Department of

Biothermodynamics and Drug Design

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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.

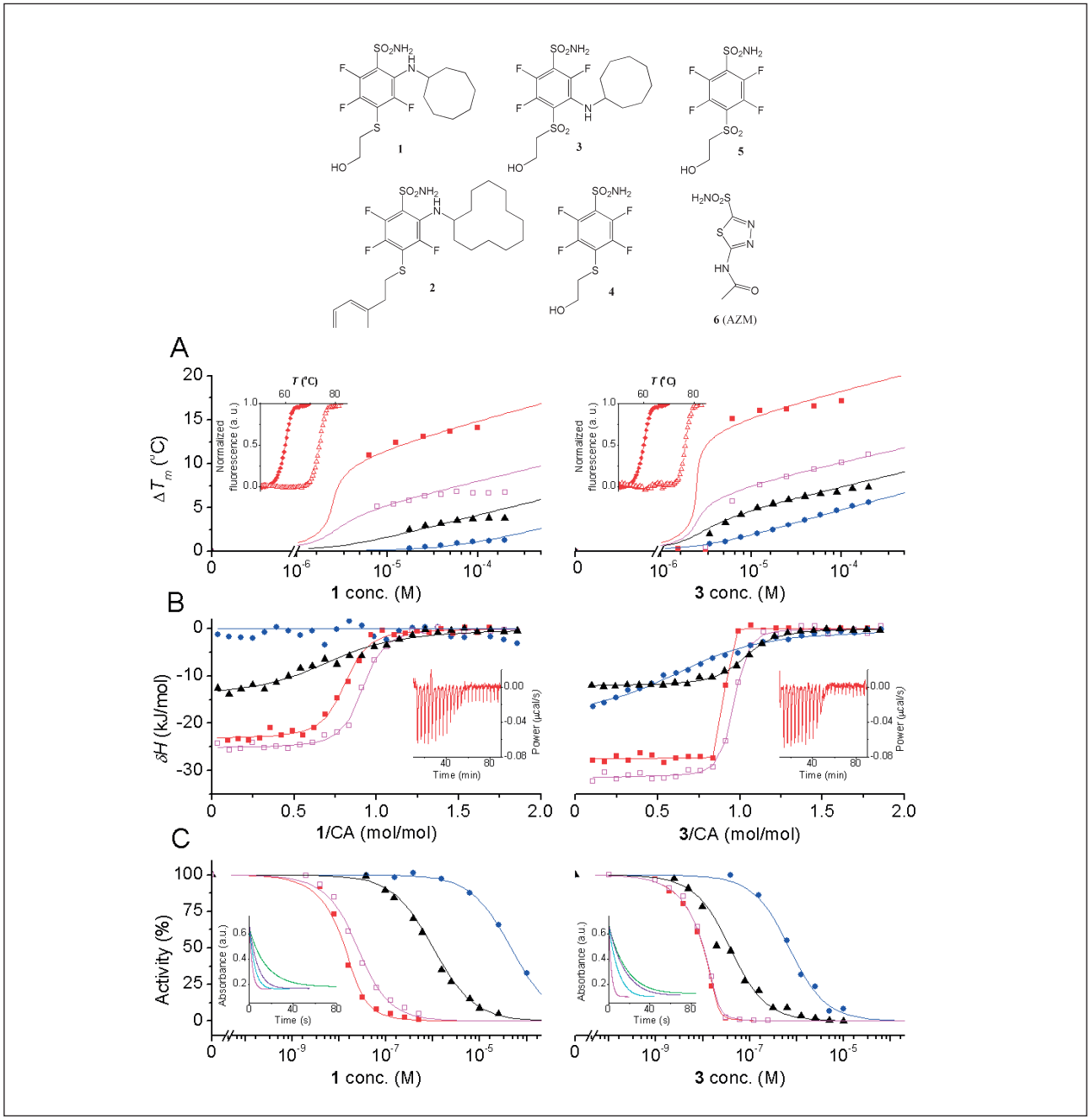


Figure 1. The top panel shows the chemical structures of CA inhibitors 1-6. Acetazolamide (6, AZM) is commonly used as a control inhibitor of CAs. Panels A, B and C show compound 1 (graphs on the left) and 3 (graphs on the right) binding and inhibition of CAs. A. Binding of compounds as determined by 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.

