized compound functional groups. Compound identity and purity is verified by NMR and HPLC-HRMS.

Part of the team of Biophysics gathered after a discussion of isothermal titration calorimetry results

The Team of Biophysics, headed by Prof. Daumantas Matulis (Ph. D. in biochemistry, molecular biology and biophysics from the University of Minnesota, USA, 1998), measures compound binding to target proteins by isothermal titration calorimetry (ITC), fluorescent thermal shift assay (DSF, ThermoFluor®), pressure shift assay (PSA), and conventional enzyme inhibition methods. The team determines intrinsic Gibbs free energies, enthalpies, entropies, heat capacities and volume of binding and measures protein stability in the presence of various excipients.

The Team of Computer Modeling, headed by Dr. Vytautas Petrauskas (Ph. D. in physics from the Vilnius University, 2008), is responsible for the application of computational methods, database management, in silico docking of large compound libraries and the analysis of X-ray crystal structures of synthetic compound – protein complexes solved in collaboration with Dr. Saulius Gražulis group at the Department of Protein – DNA interactions. Molecular modelers collaborate with the Department of Bioinformatics and use their methods to model protein structures that are not solved by X-ray crystallogphy. The group, together with several collaborating scientists is developing the software that estimates the energies of compound binding to a protein when only the crystal structure of the free protein is available.

The Team of Amyloid Research. Recently a new team has started upon the return of Dr. Vytautas Smirnovas (Ph. D. from the Technical University of Dortmund, 2007) to Lithuania in 2011. The main interests and research of his team lie in the protein aggregation and amyloidogenesis that are involved in a number of diseases, including such neurodegenerative disorders as Alzheimer's and Parkinson's.

Research Projects

The Department of Biothermodynamics and Drug Design performs fundamental and applied research focused on protein-ligand interactions and drug design. The state of the art in today's industrial drug design is still based on high-throughput approaches due to the lack of fundamental understanding of physical forces underlying such processes as protein folding and protein-ligand interactions. It is still impossible to predict and computer-model the compounds that would exhibit desired affinity and selectivity profiles towards their target proteins.

Carbonic anhydrases as drug targets

Carbonic anhydrases (CAs), a group of zinc containing enzymes, are involved in numerous physiological and pathological processes, including gluconeogenesis, lipogenesis, ureagenesis, and tumorigenicity. In addition to the established role of CA inhibitors as diuretics and drugs used to treat glaucoma and high-altitude sickness, it has recently emerged that CA inhibitors could have potential as novel anti-obesity, anticancer, and anti-infective drugs (Supuran, 2008, 2012). CAs catalyze the conversion of CO2 to the bicarbonate ion and protons.

There are 12 catalytically active CA isoforms in humans. CAs I, II, III, VII and XIII are cytosolic, CAs IV, IX, XII and XIV are membrane-bound and located on the outside of the cell, CAs VA and VB are found in mitochondria, and CA VI is the only secreted isoform found in saliva and milk. A number of CA inhibitors, mostly aromatic sulfonamides, have been designed and developed into drugs. However, most inhibitors possess low selectivity towards the target CA isoforms. It is especially important to develop highly selective inhibitors towards the novel anticancer target isoforms, CA IX and XII, that are highly overexpressed in numerous tumors and increase cancerous cell survival and metastatic invasiveness.

We have cloned and purified all human CAs or their catalytic domains in bacterial or mammalian cells. Over 600 novel compounds were designed and synthesized that bound CAs with micromolar to picomolar affinities. Four CA isoforms were crystallized in complex with numerous inhibitors and solved to high resolution thus providing structural insight into compound affinity and selectivity. A series of fluorinated CA inhibitors exhibited high affinity and great selectivity towards CA IX isoform [1]. Several other series of compounds were determined to bind various CA isoforms [2, 5, 8, 14].

However, there are several linked reactions that occur simultaneously with the binding reaction. Such linked reactions greatly influence the observed thermodynamic parameters of binding. For example, affinities are greatly dependent on pH, the enthalpies of binding – on the buffer in solution. Therefore, we determine the intrinsic thermodynamic parameters of binding that are independent of experimental conditions and could be directly correlated with structures [4, 9, 10, 15]

Discovery and characterization of novel selective inhibitors of carbonic anhydrase IX

A series of fluorinated benzenesulfonamides with substituents on the benzene ring were designed and synthesized. Several of these exhibited a highly potent and selective inhibition profile against CA IX (Figure 1, Table 1). Three fluorine atoms significantly increased the affinity by withdrawing electrons and lowering the pKa of the benzene sulfonamide group. The bulky ortho substituents such as cyclooctyl or even cyclododecyl groups fit to the hydrophobic pocket in the active site of CA IX but not CA II, as shown by the compound cocrystal structure with the chimeric CA IX (Figure 2). The strongest inhibitor of recombinant human CA IX catalytic domain produced in human cells achieved the affinity of 50 pM. However, the high affinity diminished the selectivity. The most selective compound for CA IX exhibited 10 nM affinity. The compound which showed the best balance between affinity and selectivity properties bound with 1 nM affinity. The inhibitors described in [1] provide the basis for novel anticancer therapeutics targeting CA IX.

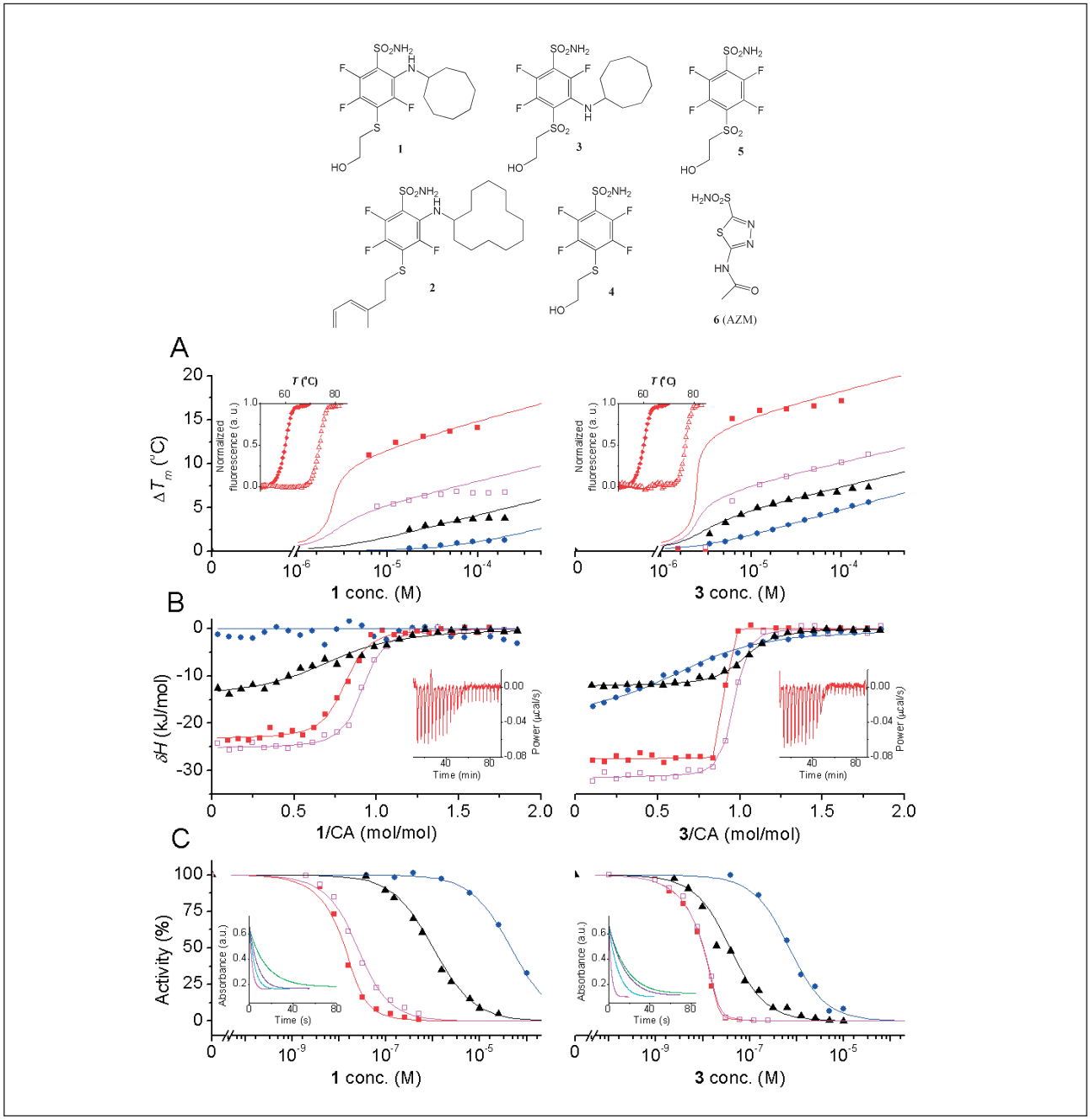


Figure 1. The top panel shows the chemical structures of CA inhibitors 1-6. Acetazolamide (6, AZM) is commonly used as a control inhibitor of CAs. Panels A, B and C show compound 1 (graphs on the left) and 3 (graphs on the right) binding and inhibition of CAs. A. Binding of compounds as determined by the thermal shift assay. Datapoints show the $\triangle T_m$ s as a function of total added compound concentration while the lines are simulated according to [42]. Red filled squares – CA IX, magenta open squares – chCA IX, black filled triangles – CA II, and blue filled circles – CA I. The largest $\triangle T_m$ shift for similar proteins corresponds to strongest binding K_d. The inset graphs show normalized raw fluorescence data as a function of temperature at zero (filled red diamonds) and 50 μM (open red triangles) total added compound concentrations. The melting midpoints correspond to the T_m. B. Binding of the compounds as determined by the isothermal titration calorimetry. Colors and symbols for CA isoforms are same as in panel A. The ITC curve fitting Kds are listed in Table 1. Insets show the raw ITC curves of the respective compound binding to CA IX. C. The inhibition of CA isoforms as determined by the stopped-flow kinetic CO_2 hydration assay. Colors and symbols for CA isoforms are same as in panel A. Datapoints correspond to % inhibition of a CA as a function of total added compound concentration. The lines are fit according to the Morrison equation as explained in the materials and methods section. Insets show raw activity curves (drop in absorbance/pH due to acidification by the CA IX) at various added compound concentration: magenta – 0 nM, cyan – 15.6 nM, violet – 31.3 nM, and green – spontaneous CO_2 hydration in the absence of CA IX. The CA IX concentration was 20 nM. All three methods conclusively indicate that both compounds 1 and 3 bound and inhibited CA IX significantly stronger than CA I and CA II. Furthermore, compound 3 bound tighter to most CA isoforms than 1. However, compound 1 exhibited greater selectivity ratio towards CA IX than 3.

