VILNIUS UNIVERSITY

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ANALYSIS OF LIGAND BINDING TO RECOMBINANT HUMAN CARBONIC ANHYDRASES I, II, VII, IX AND XIII

Summary of doctoral dissertation
Physical sciences, biochemistry (04 P)

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This study has been carried out at the Institute of Biotechnology, Vilnius University, during 2006–2012.

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VILNIAUS UNIVERSITETAS

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REKOMBINANTINIŲ ŽMOGAUS KARBOANHIDRAZIŲ I, II, VII, IX, XIII SĄVEIKOS SU LIGANDAIS TYRIMAS

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Contents

Abbreviations

AZM	acetazolamide
CA	carbonic anhydrase
$CAZnH_2O$	water molecule coordinated to Zn^{2+} in the carbonic anhydrase active site
CARP	carbonic anhydrase related protein
DMSO	dimethylsulfoxide
DTT	dithiotreitol
EZA	ethoxzolamide
GPI	glycosylphosphatidylinositol anchor
ITC	isothermal titration calorimetry
MTZ	metolazone
MW	molecular weight
ND	not determined
PG	proteoglycan domain
sCA IX	recombinant human CA IX short form
a.u.	arbitrary units
TFMSA	trifluormethanesulfonamide
TSA	thermal-shift assay

Introduction

Carbonic anhydrases (CAs) are metalloenzymes that catalyse the conversion between carbon dioxide and bicarbonate. These proteins are involved in many physiological and pathological processes. Their inhibition with small molecule ligands can be applied for treatment of different diseases, such as glaucoma, cancer, obesity, epilepsy, osteoporosis, etc. There are nearly 30 small molecule ligands that are used as drugs for carbonic anhydrase related diseases. The main challenge for generation of new CA ligands is to attain specificity towards a selected CA isozyme, as there are many CA isoforms in humans that have very similar structure.

The main tools applied for protein-ligand interaction analysis, in this dissertation, are biophysical methods, such as isothermal titration calorimetry, differential scanning calorimetry and the thermal-shift analysis. The power of these methods is their versatility. These methods can be used for nearly all protein-ligand systems without the need of a specific detection system for different enzymatic or biological activity.

Non-covalently binding small molecules are widely used as inhibitors of various protein targets to cure or prevent many diseases. One of the starting tasks in a new drug design process is to identify small molecules that interact with the target protein and to characterize the interaction. There are two main ways to identify ligands for the target protein: 1) by screening natural or synthetic compound libraries, and 2) by computer-aided modelling of new compounds using the known structure of a target protein.

The thermal-shift assay is sometimes used as a high-throughput screening method for new ligand hit identification. Yet it is also used for quantitative analysis to evaluate protein–ligand binding affinity. Protein stabilizing ligands are usually analysed, but the protein-destabilizing ligands are also available. Adaptation of thermal shift assay to the protein destabilization phenomenon further increases its applicability, as presented in this work.

The rational design of novel compounds requires detailed information about the intrinsic energies of similar ligand interaction with the target protein. However, most scientific literature lists only the observed ligand binding parameters that depend on reaction conditions such as pH and the presence of the linked reactions. By employing thermodynamic additivity methods, it is possible to dissect the intrinsic binding parameters, that do not depend on linked reactions or applied reaction conditions. One such example of the dissection, sulfonamide ligand binding to carbonic anhydrase XIII, is presented in this dissertation.

Goal of the dissertation

To analyse the interaction between recombinant human carbonic anhydrases I, II, VII, IX, XIII and sulfonamide ligands.

Specific tasks of the dissertation

- to evaluate the stability of selected human recombinant carbonic anhydrase isozymes in different experimental conditions and characterize CA IX protein;
- to extend the applicability of the thermal shift analysis method for the investigation of protein destabilizing and exceptionally tight binding ligands using CAs as model proteins;
- to evaluate the binding affinity of new carbonic anhydrase ligands and compare them to the drugs used for carbonic anhydrase inhibition;
- to analyse the binding thermodynamics of CA XIII interaction with sulfonamide ligands.

Scientific novelty and practical value

- the oligomeric structure of anticancer target CA IX was determined to be a dimer;
- increased the range of TSA method application – the ability to analyse exceptionally tight interactions and to evaluate protein destabilizing ligands;
- determined the binding parameters of 40 new compounds to human carbonic anhydrases. A group of these compounds are covered by European patent;
- the binding thermodynamics of sulfonamide ligands to CA XIII was analysed in detail. Intrinsic binding parameters, independent of the experimental conditions and linked protonation reactions, were determined.

The approbation of the results of the thesis

The original results of the dissertation were represented in 8 manuscripts published in journals listed by Thompson Reuters ISI with citation index and one article without the citation index. One European patent was obtained. The results were personally presented in three poster presentations at international conferences and at 17 conference presentations presented by co-authors.

Structure and contents of the dissertation

The dissertation contains six parts: introduction, literature survey, materials and methods, results and discussion, conclusions, and the list of 134 cited references. Overall, the dissertation consists of 106 pages. A summary of each part is presented below in this short version of the thesis.