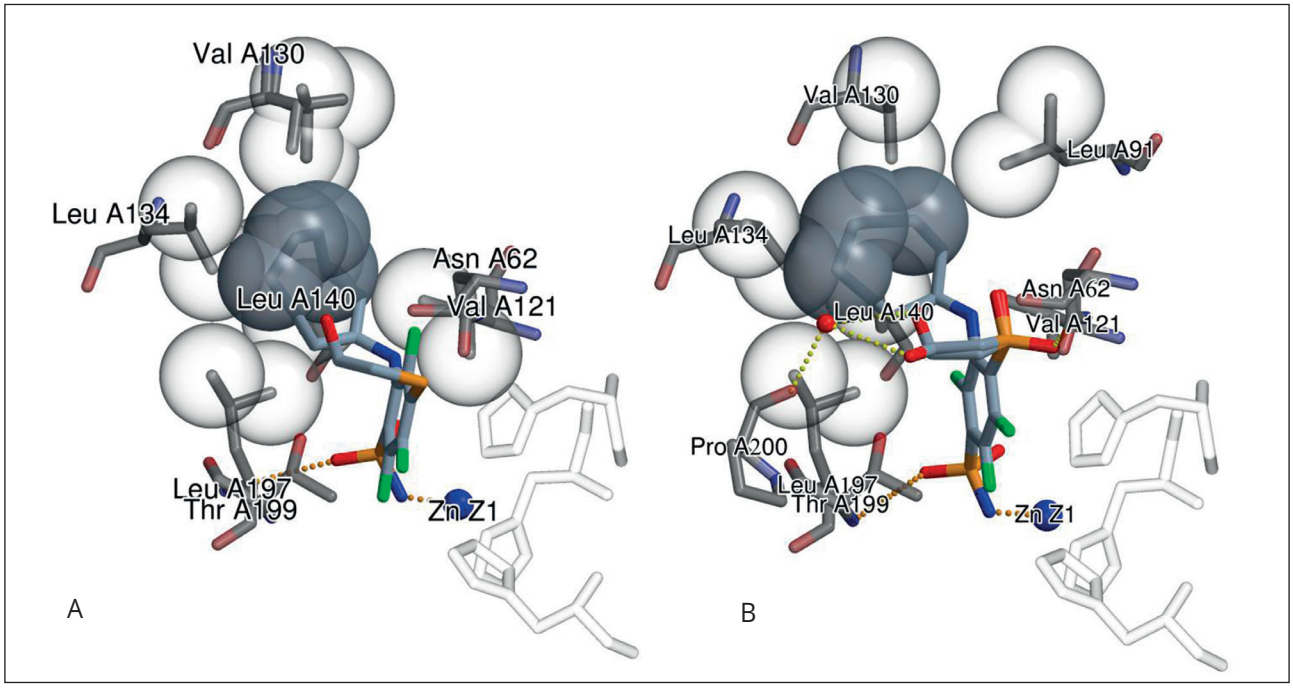


Figure 2. Compounds 1 (Panel A, PDB ID 4Q06) and 3 (Panel B, PDB ID 4Q07) bound to chCA IX as determined by X-ray crystallography. The Zn is shown as a blue sphere, while the histidine residues holding the Zn atom are transparent. The amino acids of chCA IX are shown in grey. The terminal atoms of amino 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).