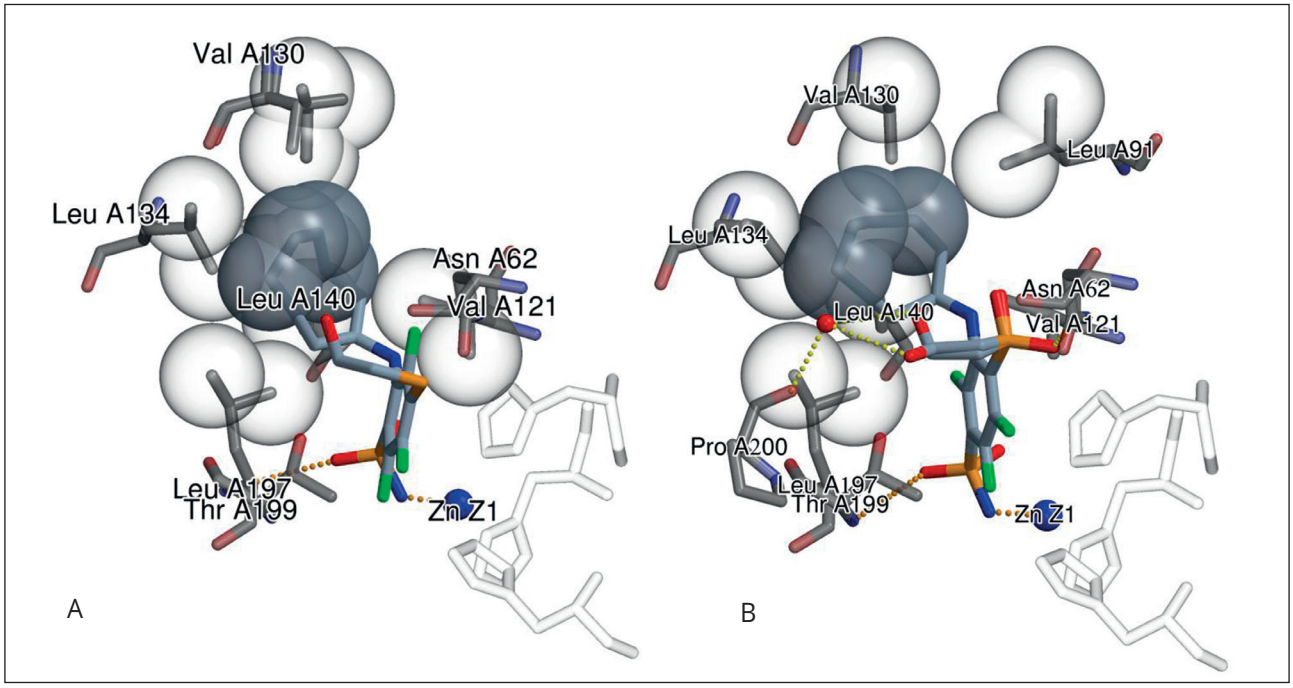


Figure 2. Compounds 1 (Panel A, PDB ID 4Q06) and 3 (Panel B, PDB ID 4Q07) bound to chCA IX as determined by X-ray crystallography. The Zn is shown as a blue sphere, while the histidine residues holding the Zn atom are transparent. The amino acids of chCA IX are shown in grey. The terminal atoms of amino acids which form the hydrophobic cavity are shown as CPK (light grey). Several atoms of cyclooctyl group are also shown as CPK (dark grey). Dashed lines connect the atoms that make hydrogen bonds or electron donor-acceptor interaction (with Zn). Water molecule is shown as a red sphere. The compounds are shown in lightsteelblue.

Compound / CA isoform	1	2	3	4	5	6
K_d determined by the fluorescent thermal shift assay, nM (37 °C, pH 7.0, Pi buffer)						
CA I	50,000	>200,000	710	0.11	0.20	1,400
CA II	1,300	>200,000	60	6.7	17	38
CA III	>200,000	>200,000	40,000	29,000	33,000	40,000
CA IV	1700	>200,000	25	590	160	100
CA VA	3,300	>200,000	2,500	330	290	1,000
CA VB	210	>200,000	5.6	1.3	22	310
CA VI	4,300	>200,000	95	200	67	310
CA VII	330	>200,000	9.8	46	7.1	17
CA IX	**1.1**	**9.5**	**0.050**	**32**	**50**	**20**
chCA IX	25	630	2.0	63	83	50
CA XII	330	>200,000	3.3	220	250	130
chCA XII	500	>200,000	6.7	310	250	330
CA XIII	140	1,700	3.6	8.3	29	50
CA XIV	26	4,300	0.16	1.3	5.0	11
X-ray crystallographic structure PDB IDs						
CA II	4PYX	ND	4PYY	ND	4PZH	ND
chCA IX	4Q06	ND	4Q07	ND	ND	ND
CA XII	ND	ND	4Q0L	ND	ND	ND
chCA XII	4Q08	ND	4Q09	ND	ND	ND
CA XIII	ND	ND	ND	ND	4HU1	ND

Table 1. Compound dissociation constants for all 12 catalytically active human CA isoforms, determined by FTSA, ITC, and stopped-flow kinetic inhibition assay. X-ray crystallographic structures PDB IDs of available structures are listed.

Observed and intrinsic thermodynamics of binding compounds to CAs

Figure 3 shows the reactions occurring simultaneously upon ligand binding to the active site of CA. Direct experimental observation of the binding will always yield only the observed parameters of binding. However, only the intrinsic parameters are meaningful if we intend to analyze any structure-activity relationships. As described in [10] and [4], the dissection of these linked reactions is a laborious process requiring numerous experiments applying not only the fluorescent thermal shift assay (differential scanning fluorimetry, ThermoFluor), but also isothermal titration calorimetry and requires large amounts of purified protein.

After detailed dissection of linked reactions and estimation of the intrinsic parameters, the maps can be drawn that compare the binding thermodynamics of similar compounds (Figure 4) and correlate the binding thermodynamics with protein-ligand crystal structures (Figure 5).

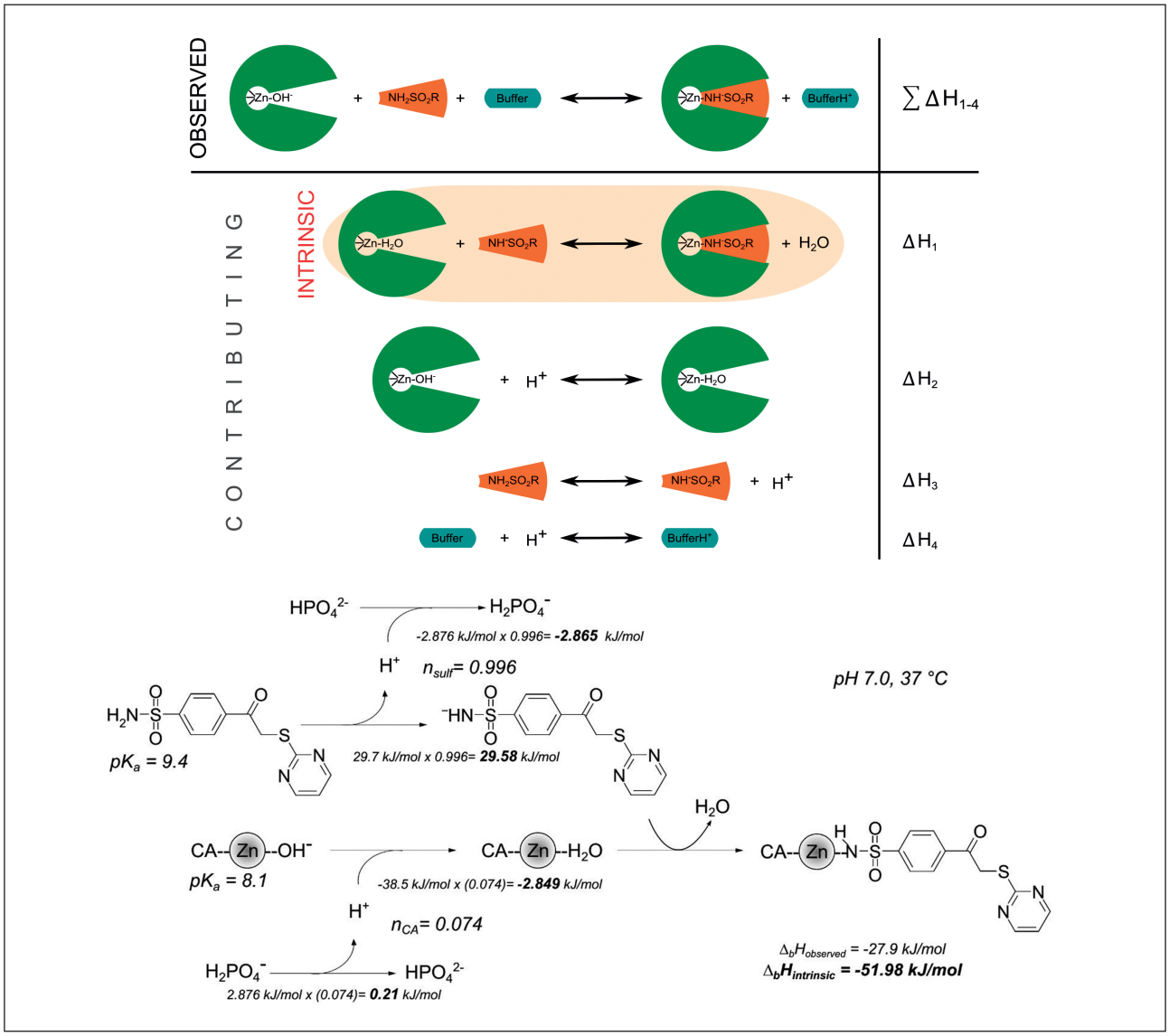


Figure 3. The observed and intrinsic binding thermodynamics. The upper panel shows the main linked reactions occurring upon ligand binding to CAs. The lower panel lists the enthalpies of all processes linked to the binding of 3b to CA I. The two left-central reactions show the binding-linked deprotonation of the inhibitor sulfonamide and the protonation of the zinc-bound hydroxide, respectively. Top and bottom lines show linked phosphate buffer (de)protonation reactions. The numbers give estimates of the enthalpies for each process multiplied by the number of linked protons (n) yielding the observed enthalpic contribution of each reaction at pH 7.0, 37 °C. The intrinsic enthalpy of binding, shown by the rightmost arrow, is equal to –51.98 kJ/mol. The observed enthalpy, estimated for phosphate buffer at pH 7.0, is equal to –27.90 kJ/mol. Zinc atom is shown as grey shaded sphere and the carbonic anhydrase protein is shown as CA.