Literature survey

In the literature survey part, an overview of carbonic anhydrases is presented. The CA types, isoforms, cellular localization, catalytic activity, structure, inhibition and activation, as well as its use as therapeutic target and model protein are discussed. Furthermore, there is a short introduction of the analysis of linked reactions.

Carbonic anhydrases (CAs) are metalloenzymes. Their main function in the organism is to catalyse the reversible hydration of carbon dioxide to bicarbonate and a proton. There are 12 catalytically active α-CA isoforms in humans that are highly homologous (sequence identity 28–60 %), but have different distribution in tissues and organs, and vary in the cellular localization.

CAs are involved in many physiological and pathological processes. Several of them are validated therapeutic targets in many diseases, such as glaucoma, disorders of nervous system, obesity, and cancer. The main type of drugs used to control CA activity are inhibitors containing a primary sulfonamide group. The major weakness of the currently used inhibitors is the lack of selectivity between different isoforms.

CA I and II are also widely considered as model proteins for the analysis of protein–ligand interaction, protein stability and protein folding.

Protein–ligand binding is often accompanied by linked reactions. Most common linked reactions are protonation-deprotonation reactions. The main hallmarks of them being present are: 1) the observed binding constant and the observed enthalpy varies when pH changes, 2) the observed binding enthalpy is different in buffers with different protonation enthalpy when all other conditions are the same.

Materials and Methods

This part consists of two main sections: **Materials** that indicate chemicals and proteins used for analysis, and **Methods** that describe applied methods and experimental conditions.

Materials

Commercially available chemicals were obtained from Sigma-Aldrich, Fluka, Bio-Rad and other suppliers.

Proteins. Recombinant human carbonic anhydrases I, II, VII and XIII (expressed in *Escherichia coli*) used in this study were prepared in the Department of Biothermodynamics and Drug Design. Recombinant human CA IX proteins (expressed in an insect cell line) were provided by Prof. Seppo Parkkila (Tampere University, Finland).

New carbonic anhydrase ligands. Novel carbonic anhydrase ligands were synthesized at the Faculty of Chemistry, Vilnius University, and the Department of Biothermodynamics and Drug design, Institute of Biotechnology, Vilnius University.

Methods

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) experiments were performed with the VP-ITC calorimeter (Microcal, Inc.).

In protein–ligand binding analysis the calorimeter cell (1.4315 mL) is filled with protein solution (5-10 µM) and the titration syringe (300 µL) is filled with ligand solution (50–100 µM). Titration is performed at selected temperature using 25–30 ligand injections with 3–4 min intervals and a stirring speed of 260 rpm. Protein and ligand solutions are prepared in identical buffer with exactly the same concentration of DMSO (usually 1 %). Experiments were repeated 2–3 times.

For ligand protonation analysis the cell is filled with ligand solution and the syringe with acid (HNO_3) solution. Injection volume 5 µL, the number of injections was 60. The protonation of deprotonated ligand is measured because the enthalpy of protonation is exothermic and it is determined with greater precision than the deprotonation with NaOH. Fully deprotonated ligand is made by adding 1.5 equivalent of NaOH and is then titrated with HNO_3 solution. Experiments were repeated 2–3 times.

For protein denaturation with acid, the analysed proteins were transferred to 10 mM NaCl solution. Desalting was accomplished by gel filtration through the gravity-flow PD10 columns (GE Healthcare). The calorimeter cell is filled with 15 µM CA in 100 mM NaCl and the syringe with 5 mM HNO_3 in 95 mM NaCl solution. The control experiments for evaluation of acid dilution were carried out using 100 mM NaCl in the cell in the absence of CA.

Sample preparation for the determination of CA XIII Zn^{2+}-H_2O pK_a and ΔH. When the ITC experiments are performed at the pH where buffer capacity is too low to ensure the expected pH value, the pH of protein and ligand samples is

adjusted by adding acid/base and verifying the pH using a micro-pH electrode and the samples are then immediately loaded to the calorimeter cell.

Data analysis. Experimental data was analysed using MicroCal Origin 5.0 ITC module which allows the determination of binding constant K_b, binding enthalpy ΔH, and the concentration correction factor n.

Fluorescent thermal shift analysis

The principle of the method, experimental design. TSA is used to analyse the equilibrium transition between the protein native state N and denatured state U by detection of fluorescence of added solvatochromic dye (equation 1), and to evaluate additional stabilization/destabilization of protein–ligand complex caused by the ligand L [1].

$$U + L_f \overset{K_U}{\leftrightharpoons} N + L_f \overset{K_b}{\leftrightharpoons} NL_b \tag{1}$$

The process is described by the protein unfolding constant K_U and the binding constant K_b:

$$K_U = \frac{[U]}{[N]} = \mathrm{e}^{-(\Delta G_U/RT)}, K_b = \frac{[NL_b]}{[N][L_f]} = \mathrm{e}^{-(\Delta G_b/RT)}, \tag{2}$$

where ΔG_U and ΔG_b are the Gibbs free energy of protein unfolding and binding process, respectively. L_f is the concentration of free, and L_b – the concentration of ligand bound to the protein. T – temperature in Kelvin, and R – the universal gas constant.