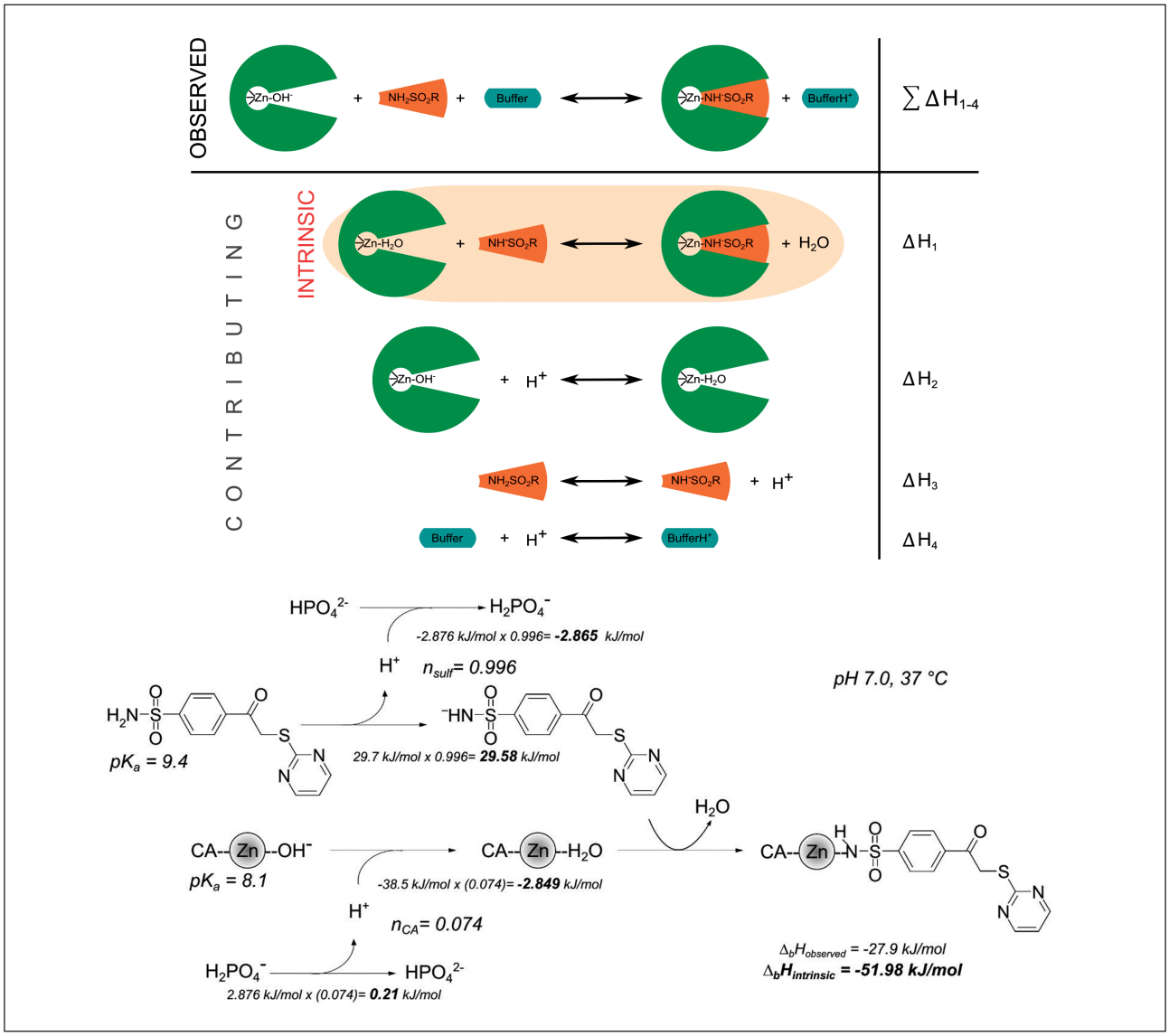


Figure 3. The observed and intrinsic binding thermodynamics. The upper panel shows the main linked reactions occurring upon ligand binding to CAs. The lower panel lists the enthalpies of all processes linked to the binding of 3b to CA I. The two left-central reactions show the binding-linked deprotonation 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.