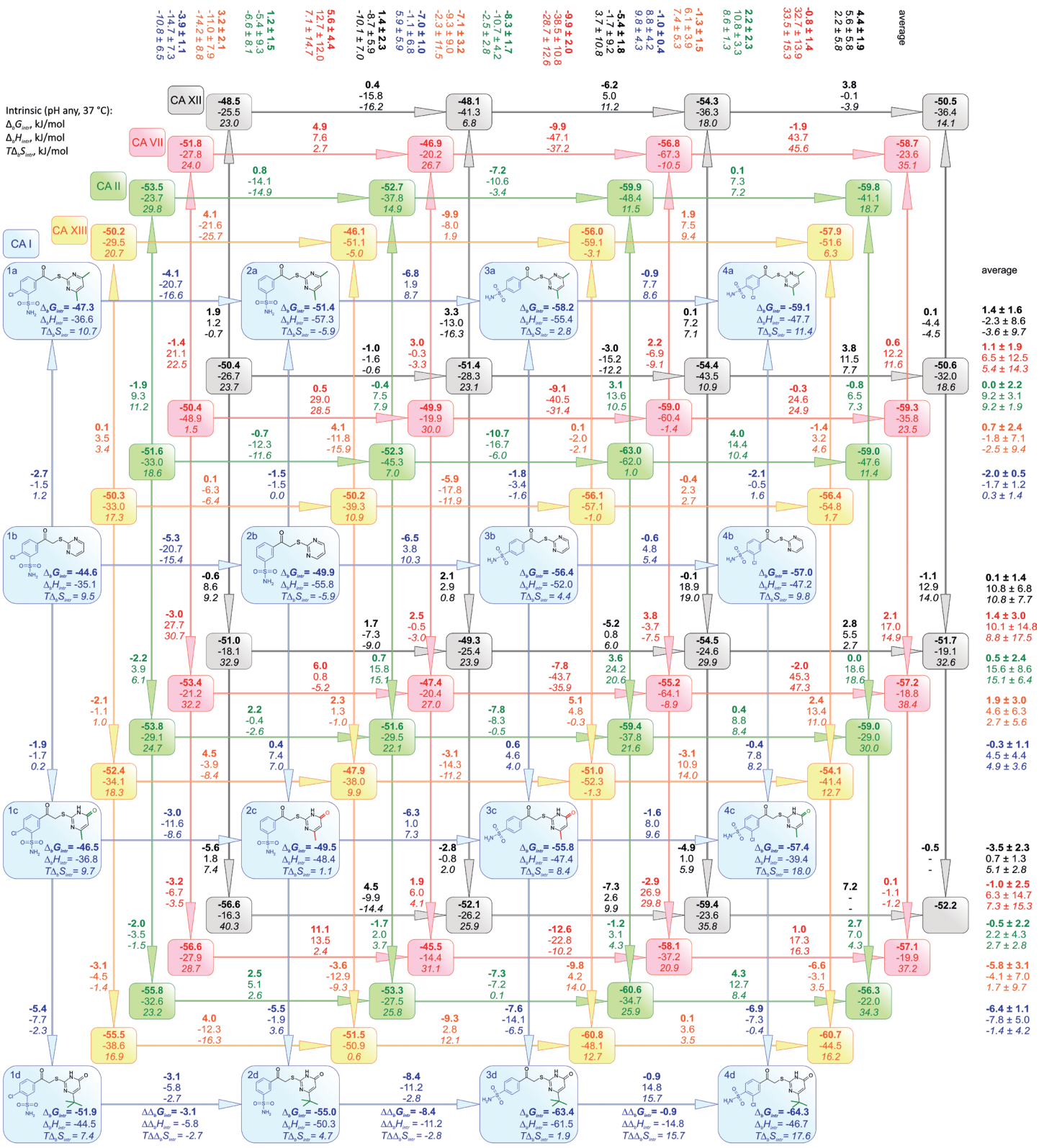


Figure 4. *Inhibitor structure correlations with the thermodynamics of binding. Intrinsic parameters of compound binding to five investigated CA isoforms are given within the shapes. Different colors represent different CA isoforms. Numbers next to arrows show the Gibbs free energy (top number, bold), enthalpy (middle number), and entropy (T bS, bottom number) of binding differences between two neighboring compounds (in kJ/mol at 37 °C). Numbers to the top and right of the map are averages between same heads and tails of the compounds. The standard deviations indicate the presence and absence of the energetic additivity of compound functional groups.*

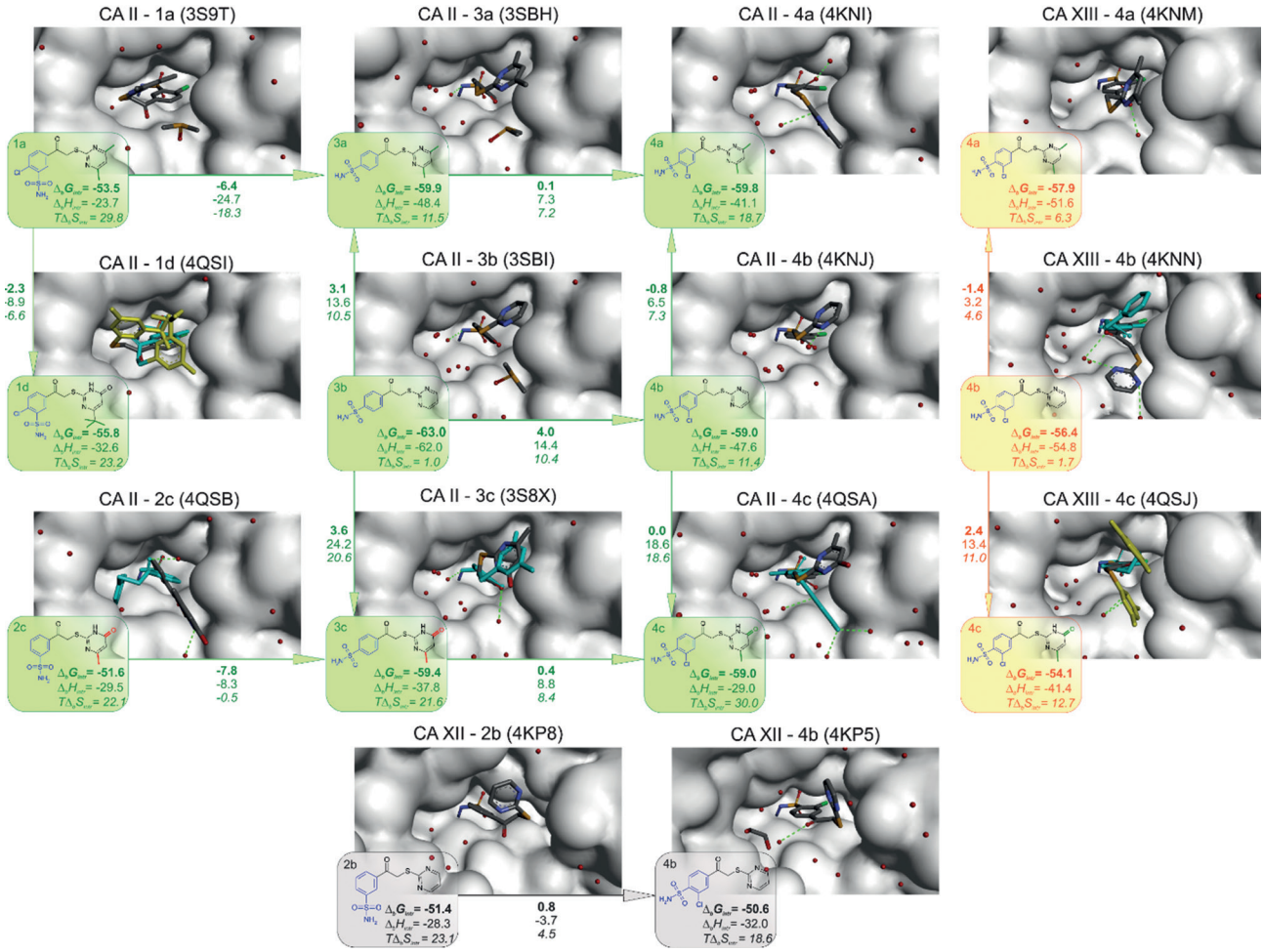
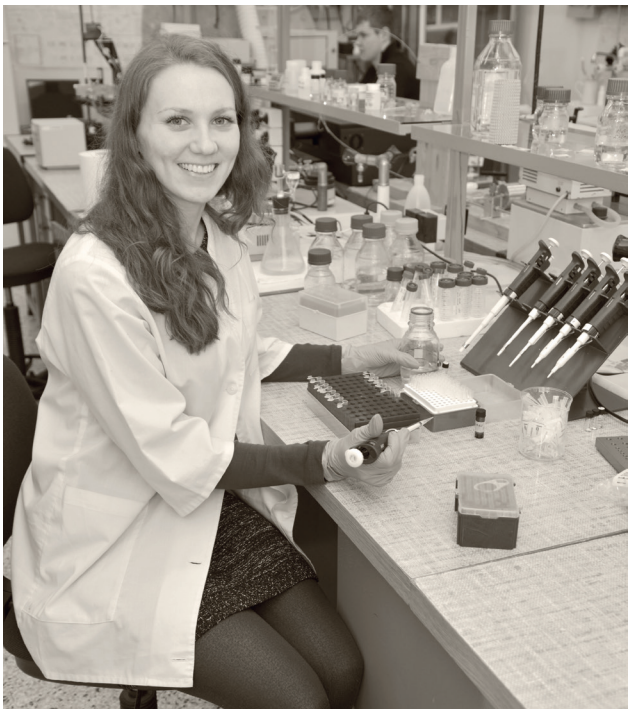


Figure 5. ***Compound chemical structure and the thermodynamics of binding correlations with the crystal structures of some compound binding mode in the active site of CAs (1a, 1d, 3a, 3b, 3c, 4a, 4b, and 4c with CAII; 4a, 4b, and 4c with CA XIII; 2b and 4b with CA XII). The thermodynamic parameters of binding and the colors of the shapes are same as in Figure 4 and indicate the CA isoform. Colors in the crystal structures are: yellow shows the pyrimidine ring that is not fixed in the crystal structure and has multiple conformations with low occupancies; blue shows the alternative conformation of the pyrimidine ring when both conformations are visible in the electron density maps.***



Doctoral student in biophysics Vaida Morkūnaitė prepares a series of protein and compound dilutions for a thermal shift assay.

Ligand binding to proteins at high pressure

The volume changes accompanying ligand binding to proteins are thermodynamically important and potentially could be used in the design of compounds with specific binding properties. Measuring the volumetric properties could yield as much information as the enthalpic properties of binding. Pressure-based methods are significantly more laborious than temperature methods and are underused. The pressure shift assay (PressureFluor, analogous to the ThermoFluor, thermal shift assay, differential scanning fluorimetry) uses high pressure to denature proteins. The PressureFluor method was used to study the ligand binding thermodynamics of Hsp90 and human serum albumin. Ligands stabilize the protein against pressure denaturation, similar to the stabilization against temperature denaturation (Figure 6).