During protein denaturation, the solvatochromic dye interacts with protein hydrophobic areas and the increase in fluorescence is observed. Protein stability is described by its melting temperature T_m which indicates a temperature when half of a protein is in the native state and the other half is denatured. The process is assumed to be fully reversible.

Experimental setup. Three different devices were used for the thermal shift analysis:

1) Real time detection system iCycler iQ (Bio-Rad) originally designed for PCR with Chroma Technology Corp. filters: excitation 405 ± 30 nm, emission 520 ± 40 nm. Analysis was performed in 96 well plates;

2) ISS PC1 spectrofluorimeter with water-thermostat ant digital thermometer TFN520 (Ebro Electronic GmbH & Co). Analysis was performed in 3 mL cuvette using excitation at 380 ± 5 nm, and monitoring the emission at 510 ± 5 nm;

3) Real time detection system Rotor-Gene 6000 (QIAGEN Rotor-Gene Q) using the Blue channel (excitation at 365 ± 20 nm, emission at $460\pm$ 15 nm). Analysis was performed in 100 µL or 200 µL sample tubes.

Heating rate of 1 ℃/min was used.

Two solvatochromic dyes were used in this study: dapoxyl (benzenesulfonic acid, 4-[5-[4-(dimethylamino)phenyl]-2-oxazolyl]-, sodium salt) and ANS (8-anilino-1-naphthalenesulfonic acid ammonium salt).

Composition of a typical sample: 10 µM protein, 50 µM ANS, 0-200 µM ligand, 2 % DMSO, 50 mM phosphate buffer, pH 7.0, 50 mM NaCl. Each experiment was repeated three times.

Determination of the melting temperature. The dependence of fluorescence intensity of protein–ligand complex on temperature is described by the equation [1, 2]:

$$y(T) = y_N + \frac{y_U - y_N}{1 + \mathrm{e}^{\Delta_U G/RT}} = y_U + \frac{y_N - y_U}{1 + \mathrm{e}^{-\Delta_U G/RT}} \tag{3}$$

where y_N and y_U describe linear parts of a denaturation curve (y_N corresponds pre-transitional line and y_U post-transitional), and $\Delta_U G$ is the Gibbs free energy of protein unfolding. The melting temperature T_m is determined by fitting the equation (3) to experimental data.

Determination of the binding constant. For the determination of the binding constant, a series of T_m's obtained at different ligand concentrations was used [3]. For ligand binding to native protein (most often used model), the dependence of T_m on added ligand concentration is described by equation:

$$L_t = (K_U - 1)\left(\frac{P_t}{2K_U} + \frac{1}{K_b}\right), \tag{4}$$

where P_t is the total protein concentration. For the calculation, the constants K_U and K_b are expressed using Gibbs free energy description: $\Delta G = -RT\ln K$. The binding constant K_b was estimated by simulated T_m dependence on L_t by varying the parameters of equation (4).

Differential scanning calorimetry

Differential scanning calorimetry (DSC) experiments were performed using the MC-2 scanning calorimeter (MicroCal, Inc., a kind gift of Prof. Vince LiCata). The scanning rate of 1 ℃/min was applied. Experimental data analysis was performed with MicroCal Origin 5.0 DSC module. Protein concentration used for DSC experiments was 50-100 µM.

Gel filtration analysis

Analytical gel filtration was performed on the chromatographic system Äkta Explorer, using TSK G2000SW column and 10 mM sodium phosphate buffer with 0.3 M NaCl, pH 7.0. Protein is loaded onto the column at the flow rate of 1 mL/min, and eluted in isocratic mode. The same conditions were used for calibration – a set of standard proteins (Bio-Rad) with molecular weights (kDa): 670, 158, 44, 17, and 1.35.

SDS-PAGE

SDS-PAGE was performed using the standard method [4] in a vertical apparatus for protein electrophoresis using 9.5–15 % polyacrylamide gels. Gels were

stained with PageBlue^{TM} dye (Fermentas) according to manufacturer's recommendations.

During the analysis of CA IX oligomeric structure, the protein samples were incubated for 2 h at 25 ℃ with different concentrations of DTT (0, 0.01, 0.05, 0.1, 0.25, 1.0, and 10.0 mM). Oligomer analysis by SDS-PAGE was performed in 9.5 % polyacrylamide gel.

Results and Discussion

The results and discussion part is composed of four sections: 1) analysis of carbonic anhydrases, 2) application of carbonic anhydrases for the development of TSA method, 3) analysis of the novel carbonic anhydrase ligands, and 4) thermodynamic analysis of sulfonamide inhibitor binding to CA XIII.

Analysis of carbonic anhydrases

Evaluation of CA stability

Protein stability measurements were performed in order to evaluate the stability at selected experimental conditions and determine the thermodynamic parameters of unfolding.