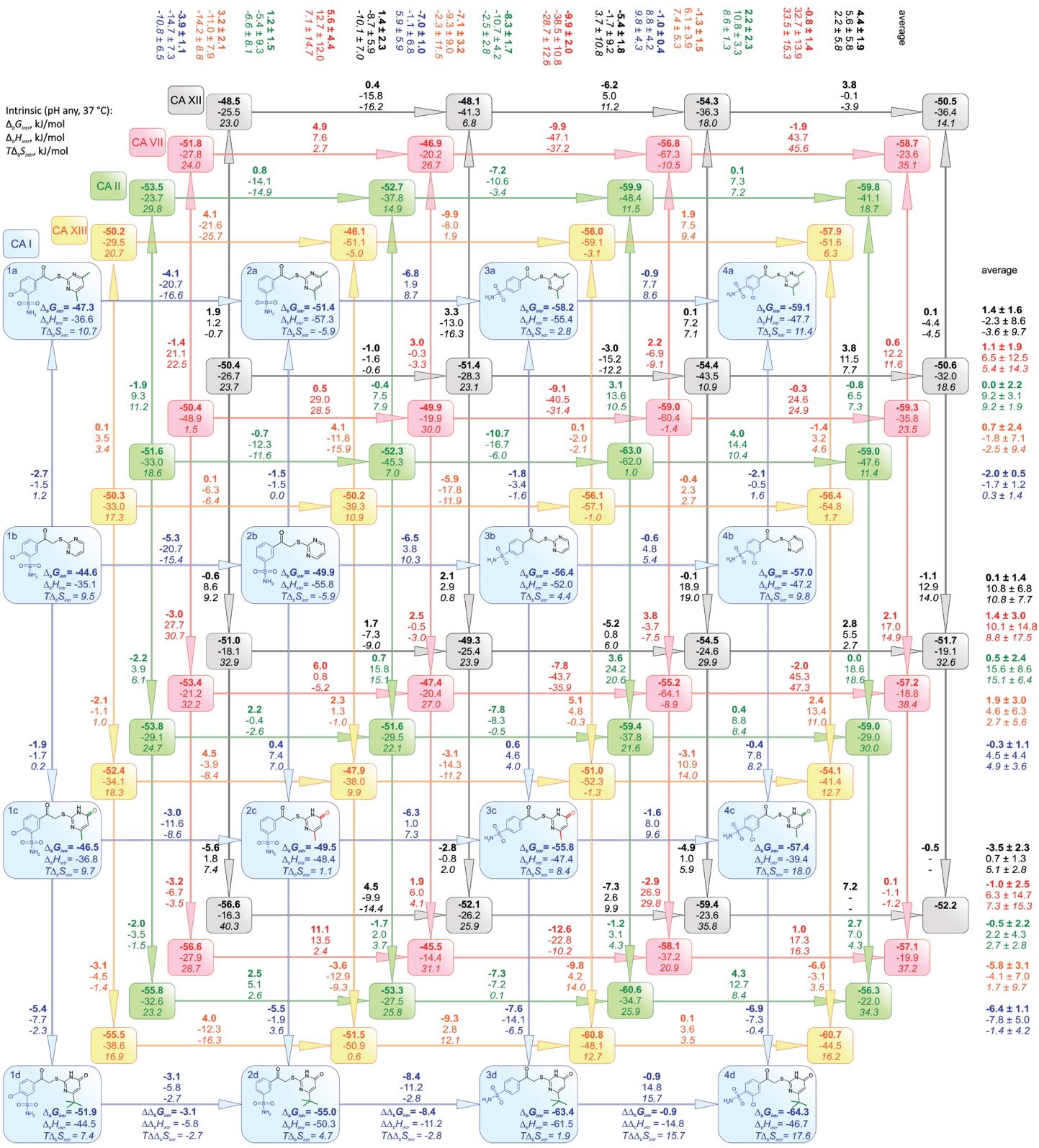


Figure 4. *Inhibitor structure correlations with the thermodynamics of binding. Intrinsic parameters of compound binding to five investigated CA isoforms are given within the shapes. Different colors represent different CA isoforms. Numbers next to arrows show the Gibbs free energy (top number, bold), 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.*