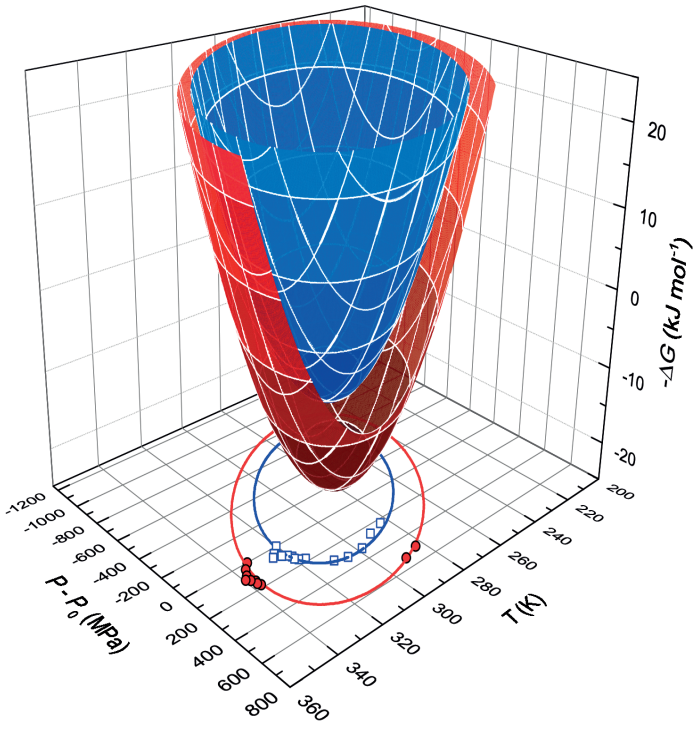
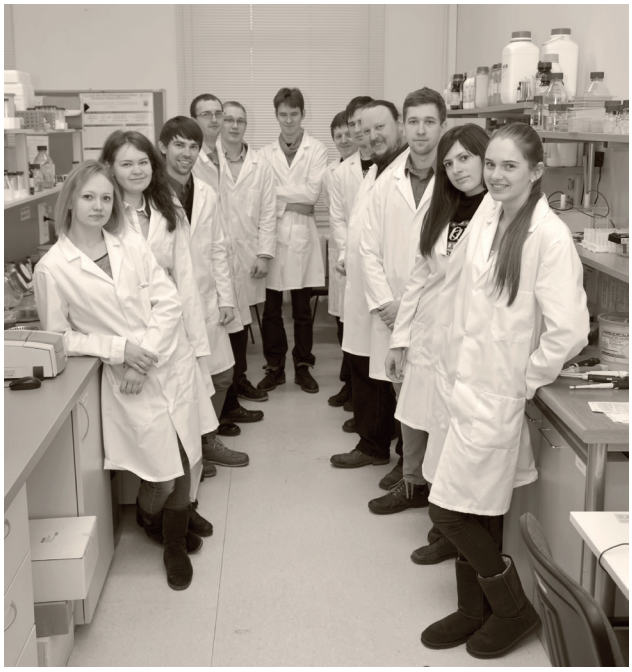


Figure 6. The Gibbs free energy dependence on pressure and temperature. Inner surface represents the ligand-free Hsp90N stability region, while outer surface shows stability region of protein-ligand system with 200 μM of added ligand [13].

The Team of Amyloid Research

We are especially interested in amyloid-like nature of prions and prion-like nature of amyloids. Protein aggregation and amyloidogenesis are involved in a number of diseases, including such neurodegenerative disorders as Alzheimer's and Parkinson's, many systemic amyloidoses and even some localized diseases such as type II diabetes or cataracts. There is an increasing evidence of amyloid nature of proteinaceous infectious particles – prions. One of the most possible ways of abnormal protein spreading is elongation of amyloid-like fibrils, thus there is a chance of all amyloid-associated diseases to be potentially infective. The same prion protein may express distinct strains. The strains are enciphered by different misfolded conformations. Strain-like phenomena have also been reported in a number of other amyloid-forming proteins. One of the features of amyloid strains is the ability to self-propagate, maintaining a constant set of physical properties despite being propagated under conditions different from those that allowed initial formation of the strain.



The team of amyloid research

Our most important findings are summarized in Figure 7. We did cross-seeding experiment using strains formed under different conditions. Using high concentrations of seeds results in rapid elongation and new fibrils preserve the properties of

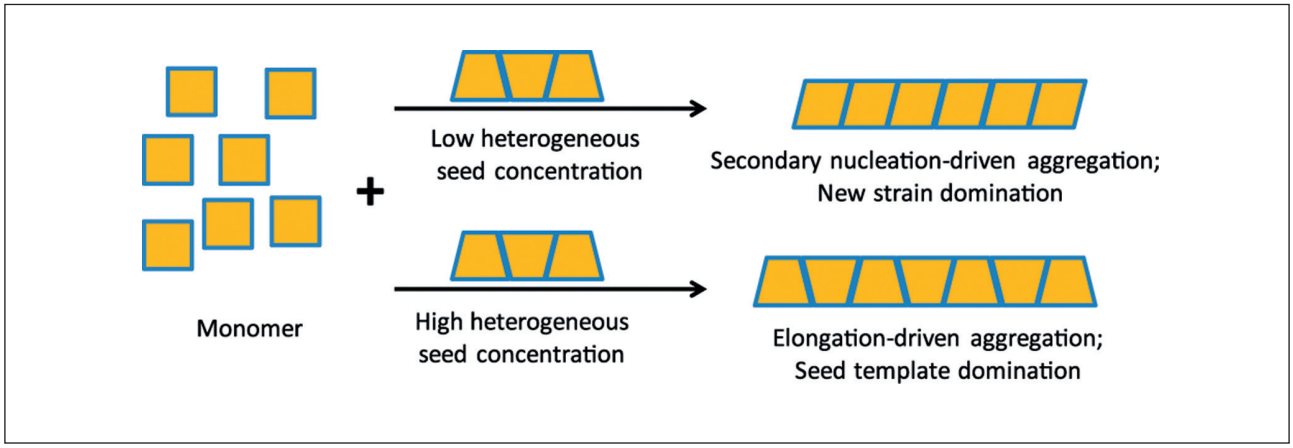


Figure 7. ***Conformational switching between amyloid fibrils.***

the seeding fibrils. At low seed concentrations secondary nucleation plays the major role and new fibrils gain properties predicted by the environment rather than the structure of the seeds. Our findings could explain infectious prion evolution in vivo and conformational switching between amyloid strains observed in a wide variety of in vivo and in vitro experiments.

Services

The DBDD is seeking to license out the compounds described in patents and patent applications. The DBDD is interested in collaborations where our expertise in recombinant protein production and the determination of compound – protein binding thermodynamics and recombinant protein stability characterization could be applied. Protein – ligand binding constants and protein thermal stability profiles at hundreds of conditions may be determined in a single experiment by consuming microgram quantities of protein.

Conferences

The DBDD regularly participates in many international conferences and symposiums, including:
Biophysical Society Annual Meeting
European Biophysics Congress
Gibbs Conference on Biothermodynamics
International Conference on the Carbonic Anhydrases
International Conference on High Pressure Bioscience and Biotechnology
International Conference on the Hsp90 chaperone machine
International Conference of the Lithuanian Biochemical Society (in 2014 Prof. D. Matulis was elected President of the Society and will be the Chairman of the conference in 2016)

Collaboration

The DBDD has ongoing collaborations with a number of research laboratories and industry worldwide, including:
Prof. John Ladbury, Houston Methodist Research Institute, USA
Prof. Nadine Martinet, University of Nice, France
Prof. Martin Mayer, Institute of Organic Chemistry, University of Tubingen, Germany
Prof. Seppo Parkkila, Institute of Medical Technology, University of Tampere, Finland
Dr. Martin Redhead, Sygnature Discovery, Nottingham, UK
Prof. Catherine Royer, Centre for Structural Biochemistry, Montpellier, France
Dr. Vladimir Sirotkin, Butlerov Institute of Chemistry, Kazan Federal University, Kazan, Russia
Prof. Claudiu Supuran, University of Florence, Italy
Dr. Matthew Todd, Janssen Pharmaceuticals, Johnson&Johnson, USA
Prof. Peteris Trapencieris, Institute of Organic Synthesis, Riga, Latvia
Prof. Nicolas Westwood, St. Andrews University, UK
Prof. Maciej Zylicz, International Institute of Molecular and Cell Biology, Warsaw, Poland

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Publications 2013-2014

Journal articles

1. **Dudutienė V., Matulienė J., Smirnov A., Timm D.D., Zubrienė A., Baranauskienė L., Morkūnaitė V., Smirnovienė J., Michailovienė V., Juozapaitienė V., Mickevičiūtė A., Kazokaitė J., Bakšytė S., Kasiliauskaitė A., Jachno J., Revuckienė J., Kišonaitė M., Pilipuitytė V., Ivanauskaitė E., Milinavičiūtė G., Smirnovas V., Petrikaitė V., Kairys V., Petrauskas V., Norvaišas P., Lingė D., Gibieža P., Čapkauskaitė E., Zakšauskas A,, Kazlauskas E.**, Manakova E., Gražulis S., Ladbury J.E., **Matulis D.** Discovery and characterization of novel selective inhibitors of carbonicanhydrase IX. J Med Chem 2014, 57(22):9435-9446.

2. Rutkauskas K., **Zubrienė A.**, Tumosienė I., Kantminienė K., Kažemėkaitė M., **Smirnov A., Kazokaitė J., Morkūnaitė V., Čapkauskaitė E.**, Manakova E., Gražulis S., Beresnevičius Z.J., **Matulis D.** 4-Amino-substituted Benzenesulfonamides as Inhibitors of Human Carbonic Anhydrases. Molecules 2014, 19(11):17356-80.

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7. **Petrikaitė V., Matulis D.** Inhibitor binding to hsp90: a review of thermodynamic, kinetic, enzymatic, and cellular assays. Curr Protein Pept Sci 2014, 15(3):256-82.

8. **Zubrienė A., Čapkauskaitė E., Gylytė J., Kišonaitė M.**, Tumkevičius S., **Matulis D.** Benzenesulfonamides with benzimidazole moieties as inhibitors of carbonic anhydrases I, II, VII, XII and XIII. J Enzyme Inhib Med Chem 2014, 29(1):124-31.

9. **Pilipuitytė V.** and **Matulis D.** Intrinsic thermodynamics of trifluoromethanesulfonamide and ethoxzolamide binding to human carbonic anhydrase VII. J Molec Recogn 2014. doi: 10.1002/jmr.2404.