Thermal shift analysis – pH, salts. The pH stability profile and the influence of different salts to CA isoforms was evaluated by the TSA method. It was determined that the pH stability interval is wide (T_m is close to max value at pH 5–9) for all tested bacterially expressed CA isofroms, and the insect cell expressed CA IX is also stable in slightly acidic pH. This agrees well with its biological function – this isoform is active in acidic media during hypoxia.

The tested CA isoforms are not sensitive to buffer salts except for carboxylates that destabilize these proteins. Therefore, for instance, citrate should not be selected as a buffer material. The addition of sulfate destabilizes CA I, II and XIII, and chloride has slight destabilizing effect on CA II and CA VII, and conversely – it stabilizes CA I and CA XIII. CA VII is not very sensitive to both salts (data is not shown).

Despite the fact that all CA isoforms are very homologous and have similar structure, their thermal stability varies: CA VII has lower stability and CA IX dimer is far more stable than its monomer (oligomeric forms of CA IX will be discussed below in greater detail).

Determination of the protein unfolding enthalpy (DSC, acid-ITC) Thermodynamic parameters of protein unfolding (ΔH and ΔC_p) were determined by DSC (Fig. 1**A**), and acid-ITC [7*] (Fig. 1**B**) – methods, that allow determination of protein unfolding enthalpy ΔH. DSC also identifies protein melting temperature T_m.

Both methods – DSC and acid-ITC – provide protein unfolding enthalpies that linearly depend on protein melting temperature (Fig. 2). Therefore, it is likely that both thermal and acid denaturation of carbonic anhydrases result in a similar denatured state of the protein.

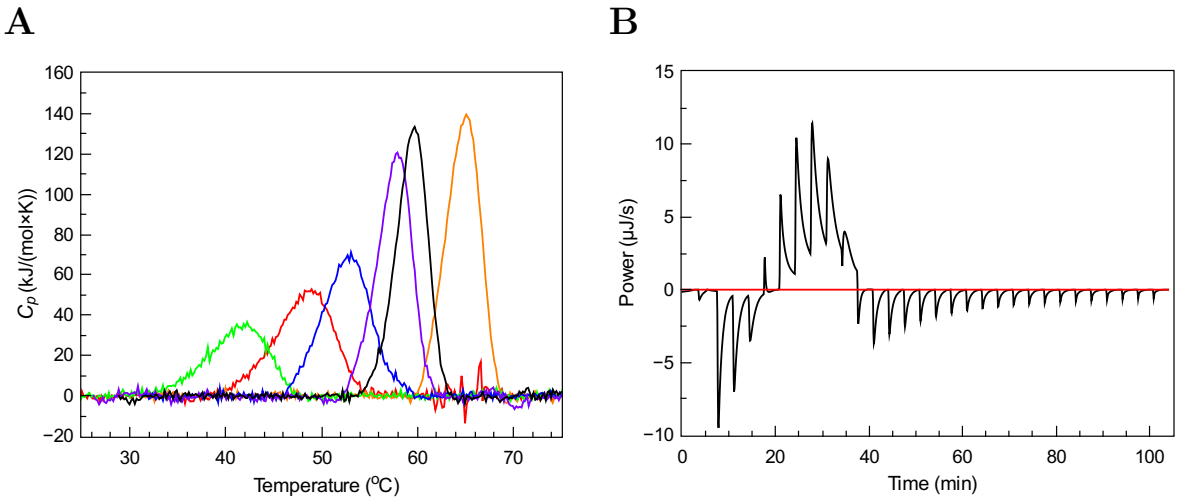

Figure 1. Examples of raw data of protein denaturation enthalpy determination: **A** – DSC, **B** – acid-ITC. **A** – 50 μM CA XIII denaturation at pH 4.5 (green), 4.75 (red), 5.0 (blue), 5.5 (purple), 6.5 (black) in universal buffer mix, and orange line – with 500 μM of p-carboxybenzensulfonamide in 50 mM phosphate with 50 mM NaCl at pH 7.0. **B** – CA XIII acid-ITC. First four endothermic injections show acid binding to protein, 5–10 injections show protein unfolding (endothermic process), next exothermic injections correspond to acid dilution process.

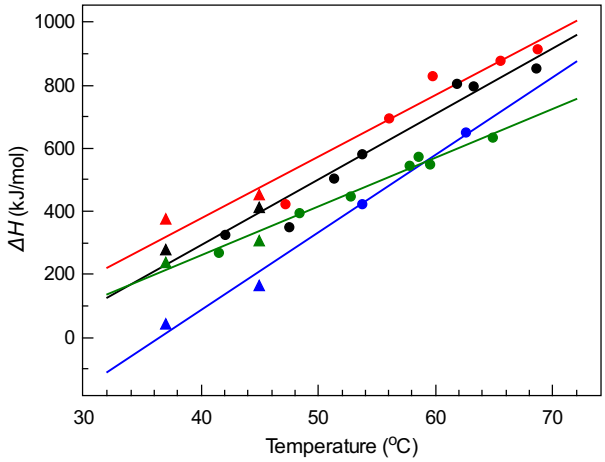

Figure 2. The dependence of carbonic anhydrase unfolding enthalpies on temperature: CA I (black), CA II (red), CA VII (blue), and CA XIII (green). Circles represent DSC data, triangles – acid-ITC data. Lines represent linear regression of experimental data derived by least squares method.