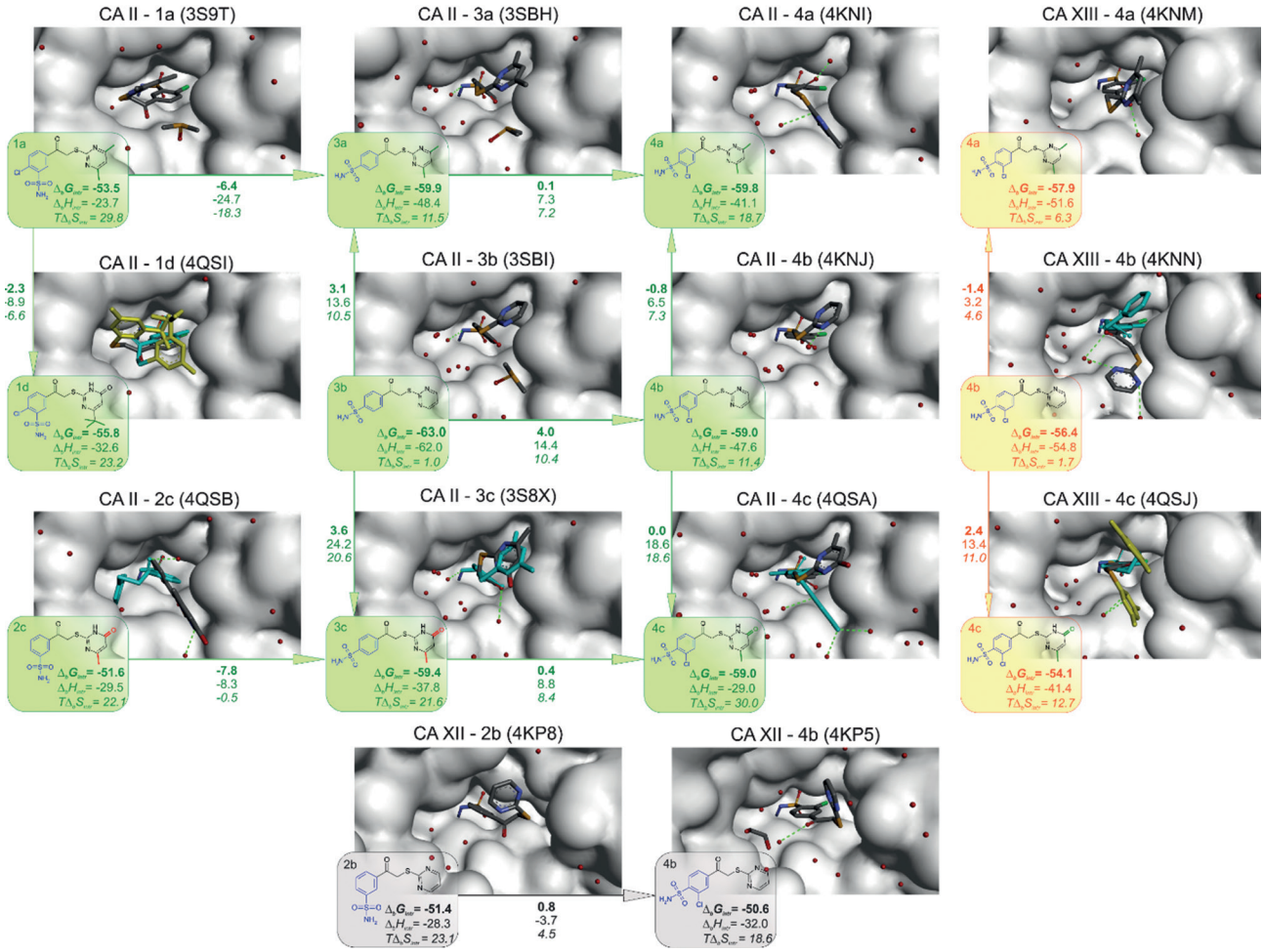
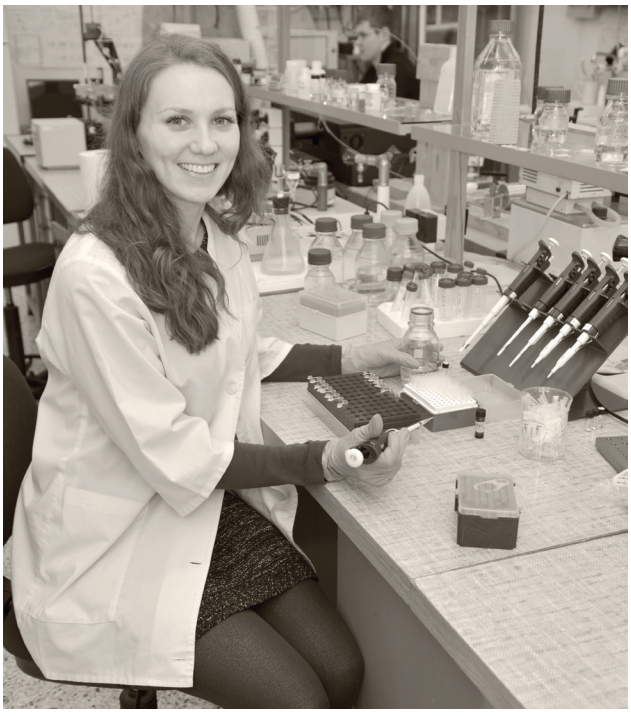