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14. **Dudutienė V., Zubrienė A., Smirnov A., Gylytė J., Timm D.D., Manakova E.**, Gražulis S., **Matulis D.** 4-Substituted-2,3,5,6-tetrafluorobenzenesulfonamides as inhibitors of carbonic anhydrases I, II, VII, XII, and XIII. Bioorg Med Chem 2013, 21(7):2093-106.

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The team of amyloid research:

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17. **Milto K., Botyriūtė A., Smirnovas V.** Amyloid-like fibril elongation follows Michaelis-Menten kinetics. PLoS One 2013, 8(7):e68684.

Sector of

Applied Biocatalysis

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Antanas Karalius, B.Sc.

Affiliated scientist
Prof. Gervydas Dienys, PhD, Dr. Habil.

Sector of Applied Biocatalysis was established in 2007 as a group of Industrial Biotechnology in conjunction with the start of the National Programme on the Development of Industrial Biotechnology in Lithuania 2007-2010. In 2010 the group was transformed into the Sector of Applied Biocatalysis and is headed by Inga Matijošytė (PhD in biochemistry and biocatalysis from Delft University of Technology, The Netherlands, 2008). The limited number of suitable and well characterized biocatalysts delays the progress in the application of enzymes in the synthesis of compounds for materials, pharmaceuticals and chemicals. Sector's research is directed towards the search for enzymes with new functionalities and their development towards applied biocatalysis. The research focuses on developing of biocatalytic systems employing oxidative, lypolytic, hydrolytic, proteolytic enzymes. Recently, the sector is orienting the research towards discovery of new novel biocatalytic routes for high-added value products from bio-based raw materials – biopolymers. We strive to meet scientific challenges in combination with application-oriented research.

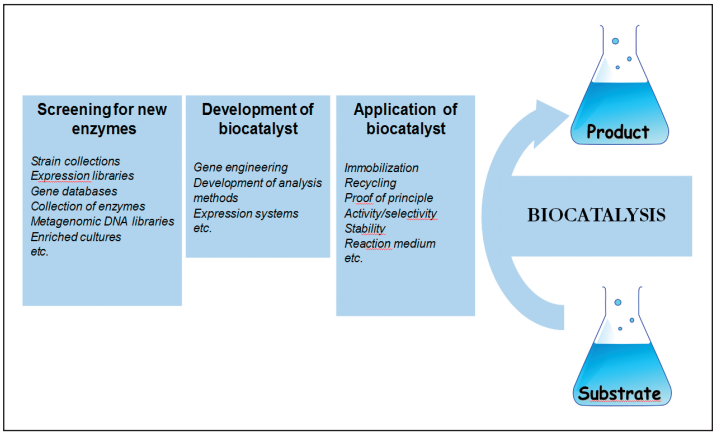

Figure 1. Research areas of Sector of Applied Biocatalysis. Sector of Applied Biocatalysis seeks to identify biocatalysts with novel activities by screening for enzymes, development of biocatalyst and application of biocatalyst

Screening for new enzymes

• Metagenomic DNA libraries

The demand for enzymes with new or specific characteristics and functionalities is constantly increasing. A vast majority of microbial species producing enzymes of interest are living in some unique environments and it is difficult or sometimes impossible to cultivate them at the laboratory conditions. Well known that less than 1 % of the microorganisms found in the nature could be cultivated under the laboratory conditions, that is why only a miserable part of their genetic potential can be covered by standard microbiological methods. Metagenomics approach is one of the modern methods for searching and investigation of new enzymes, thus, reducing the cultivation problem. This method is based on the direct extraction and analysis of total DNA from the environmental samples. We are able to produce high quality metagenomic DNA libraries from DNA originated from sludge, soil and polluted water samples. Functional analysis of some constructed libraries indicated presence of lipase, hydrolase, esterase, peroxidase, epoxidase and laccase activities. Sequencing of some inserts revealed the presence of enoyl ACP reductase, 1-deoxy-D-xylulose-5-phosphate synthase (DXP synthase), chorizmate mutase/prephenate dehydrogenase, diaminopimelate decarboxylase, acetyltransferase, NADH dehydrogenase (ubiquinone), histidinol phosphate amino transferase.

• Microorganisms from environmental samples

Another way for the discovery of microorganisms with distinguished features is accomplished by the enrichment cultivation of the environmental samples on the delicately selected substrates. Using this approach we have already isolated Bacillus pumilus, Pseudomonas pertucinogena, four strains of Pseudomonas putida, four strains of Pseudomonas aeruginosa, Alcaligenes faecalis, Alcaligenes sp., etc. Valuable enzymes such as secondary alcohol oxidase, laccase, cytochrome C oxidase, lipase were identified in the isolated microorganisms.

Development of biocatalysts

• Gene engineering

The project BIOLUBRICANT was performed in collaboration with a company JSC Biocentras with the main goal to develop a biocatalyst – lipase for the production of oil based lubricant, due to the price of commercial lipase which effects the whole process significantly. The developed and constituted purification scheme for lipolytic enzymes using single-step purification enables to reduce costs and consumption of reagents and energy, which are needed for purification procedures in a large scale. A new bacterial lipase from Serratia sp. was cloned. Bacterial gene was synthesized de novo by GenScript, using OptimumGene ™ codon optimization technology. The new gene was translocated into the original heterologous protein expression system in the yeast Kluyveromyces lactis, based on a strong promoter and signal

peptide usage. In both cases, the attempts gave desirable results – only extracellular lipase activity was determined. Following studies for estimation of optimal cultivation conditions showed that K. lactis can be successfully grown in the medium containing industrial waste the whey – a cheap raw material. Futhermore, both variants of lipases (mG1 and sG1) expressed in K. lactis showed promising characteristics for application in biocatalytic systems. Also, our study showed that de novo synthesis technology used for production of targeted biocatalyst directly from genome sequence data has undoubtedly the future: it can improve the expression level in the system of choice for production of sufficient amount of biocatalyst; also, it may save time required for screening and isolation of enzymes.

Expression systems

Several systems for the expression of the genes of the metagenomic origin is employed in our laboratory so far - in E.coli and in unique heterologous protein expression system in yeast. The latter let us express heterologous genes in several yeast species, including Kluyveromyces lactis, Kluyveromyces marxianus and S. cerevisiae.

Development of analysis methods

Enzyme collections are typically of environmental origin, such as microbial strain collections and/or metagenomic libraries. Screening systems are clasified into three different classes: i) growing cells (colour test, genetic selection, etc.); ii) microtiter plate/microarray (enzyme coupled assays, labeled substrates, product sensors); iii) instruments (GC, HPLC, TLC, izotope labels, etc.). We are keen on development of screening systems for growing cells aiming to search for targeted enzymes. Enzyme assays based on coloured substrates or products can be applied to identify colonies producing active enzymes which can be then be picked for future studies. We have developed such assays for epoxidation, hydrolysis, lypolytic, oxidation (laccase) enzyme activities. Also, we are developing direct and indirect assays for soluble and immobilized enzyme activities or identification of different functional groups of various compounds using Oxygraph instrument. It was designed and optimized assay for phenol functional group determination in fat, oil and aqueous originated samples. Also, it was developed an assay for free fatty acid determination in fats and oils. The assay for determination of the amount of reduced sugars was adopted for different stages in biodiesel production process.

Application of biocatalyst

Synthesis of biopolyols

Traditional bulk chemical manufacturing abounds with classical "stoichiometric" technologies. Conversely, the introduction of biocatalytic reactions is well suited to cost reduction and higher quality products, as well as to more sustainable processes. As safety, health and environmental issues are key drivers for process improvements in the chemical industry, the development of biocatalytic reactions or pathways replacing hazardous reagents is a major focus. The aim of this project is to improve the economic aspects of a green chemistry-based polyurethane production, whereas polyols are important starting materials for the manufacture of polyurethanes. We have developed a chemo-enzymatic synthesis reaction involving epoxidation and hydrolysis. For the first step commercial immobilized lipase (Novozym 435) is applied and for the second step commercial ion exchange resin based catalyst Amberlyst 15 is used. Application of other enzymes with epoxidizing and hydrolyzing activities can achieved more economical and environmental benefits for biopolyol production. Recently, we have isolated several microorganizms from environmental samples which are able to synthesize polyols from rapeseed oil. The research is directed towards characterization of particular enzymes catalyzing this reaction and scale-up optimization of the process.

Immobilization

Immobilization allows for an easy recovery and subsequent reuse of enzymes, thus further reducing the costs of enzymatically catalyzed processes. In general, immobilization is clasified into the main three methods: i) binding to a support (carrier), ii) entrapment (encapsulation) and iii) cross-linking.

PhD student Milda Šulcienė during implementation of the collaborative SCIEX project at University of Applied Sciences (Switzerland) performed immobilization of enzymes with epoxidizing and hydrolyzing activities on solid surface of amino-modified silica nanoparticles and the subsequent covalent cross-linking using two linkers: glutaraldehyde and triacryloylhexahydrotriazine. Seven objects were chosen for immobilization: four bacterial strains (B. pumilus, P. pertucinogena, E10 and E12 (from metagenomic library) and three commercial yeast strains (Y. lipolytica DSMZ 1345, Y. lipolytica DSMZ 3286, C. cylindracea DSMZ 2031). Optimal conditions for

immobilization of samples from bacteria and yeasts origin were determined. Influence of carrier, pH, sorption time, concentration of linker, cross-linking time for immobilization were estimated. Further, the stability of immobilized derivatives under optimal conditions was investigated.

We are also developing a carrier-free immobilization methods (CLE, CLEA, „layered" CLEA). The studies on immobilization of wild type lipase from Serratia sp. and its prototypes by CLEA method revealed that it can be successfully applied for the biocatalytic synthesis of 2-ethyl-1-hexyl oleate (biolubricant). Immobilization of alcohol oxidase from Pichia pastoris by CLEA method was initiated and is under development.

Collaboration

dr. Boris Kovenbach, University of Applied Sciences, Muttenz, Switzerland
Prof. Dr. P. Corvini, University of Applied Sciences, Muttenz, Switzerland
Prof. R.A. Sheldon, JSC CLEA technologies, the Netherlands
Prof. I.W.C.E. Arends, Delft University of Technology, the Netherlands
Nathalie Berezina, Materia Nova, R& D Centre, Belgium
dr. Patrizia Cinelli, University of Pisa, Italy
prof. Vytas Švedas, Laboratory of Biocatalysis and Enzyme Engineering, Belozersky Institute, Moscow, Russian Federation

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COST

Research Contracts

JSC Bioenergy
Lithuanian University of Health Sciences

Inovation Vouchers

JSC Lutora
JSC Akses
JSC Noventus

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Book Chapter

Buchovec I., Pamedytyte V., **Gruskiene R.**, Luksiene Z. Novel approach to the microbial decontamination of wheat sprouts: photoactivated chlorophillin-chitosan complex // Industrial, medical and environmental applications of microorganisms. Current status and trends. ISBN: 978-90-8686-243-6. Editor: Antonio Mendez-Vilas. Wageningen Academic Publishers 2014, 352-356.