The estimated protein unfolding heat capacities (ΔC_p) are: CA I 20.8, CA II 19.6, CA VII 24.6, CA XIII 15.5 J/(K×mol). The results are within the range of literature data where statistical distribution of heat capacities on the protein chain length are presented [5]: an average presumable ΔC_p for ∼260 amino acid protein should be about 16 kJ(K×mol).

CA IX biochemical characterization

CA IX characterization was performed in collaboration with scientists from Finland, Italy and Slovakia. CA IX is exceptional carbonic anhydrase isoform – its expression significantly increases in various tumors [6].

Mature CA IX form is a transmembrane protein composed of a proteoglycan domain (PG), a CA catalytic domain, a transmembrane helix (TM), and a short intracellular tail in the C-terminus (Fig. 3 **A**). The signal peptide (SP) is cleaved during protein production in the cell.

Recombinant CA IX was produced in an insect (*Spodoptera frugiperda*) cell line using baculovirus expression system Bac-to-Bac® by Prof. S. Parkkila group, at University of Tampere. Two protein constructs were used [5*]: CA IX (Fig. 3 **B**) composed of proteoglycan and catalytic domains, and sCA IX (Fig. 3 **C**) which has only the catalytic domain and a C-terminal His-tag.

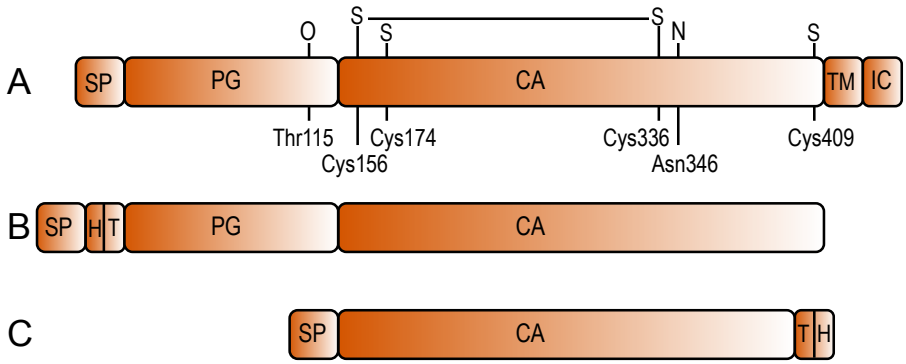

Figure 3. Schematic figure of human CA IX and the recombinant CA IX proteins used in this study. **A** – full legth human CA IX, **B** and **C** – recombinant forms used in this study, SP – signal peptide which is cleaved during protein maturation, PG – proteoglycan domain, CA – catalytic domain, TM – transmembrane domain, IC – intracellular tail, H – polyhistidine tag, T –thrombin cleavage site.

The thermal shift analysis of CA IX samples yielded double transitions. This indicates that either denaturation of this protein is a double step process or the sample is a mixture of two components that differ in their stability.

SDS-PAGE under reducing and non reducing conditions revealed that both CA IX and sCA IX samples were mixtures of monomer and covalently linked oligomer forms. Figure 4 shows SDS-PAGE of sCA IX (left) and CA IX (right) samples with different amounts of the reducing agent. Densitometric analysis showed that the initial CA IX sample was composed of ∼60 % oligomer and 34 % monomer, and sCA IX of 54 % oligomer and 46 % monomer respectively. According to molecular weight standards in SDS-PAGE, the oligomeric form of sCA IX is a dimer (theoretical MW of monomer is 29598.4, observed MWs are ∼30 kDa and ∼60 kDa). However, the observed MW of CA IX monomer differs from theoretical (∼50 kDa vs 41.1 kDa) and the oligomeric form could be a trimer (observed MW>120kDa).

Two oligomeric states of CA IX were separated by analytical gel filtration (Fig. 5 **A**). Observed molecular weights according to gel filtration standards