Figure 5. ***Compound chemical structure and the thermodynamics of binding correlations with the crystal structures of some compound binding mode in the active site of CAs (1a, 1d, 3a, 3b, 3c, 4a, 4b, and 4c with CAII; 4a, 4b, and 4c with CA XIII; 2b and 4b with CA XII). The thermodynamic parameters 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).

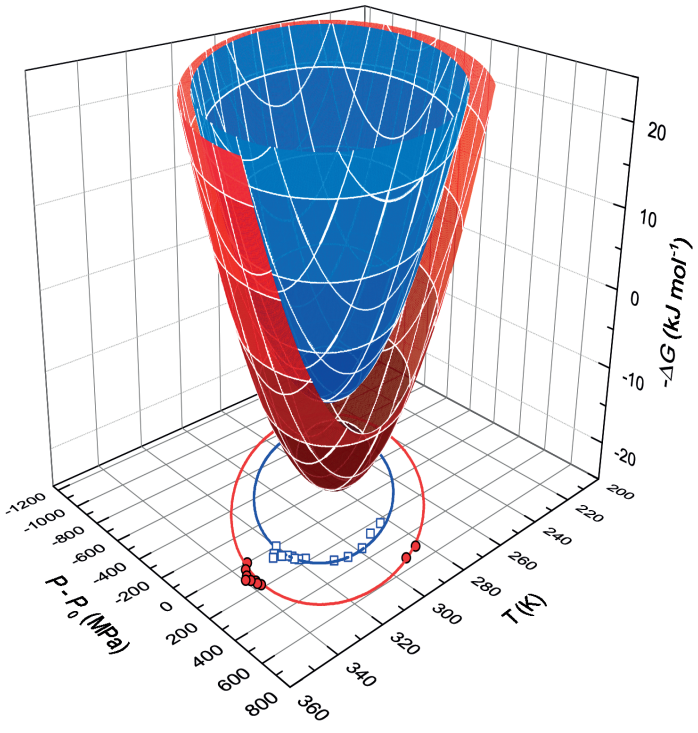
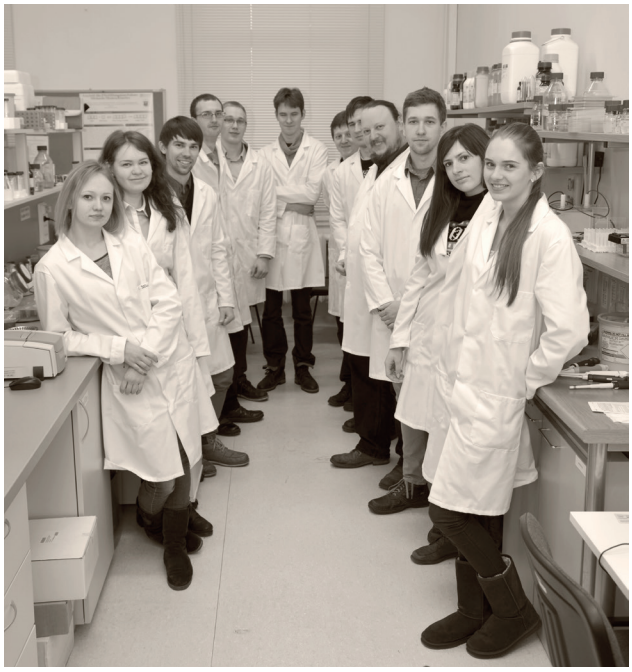


Figure 6. The Gibbs free energy dependence on pressure and temperature. Inner