Sector of

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Greta Stonytė

Microfluidic systems can overcome many of the limitations of existing techniques for single-cell analysis and manipulation, drug screening and cell-based directed evolution experiments. Compartmentalization of single-cells and into pico- or nano-liter volume droplets allows millions of individual cells to be analyzed and sorted at ultra-high-throughput rates. Cells stay alive for extended periods of time in droplets, and secreted molecules from single compartmentalized cells rapidly achieve detectable concentrations due to the small volume of the droplets. Alternatively, encapsulated cells can be lysed and intracellular biomolecules essayed. This enables the genetic make-up of cells to be analyzed as well as the biochemistry, since the released DNA or RNA can be amplified in the droplets. Thus, analysis is highly flexible, and not just limited to the detection of cell-surface markers. In addition to single-cell screening applications we are also involved in two other areas of research namely directed evolution of in silico engineered enzymes and development of new drug delivery systems based on degradable biopolymers.

The main research areas of our laboratory include:

1. *High-throughput screening of single-cells*
2. *Single-cell transcriptomics*
3. *Organs-on-a-chip*
4. *Directed evolution*
5. *Development of drug delivery systems*

1. High-throughput single-cell screening

Compartmentalization of single cells in droplets allows the analysis of proteins released from or secreted by cells, thereby overcoming one of the major limitations of traditional flow cytometry and fluorescence-activated cell sorting (FACS). In collaboration with Dr. John Heyman (Harvard University) and Prof. Andrew Griffiths (Paris ESPCI) we have developed a binding assay to detect antibodies secreted from single cells compartmentalized in 50 pL droplets (Figure 1). Secreted antibody is detected after only 15 minutes by co-compartmentalizing beads coated with anti-mouse IgG antibodies and a fluorescently-labeled probe: when the antibody captured on the bead binds to the probe the fluorescence becomes localized on the beads, generating a clearly distinguishable fluorescence signal allowing droplet sorting at ~ 200 s-1 rate and cell enrichment. The microfluidic system described is easily adapted to screen other intracellular, cell-surface or secreted proteins and to screen for catalytic or regulatory activities.

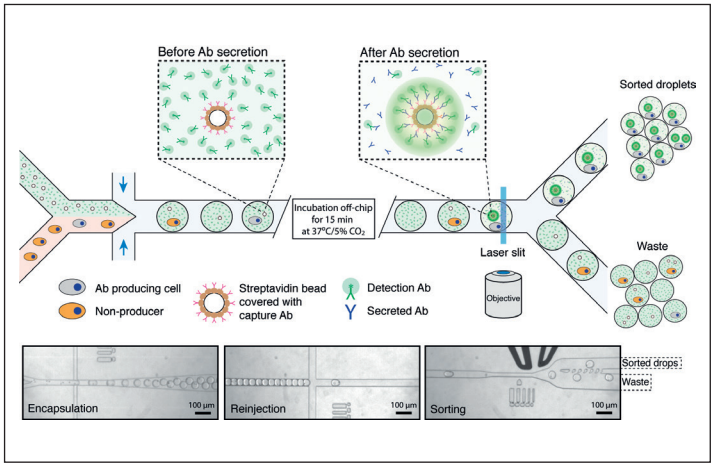

***Figure 1.** Principle of droplet microfluidics platform for single-cell screening. A cell suspension is introduced into a microfluidic device together with a bead suspension, containing fluorescently-labeled goat detection antibodies (green) and orange fluorescent beads coated with goat anti-mouse-Fc capture antibodies (red). Droplets are created at the flow-focusing junction with fluorinated oil containing fluorosurfactant and then collected off-chip at 4 °C. After incubation for 15 min at 37 °C and 5% CO_2, those beads that are co-encapsulated with an antibody-producing cell become highly fluorescent, due to the capture of secreted antibodies on the bead by the anti-mouse Fc antibody and binding of the green-fluorescent detection antibodies to the captured antibodies in a sandwich assay. The emulsion is then introduced into a second microfluidic device and droplets containing green fluorescent beads are sorted using a fluorescence-activated droplet sorter. Hence, droplets containing no bead, no cell, a cell which does not secrete antibody, or an antibody producing cell but no bead are discarded (no green fluorescent bead is present), whereas droplets containing an antibody-producing cell and a bead (which becomes fluorescent) are collected. The three micrographs show i) co-encapsulation of cells with beads, ii) droplet reinjection after incubation off-chip and iii) droplet sorting. (Adapted from Nature Protocols 2013 http://www.nature.com/nprot/journal/v8/n5/full/nprot.2013.046.html)*

2. Single-cell transcriptomics

In collaboration with Dr. Allon Klein (Harvard Medical School) we developed a novel technique named for parallel barcoding of thousands of individual-cells in a single tube. The principle of this technique relies on encapsulation of individual cells into microfluidic droplets together with beads carrying ssDNA barcoded primers and RT/lysis reagents. The mRNA released from the lysed cells remains entrapped inside the droplet and is tagged with cellular and molecular barcodes during RT reaction. Using such approach simultaneous barcoding of thousands of cells becomes possible. We exemplified the use of the technique to barcode and sequence over

6000 cells (manuscript submitted to Cell), but the technology is highly flexible and can be readily adapted to other applications requiring barcoding of RNA/DNA molecules, such as Chip-Seq, RNA- and DNA-Seq or Hi-C, for example.

3. Organs on a Chip

Platelet transfusions are critically important in biomedicine and patient care. Their demand reach total of >2.17 million apheresis-equivalent units a year in the United States only and are derived entirely from human donors despite clinically significant immunogenicity, associated risk of sepsis, and inventory shortages due to high demand and 5-day shelf life. To take advantage of known physiological drivers of thrombopoiesis in collaboration with Dr. Johnathan Thon and Joseph E. Italiano Jr. (Brigham and Women's Hospital, USA) we have developed a microfluidic human platelet bioreactor that recapitulates the main functions of bone marrow such as stiffness, extracellular matrix composition, micro-channel size, hemodynamic vascular shear, and endothelial cell contacts, and supports high-resolution live-cell microscopy and quantification of platelet production. Physiological shear rates improved proplatelet initiation and release from 10% to 90%, reproduced ex vivo bone marrow proplatelet production, and generated functional platelets. Modelling human bone marrow composition and hemodynamics in vitro obviates risks associated with platelet procurement, storage and may fulfil growing transfusion needs.

4. Directed evolution

In this project we are seeking to combine the latest advances of droplet-based microfluidics technology to explore the directed evolution approach for the optimization of computationally designed enzymes (Figure 3). In collaboration with Prof. Donald Hilvert group (ETH Zurich) we are investigating the mechanisms and strategies for optimizing artificially created enzymes. By applying droplet-based microfluidics technology, in which biochemical reactions are performed inside micrometer size vessels, we are developing a powerful tool enabling large number of libraries (>106) to be screened at ultra-high-throughput rates and using conditions that are incompatible with in vivo systems. Our experimental approach could lead to a deeper understanding of the enzymes catalysis and should afford valuable insights into the evolvability of promiscuous protein functions and scaffolds.

5. Development of drug delivery systems

We are applying droplet microfluidics devices for production of highly monodisperse droplets, particles and vesicles for allow encapsulation of therapeutic molecules, pharmaceuticals and drugs. Additional advantage of using microfluidics is that inner and outer parts of the particles can be tuned by control-

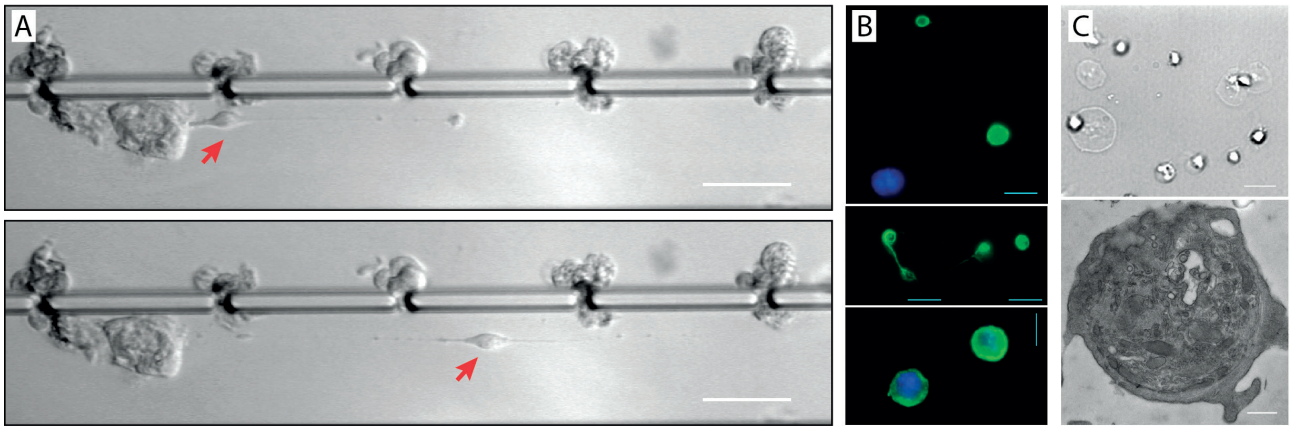

Figure 2. Platelet production using microfluidic bioreactor. A) Megakaryocyte cells trapped at the 2 μm gaps between the posts are releasing proplatelets (red arrow) under physiological shear stress (600 mPa) conditions. B) Bioreactor derived proplatelets are morphologically similar to human blood platelets, and display comparable microtubule expression (green). C) Bioreactor derived human platelets form filpodia/ lamellipodia on activation and spread on glass surface. They are also ultrastructurally similar to human blood platelets and contain a cortical microtubule coil, open canalicular system, dense tubular system, mitochondria, and characteristic secretory granules.