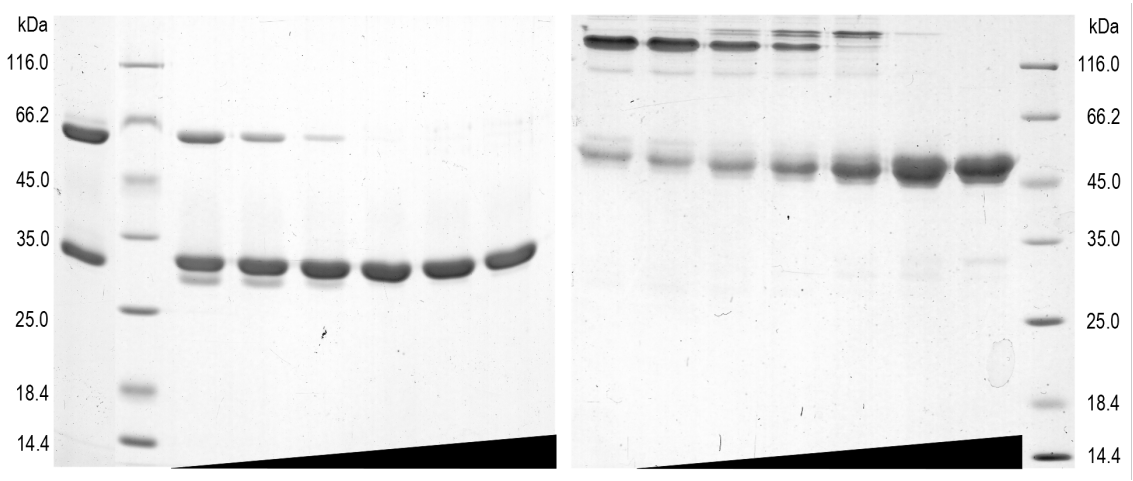

Figure 4. Influence of DTT to electrophoretic mobility of sCA IX (left) and CA IX (right). Black triangles indicate increasing concentration of DTT.

were 297 kDa and 115 kDa of the first and second peak, respectively. Mass spectrometric analysis (Italy) results indicated the molecular weights of 86 kDa and 43 kDa. Therefore, during gel filtration, the first peak corresponds to dimer and the second – to monomer. Exaggerated molecular weights observed in SDS-PAGE and especially in gel filtration analysis are likely to be caused by a non-globular structure of the proteoglycan domain of CA IX.

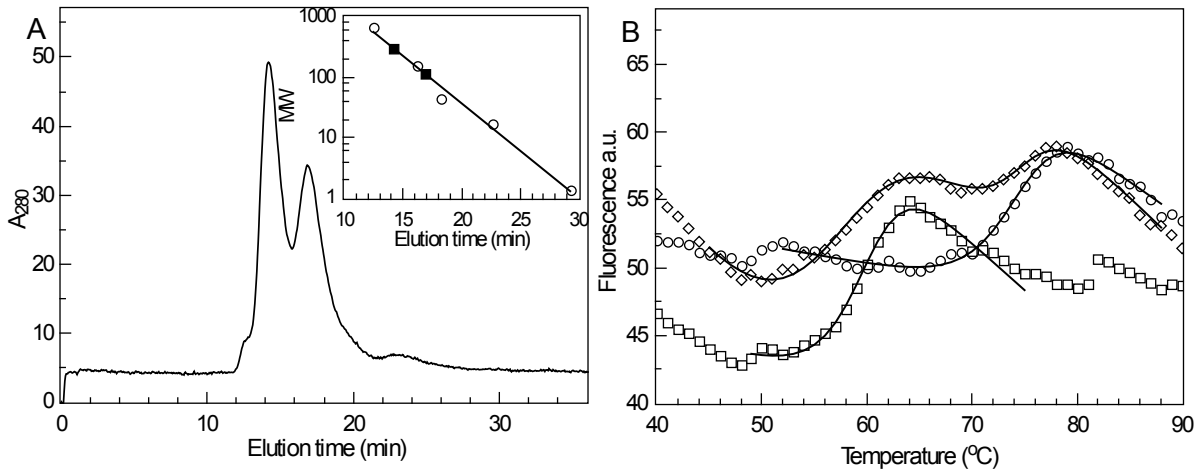

Figure 5. Separation of monomers and dimers by gel filtration. **A** – chromatography data, **B** – thermal shift assay curves of (○) first peak (dimer), (□) second peak (monomer) and (⋄) the unseparated sample.

Separation of monomer and dimer forms was also confirmed by SDS-PAGE under reducing and non-reducing conditions (data not shown) and the thermal shift analysis (Fig. 5 **B**).

Due to sufficiently different stability between CA IX oligomeric forms, it was possible to use the TSA method for the determination of ligand binding constants to both the monomeric and dimeric forms in the same sample.

Application of carbonic anhydrases for the development of TSA method

During the analysis of different protein-ligand bindings, it was realized that the currently used model for thermal-shift analysis does not fully describe some processes, such as protein destabilization by ligand, or doubling of the transitions in the a protein melting curve. Therefore, the model was expanded and the illustrating experimental results are shown using carbonic anhydrase as a model protein.

CA II-EZA as a model system for analysis of strong interactions

Direct ITC titration cannot be used to determine the binding constants of extremely tight interactions ($K_b > 10^9$ M^{-1}). In such cases, the displacement method is used. However, this method has a lot of limitations, mainly because of the requirement to have a weakly binding ligand competing for the same position with the ligand of interest [7].

The change in protein stability upon ligand binding can be used to estimate the binding affinity [8]. The TSA, as well as DSC, can be used to measure protein melting temperature. Moreover, TSA is a fast and efficient method for high-throughput screening [9–11].

When ethoxzolamide (EZA) binds to CA II, and ligand concentration is lower than the protein concentration, the melting curve in TSA experiment is untypical – the transition doubles (Fig. 6 A).