surface represents the ligand-free Hsp90N stability region, while outer surface shows stability region of protein-ligand system with 200 μM of added ligand [13].

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

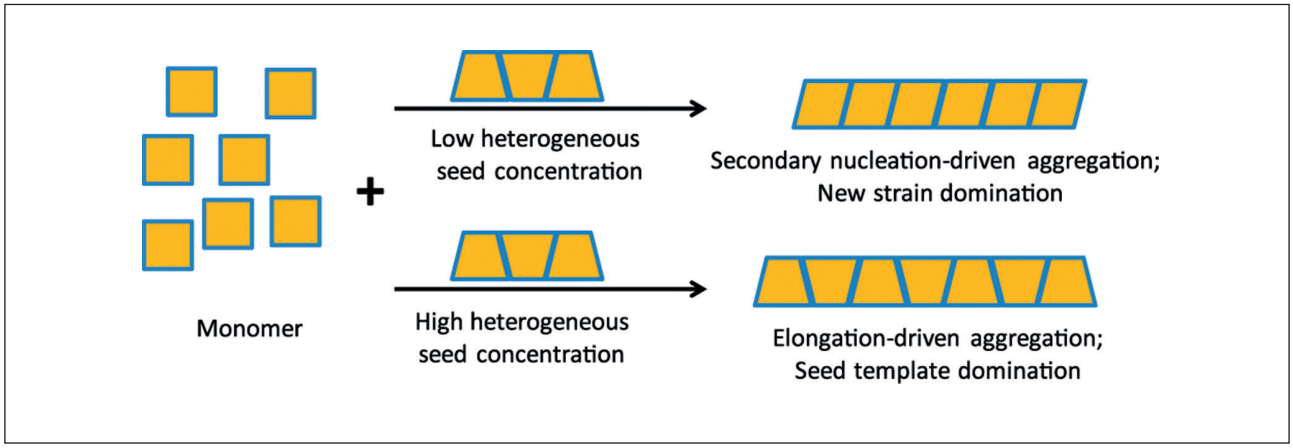


Figure 7. ***Conformational switching between amyloid fibrils.***

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.

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The team of amyloid research:

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