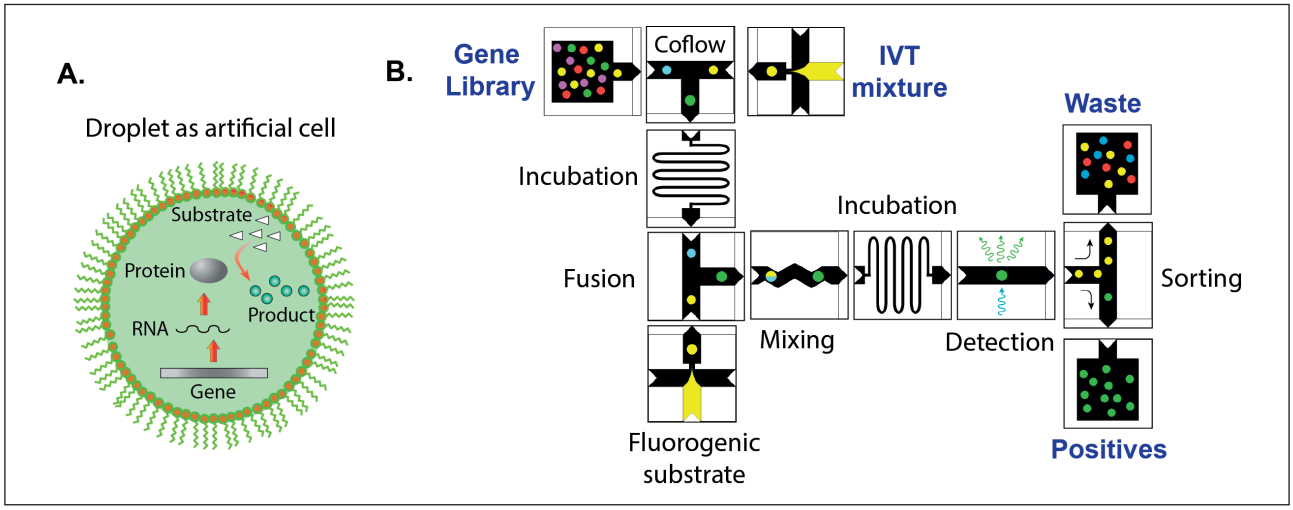

Figure 3. Concept of directed evolution approach in droplet microfluidics. A) Droplets containing single-genes with all ingredients necessary for in vitro expression will serve as artificial cells that can be selected for a desirable phenotype under conditions that are not feasible in living systems. B) Schematics of the integrated droplet-based microfluidics platform for directed evolution.

ling the flow parameters, thus allowing precise control over the size and thickness of the shell. We are producing particles with both, solid biodegradable polymer composed of PLGA (lactic-co-glycol acid) and semi-solid shell composed of alginate.

Collaboration

Prof. David Weitz, Harvard University, USA
Prof. Andrew Griffiths, Paris-ESPCI, France
Prof. Donald Hilvert, ETH Zurich, Swiss
Prof. Andrew deMello, ETH Zurich, Swiss
Prof. Martin Melis, EPFL Lausanne, Swiss
Dr. Thon Jonathan, Brigham and Women's Hospital, USA
Dr. Helder Santos, Helsinki University, Finland

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Research Council of Lithuania
Agency for Science, Innovation and Technology

Publications 2013-2014

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Patent Application

Mazutis L. Microfluidic system and method for production of biopolymer-based droplets and particles. PCT/LT2014/000013. 2014/12/03

DNA Sequencing Center

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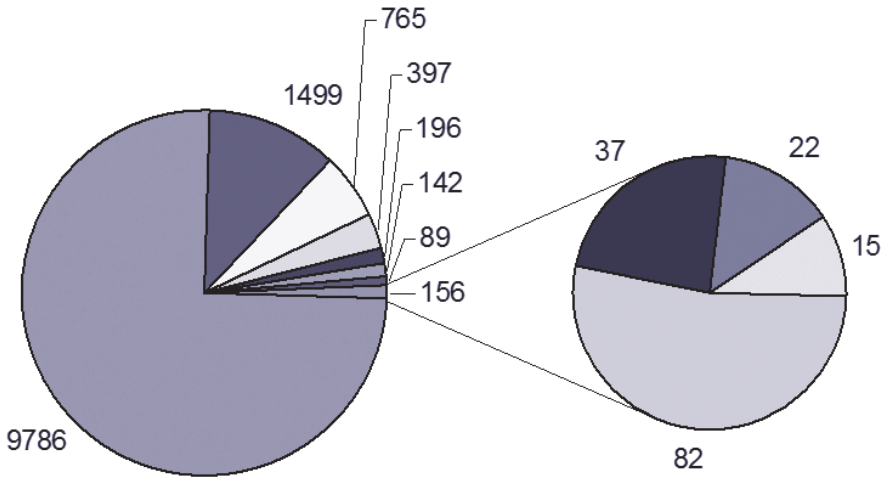
DNA Sequencing Center (SC) of the Institute of Biotechnology (IBT) is successfully running since March 27 of 2003. SC was founded to help researchers, both at IBT as well as other institutions in Lithuania, process DNA samples in an efficient and economical manner. The Center is equipped with the Applied Biosystems 3130xl Genetic Analyzer 16-capillary automated DNA sequencer that yields 700 to 1000 bases per template. It performs cycle sequencing reactions using fluorescent dye terminators ABI Big Dye® Terminator v3.1 on any kind of DNA (plasmid, phage or PCR product) provided by the users. We also run the user's reactions. Usually, turnaround time takes 2-3 days after the receipt of samples. Sequencing of the larger samples may take longer.

Services provided by the DNA SC include:

- Custom DNA Sequencing
- Sequencing, PCR troubleshooting and training workshops

We are committed to giving every user satisfactory sequence.

Number of DNA sequencing reactions performed by SC for the Lithuanian institutions in 2013-2014



Start-ups

ThermoPharma

ThermoPharma Corporation, Inc. – a pharmaceutical company established in 2012 in USA and ThermoPharma Baltic UAB – a company established in 2014 in Lithuania to develop compounds discovered in the Department of Biothermodynamics and Drug Design (DBDD), Institute of Biotechnology, Vilnius University, Lithuania. Scientists at the department have designed, synthesized and evaluated over 700 novel chemical entities as drug candidates.

The main therapeutic areas are cancer, glaucoma, obesity, and other. Research is primarily target-oriented with opportunities in various therapies. The group has designed and patented compounds that inhibit human heat shock protein 90 (Hsp90) and shown compound efficacy in xenograft mice. We have also designed and patented compounds that specifically inhibit several carbonic anhydrases (CAs), especially CA IX – an isoform overexpressed in numerous cancers. The group has also made anti-CA XII monoclonal antibodies and various other recombinant proteins, mostly epigenetically gene-modifying enzymes, histone deacetylases, sirtuins, methyl transferases and other.

List of patents and publications is available at the DBDD website: http://www.ibt.lt/en/laboratories/laboratory-of-biothermodynamics-and-drug-design.html .

Contact: info@thermopharma.lt

MoBiLi

Strengthening and Sustaining the European perspectives of Molecular Biotechnology in Lithuania (MoBiLi)

MoBiLi is funded by the European Union, Research Potential Call FP7-REGPOT-2009-1

Mission of the MoBiLi:

MoBiLi is a support action to strengthen the research capacities and to mobilize human resources in molecular biotechnology at the Institute of Biotechnology (IBT) Vilnius, Lithuania. The MoBiLi, dedicated to the strengthening and sustaining the European perspectives of Molecular Biotechnology in Lithuania, has been selected for funding by the EU FP7 Capacities programme. The latter coordination and support action (call FP7-REGPOT-2009-1) was very competitive: 312 projects were received by the Commission and only 16 were selected for funding (MoBiLi ranked 7-th).

Purpose of the project

is to build up scientific excellence and human potential of IBT thereby transforming it into an excellence centre in molecular biotechnology and a significant player in the European Research Area.

The major objectives:

Human capital building for research and technological development (RTD) in the field of state-of-the-art molecular biotechnology

Networking of IBT with major centres of excellence in the EU via joint research and mobility of researchers

Upgrading and modernisation of research infrastructure in line with emerging thematic priorities in the field

The objectives of the project will be fulfilled by 7 Work Packages via collaboration with the project core partners:

The European Molecular Biology Laboratory (EMBL)
Karolinska Institutet, Stockholm (KI)
Justus Liebig University Giessen (JLU)
University of Edinburgh (UE)
The Swiss Institute of Bioinformatics (SIB)

Scientific priority areas of collaboration with the core partners cover topics like protein structure, interactions and cellular networks (JLU, EMBL, SIB, UE) and cellular imaging and high-throughput approaches to study human diseases (EMBL, KI, SIB, UE).

Project progress (January – November 2013) Exchange of Know-How and Experience

The purpose of exchange programme is to strengthen the expertise and know how of IBT.

During the period concerned, 6 scientists came to the IBT to do collaborative research and researchers from the IBT had made 21 visits to foreign partners. Since the beginning of the project, 76 scientists from the IBT visited partner institutions and 24 scientists from foreign institutions had visited IBT, including Prof. Robert Huber, the Nobel Prize Laureate, who had visited the IBT repeatedly after giving a seminar on March 2011.

Most significant results of the exchange programme: four visitors, M. Juozapaitis, E. Mažeikė, Z. Liutkevičiūtė and M.

Tomkuvienė, made a considerable progress in their Ph.D. studies during their visits. As a result, all of them had defended their thesis successfully. Moreover, a number of new techniques became available to the researchers of the IBT, the methods and research tools (e.g. antibodies) developed at the IBT had been successfully tested at the partner institutions, new applications of joint research projects had been discussed and initiated, junior researchers from the IBT had possibility to get new knowledge and expertise at the partner institutions. All these measures had definitely increased IBT's visibility on the European level, laid the foundations for a closer further collaboration as well as had led to improvement of skills of the IBT staff.

Recruitment of Incoming Experienced Researchers

This work package includes measures for attracting researchers and establishment of new research trends.

2 group leaders, Prof. Irutė Meškienė and Dr. Linas Mažutis, and 5 experienced researchers (postdoctoral associates), Dr. Ieva Mitašiūnaitė-Besson, Dr. Rasa Rakauskaitė, Dr. Vytautas Smirnovas, Dr. Simonas Laurinavičius and Dr. Visvaldas Kairys, had been hired to established new research groups in line with the priority areas of MoBiLi as well as to join the existing laboratories with the goal to strengthen their scientific potential.

Most significant results of the recruitment programme was attraction of new research personnel being well trained and experienced. It is important to mention that 3 out of 7 hired researchers were females. The hired group leaders Prof. I. Meškienė and Dr. L. Mažutis as well as 2 post-docs, Dr. V. Smirnovas, Dr. R. Rakauskaitė and Dr. S. Laurinavičius, had won a number of competitive grants both from international (2 from the Lithuanian-Swiss cooperation programme "Research and Development" and 2 Marie Curie Career initiative grants) and national (1 Global Grant funded by the Research Council of Lithuania, 1 from the Agency for Science, Innovation and Technology, and 5 from the Research Council of Lithuania) funding sources. Moreover, both hired group leaders not only established new research groups, but had attracted to the IBT novel techniques on plant biotechnology and microfluidics. These two new areas of research at the Institute of Biotechnology have been positively evaluated by the MoBiLi Advisory Board. The sector of microfluidic technologies, established by Dr. L. Mažutis, has been very successful in attracting funding from the Lithuanian Research Council and also enjoyed private equity attention and financial support (aprox. 300,000 €) that is quite unusual in the Lithuania research environment. These facts illustrate clearly that the IBT had managed to attract and recruit strong scientists. Thus, the implementation of the workpackage 2 of MoBiLi project had helped to strengthen the existing research directions at the Institute of Biotechnology and establish new research laboratories that should be instrumental for adaption to the dynamic and rapidly changing research landscape in the field of molecular biotechnology. It had been a great success and support for the IBT to have agreed with the European Commission to postpone the end date of implementation of this WP until the end of May 2013 at no additional cost. This measure allowed the Institute to support the hired personnel by paying them salary for additional 6 months. All 7 recruited scientists continue working at Vilnius University after the MoBiLi project had ended.