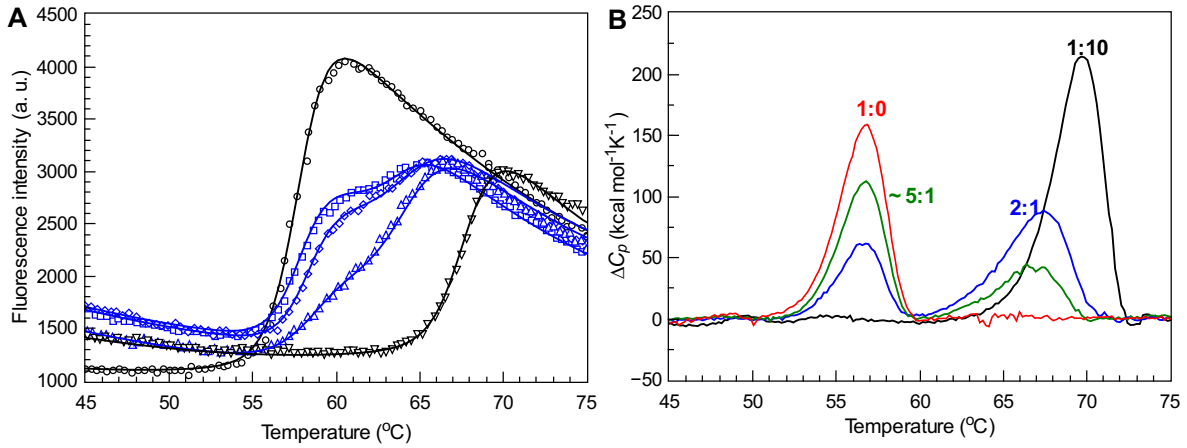

Figure 6. Separation of protein and protein–ligand transitions: **A** – TSA data. CA II binding to EZA in 50 mM phosphate buffer with 50 mM NaCl, pH 7.0. Protein concentration 5 µM, ligand concentration: (○) – 0 µM, (□) – 1.5 µM, (◇) – 2 µM, (△) – 3 µM, (▽) – 50 µM. Symbols correspond to experimental data points, lines – model, described by 5 equation. Blue lines show curves with double transition while black lines show conventional thermal denaturation curves with single transition. **B** DSC data. Protein concentration 100 µM, red line – CA II without ligand, green – with 22 µM EZA, blue – with 50 µM EZA, black – with 1 mM EZA. The numbers list the protein:ligand ratio.

When the same system is analysed by the DSC method, two protein unfolding peaks are observed: 1) at the unliganded protein unfolding T_m, and 2) at higher

temperature, the unfolding transition of the protein–ligand complex. The relative size of the peaks changes proportionally to the added protein:ligand ratio (Fig. 6 B). Separation of protein and the protein–ligand complex denaturation into two separate peaks was described by J. F. Brandts and L.-N. Lin [8]. By analogy to DSC data, the two transitions in the TSA melting curve were attributed to protein and protein–ligand melting. For description of the double transition Equation 5 was derived [4*]:

$$y(T) = y_N + (y_U - y_N)((1-n)P_U + nP_U^L) \tag{5}$$

Where y_N and y_U are fluorescence intensities of native and unfolded protein respectively. The fluorescence intensities depend on temperature. P_U is the probability for protein to be unfolded. P_U^L is the probability for the protein–ligand complex to be unfolded. Parameter n varies from 0 to 1 and indicates the fraction of ligand bound protein.

When strong binding of protein and ligand is analysed in a TSA experiment, two distinct fractions of different stability are separated: protein and protein–ligand complex (Fig. 7). Using this method for the CA II–EZA system, K_d ∼2 nM was obtained. Literature lists the K_i =8 nM as measured by the stop-flow method [12].

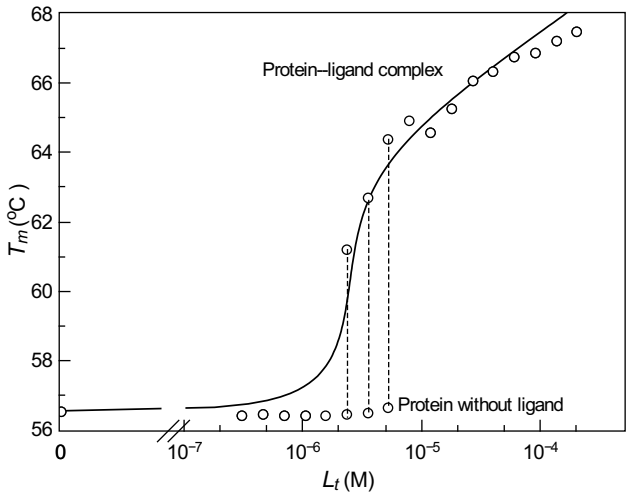

Figure 7. CA II–EZA TSA results – determination of the binding constant. Dashed lines connect data points which are obtained from the double transitions. Protein concentration used is 10 µM. When ligand concentration exceeds the protein concentration, only the denaturation of the protein–ligand complex is observed.