Acquisition, Development, Maintenance or Upgrading of Research Equipment

The MoBiLi project is aimed to create a stimulating, multidisciplinary environment promoting research of excellence in biomedicine at the interface between structural biology, chemistry and biology. Therefore IBT had purchased the following equipment: Universal X-Ray Diffractometer, HPLC-MS System, Cell sorting system for high performance analytical and preparative flow cytometry and High performance computing (HPLC) Linux cluster. Total value is 600.000 Euro.

This research equipment has been invaluable for carrying out research projects (notably, some of the results obtained by using them had been published by the end of the MoBiLi project) and give a solid future for further developments. This funding had allowed attraction of another € 2.1 million from the EU Structural Funds for support of infrastructure development and also € 280,000 from a private fund to acquire equipment for microfluidics.

The research equipment acquired through MoBiLi funding helped to create the X-ray crystallography unit which is the only such unit in the Baltic countries. It is planned that in the Joint Center for Life Sciences the X-ray crystallography unit

would be further developed into a Center for the structural studies of biomolecules. This center would carry structural studies of biomolecules important for biotechnology and biopharma and also would provide services for the local research community and industries. It has a potential to further develop into the regional center that would serve for the wider research community of Baltic countries and become a local hub of a larger pan-European network in structural biology.

International Seminars & Workshops

The aim of this WP is to increase the international visibility of IBT, dissemination of scientific information obtained at IBT and exchange of know how with potential collaboration partners. 2 experienced researchers had visited IBT and gave their presentations on 2013 (25 high-level scientists including Nobel Prize Laureate Prof. Robert Huber since the beginning of the project). Furthermore, IBT researchers had attended 17 international conferences and workshops on structural and computational biology and biomedicine. Altogether, 54 scientists from the IBT had attended international meetings. Of 54 IBT's employees, 16 were junior researchers, 9 researchers, 27 senior researchers, and 2 MoBiLi post-docs (Dr. V. Smirnovas and Dr. S. Laurinavičius).

Most significant results of the international seminar series at the IBT is that Prof. Robert Huber, the Nobel Prize Laureate, had visited the IBT. Received a permission to cover partial expenses for conference attending instead of covering all expenses only, had resulted in a higher number of the meetings attended, a higher number of the IBT researchers being able to attend them and a higher number of presentations made which had increased the IBT's international visibility.

Dissemination and Promotional Activities

Dissemination activities facilitated dissemination and transfer of knowledge at regional, national and international level involving both the own research/PR staff and invited specialists from other countries as well as increased the international knowledge/ experience exchange capacity and reputation of IBT.

MoBiLi had communicated its activities through a variety of communication channels, including:

- Publication of 11 open-access articles in scientific journals and 5 scientific articles with acknowledgement to the MoBiLi project
- Instant highlighting of research achievements on the IBT and MoBiLi web pages and with the media
- Production of biennial reports 2008-2010 and 2011-2012 on IBT research achievements and services offered to the community in connection with the MoBiLi project
- Meeting with local biotech SME (UAB Sorpo, UAB Profarma, UAB Biocentras, UAB Fermentas) and Lithuanian Biotechnology Association (LBTA)
- Meeting in the Diagnostic Centre of Vilnius University Hospital
- MoBiLi project had been presented at the international event that took place in Vilnius on May 5th, 2011. The audience of the meeting included the representatives from the Parliament of the Republic of Lithuania, Ministry of Education and Science, Ministry of Economy, representative from the programme "Eureka", Swedish government agency VINNOVA and the Finnish Funding Agency for Technology and Innovation (TEKES)
- short presentation of the project had been published by the European Commission (Science at the service of Europe: A guide to the 2009 Annual Activity Report of the European Commission's Directorate-General for Research", p. 20.

Announcements of the MoBiLi seminars are distributed to the target groups. A MoBiLi website (http://www.mobili.ibt.lt/) has been launched and is constantly updated. There is a link to the MoBiLi website at the IBT's site (http://www.ibt.lt/en/title.html) as well.

External Evaluation

To check and control the achieved research quality and scientific excellence at the project's end, an independent evaluation will be implemented. External evaluation facility is foreseen to take place after the end of the implementation in order to evaluate the applicant's overall research quality and capability (including management and infrastructure). European Commission had appointed 4 Experts for external evaluation of the project: Prof. dr. hab. Jan Żeromski, Poznan

From left to right the experts of External evaluation Dr. Urszula Bialek-Wyrzykowska, Prof. Jan Zeromski and Dr. Joaquin Guinea at the MoBiLi meeting at the Institute of Biotechnology on April 26, 2013

University of Medical Science, Poland; Prof. Toivo Maimets, University of Tartu, Estonia; Dr. Urzula Bialek-Wyrzykowska, Institute of Biotechnology, Poland; Dr. Joaquin Guinea, INNOVATEC/ZF BIOLABS, Spain.

First meeting of the external Evaluation Board was held on April 26th, 2013, final meeting took place at Vilnius University main campus on September 25th, 2013. On November 19th, 2013, the experts had produced and submitted **the MoBiLi project Independent expert evaluation Report. The Report says:**

The Expert Group approached the MoBiLi project from different perspectives. However, we all came to the conclusion that financial support received by the Institute of Biotechnology University of Vilnius for the development of research potential had been used in a very efficient way. The investment of € 1.6 million resulted in opening of new research areas at IBT, increased significantly human potential, strengthened the equipment base and established international contacts. Researchers involved in MoBiLi project, both, previously employed and recruited for the MoBiLi, had shown commitment to their work and the willingness to contribute to the development of research in Lithuania.

The implementation of MoBiLi coincided with the integration of IBT into Vilnius University. The Expert Group is convinced that in a long-term this was a very positive development that will open new possibilities for the Institute. One of them is the development of a Joint Center for Life Sciences at Santara Valley – an integrated science, studies and business centre in Vilnius. The IBT is now well prepared to participate successfully in Horizon2020 and other research supporting programs.

Conclusions

1. The goals formed at the time of application for the MoBiLi project were mostly reached.
2. Funds provided by EC were spent and used in an appropriate, economical and logical way.
3. IBT position and prestige in scientific environment of Europe has risen significantly, due to personal contacts, high-ranked publications and participations in international conferences.

Project Management

To ensure successful implementation and professional administration, vigorous and excellent project management is necessary. The internal decision making were done by the MoBiLi Management Board in close agreement with the IBT Council members. To ensure best possible scientific, administrative and managerial decisions, the MoBiLi Management Board consisted of Mr. Leonas Pašakarnis, (Deputy Director of Institute of Biotechnology Vilnius University), Prof. Saulius Klimašauskas, (Head of Department of Biological

Joint meeting of the MoBiLi Advisory Board and External evaluation experts at the Institute of Biotechnology on April 26, 2013

From left to right Dr. Joaquin Guinea, Prof. Henri Grosjean, Prof. Stephen Halford, Prof. Miguel Quiros Olozabal, dr. Saulius Gražulis, Prof. Bo Samuelsson, Prof. Alfred Pingoud, Prof. Jan Zeromski, Dr. Česlovas Venclovas and Dr. Urszula Bialek-Wyrzykowska after the MoBiLi meeting at the Institute of Bitoechnology on April 26, 2013

DNA Modification), Dr. Daumantas Matulis, (Head of Department of Biothermodynamics and Drug Design), Dr. Gintautas Žvirblis, (Head of Department of Eukaryote Genetic Engineering), Prof. Aurelija Žvirblienė, (Head of Department of Immunology), Dr. Česlovas Venclovas, (Head of Department of Bioinformatics), and Prof. Virginijus Šikšnys, (Head of Department of Protein-Nucleic Acids interactions).

To ensure qualified strategic guidance and impartial critique for the project, an Advisory Board had been set up. The prime role of the Advisory Board was to monitor and evaluate the progress of the MoBiLi project from the European perspective. The Advisory Board comprised of six prominent EU scientists representing a wide spectrum of relevant research fields and four representatives from the national biotechnology arena. The Advisory Board members are Prof. A. Tramontano (University of Rome "La Sapienza", Italy), Prof. A. Pingoud, (Justus-Liebig-Universität, Germany), Prof. L. Poellinger, (Karolinska Institutet, Sweden), Prof. S. Halford, (University of Bristol, U.K.), Prof. B. Samuelsson, (University of Gothenburg, Sweden), Prof. H. Grosjean, (University of Paris-South, France), Prof. E. Butkus, (Research Council of Lithuania), Mr. A. Markauskas, (Fermentas, CEO, Lithuania), Dr. A. Žalys, (Ministry of Education and Science, Lithuania), and Prof. G. Dienys, (Lithuanian Biotechnology Association).

The Final Report of the MoBiLi project Advisory Board of September 25th, 2013, says:

The impressive fact is that these scientists have been recruited from prestigious foreign Academic Institutions in the United States and Western Europe, and that these scientists themselves have already been very successful in publishing high visibility papers and securing external funding for their research after arrival in the IBT. This reflects the high academic

and scientific competence of the recruitments. In conclusion, this objective of Human Capital Building the MoBiLi project has been very successfully met and implemented.

There have been very high profile seminar speakers in the IBT, and very active PhD student exchange programs with a number of foreign Universities. The positive impact of both the high level seminars on the research education of the students and the initiation of new projects by the student exchange is appreciated.

It is apparent that the infrastructure of the IBT has been very significantly improved and upgraded by the help of the MoBiLi funds. This will make the Institute more attractive to outside laboratories for collaboration, and, most importantly, will certainly increase the scientific competitiveness of the IBT.

In conclusion, all three objectives of the MoBiLi project have been fully met and to this end very good use of the MoBiLi funds has been made. This improvement of the impressive quality of the IBT will therefore not only benefit Lithuania but the whole European scientific community.

Most significant results of the project management: it had been agreed with the European Commission to postpone the deadlines of implementation of workpackages 1-5 for additional 6 months until May 31st 2013. The second achievement is that the European Commission had accepted our request for Amendment No. 2 to the Grant Agreement extension which had led to extension of implementation of workpackages 1, 3-7 for additional 6 months at no extra cost until November 30th 2013.

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