The application of the extended model enables significantly more precise determination of the binding constants and the protein concentration.

Ligand effect on protein stability: stabilization and destabilization

Most ligands stabilize proteins upon binding, i.e. increase the protein T_m. However there are some ligands that have the opposite effect – they destabilize proteins. The traditional TSA model [3] does not consider this option. Therefore,

for the analysis of destabilizing ligands, TSA model was extended. Protein destabilization was described as ligand binding to unfolded protein. In the extended model, ligand (L) can bind both to the native (N) and unfolded (U) protein:

$$UL \overset{K_{bU}}{\rightleftharpoons} U + L \overset{K_U}{\rightleftharpoons} N + L \overset{K_{bN}}{\rightleftharpoons} NL, \tag{6}$$

where K_U is the equilibrium constant of protein unfolding, and K_{bU} and K_{bN} are ligand binding constants to the unfolded and native protein, respectively.

The equation describing this extended model was derived as [6*]:

$$L_t = (1 - K_U)\left(\frac{P_t}{2}\frac{K_{bN} + K_{bU}K_U}{K_U(K_{bU} - K_{bN})} + \frac{1}{K_U K_{bU} - K_{bN}}\right) \tag{7}$$

here L_t is the total added ligand concentration. This model is only valid when protein and ligand binds with 1:1 stoichiometry.

When ligand binds primarily to the native protein ($K_{bU} \longrightarrow 0$), a stabilization phenomenon is observed, described by the classical TSA model (equation 4). When ligand binds to the unfolded protein ($K_{bN} \longrightarrow 0$), equation 7 is simplified to:

$$L_t = (1 - K_U)\left(\frac{P_t}{2} + \frac{1}{K_{bU}K_U}\right) \tag{8}$$

This equation can be used to evaluate the effect of such non-specific ligands as malate, citrate, and isocitrate on the stability of CA II (Fig. 8 **A**).

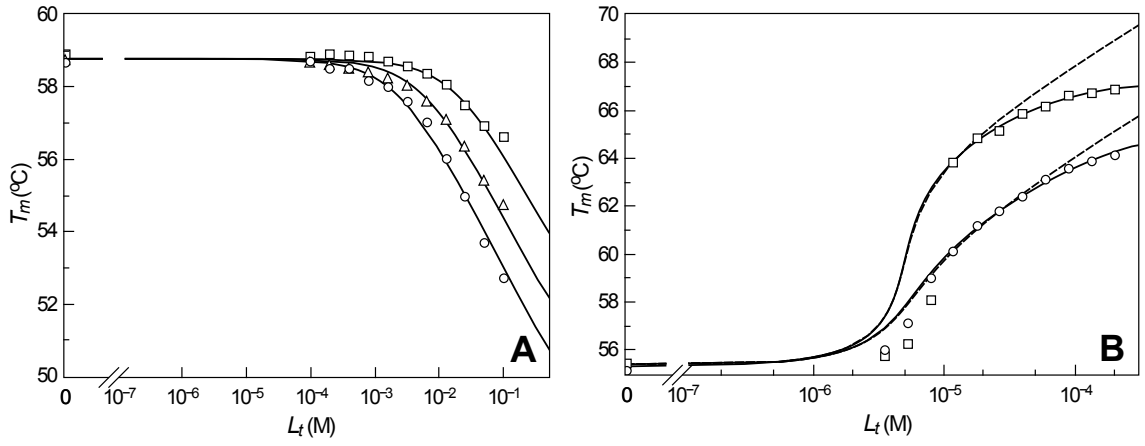

Figure 8. TSA results. **A** – CA II destabilization by: (□) malate ($K_d \sim 7.7$ mM), (△) isocitrate ($K_d \sim 2.3$ mM), and (○) citrate ($K_d \sim 0.9$ mM), **B** – CA II stabilization by: (□) EZA ($K_d \sim 2$ nM) and (○) TFMSA ($K_d \sim 1.2$ µM). Solid line represents the expanded TSA model, while the dashed line – the classical TSA model.

The expanded TSA model, that includes both the stabilization and destabilization, can also be applied to describe the saturation effect. This effect is observed when T_m increases to a lower extent than predicted by the classical TSA method (Fig. 8 **B**). If the saturation effect is observed, the binding constants

are underestimated by applying the classical TSA model. Other methods then yield significantly tighter binding constants. In the case of EZA and TFMSA binding to CA II, the classical model yields the K_b values 1×10^8 M^{-1} and 8×10^6 M^{-1}, respectively, while the expanded model yields the $K_{bN} = 1.3 \times 10^8$, $K_{bU} = 1.2 \times 10^5$ for EZA and $K_{bN} = 9.2 \times 10^6$, $K_{bU} = 2.8 \times 10^4$ for TFMSA.

Analysis of novel ligand binding to carbonic anhydrases

In this study, 40 novel primary sulfonamide group bearing compounds were synthesized at Vilnius University and tested as carbonic anhydrase inhibitors.

4-[*N*-(Substituted 4-pyrimidinyl)amino]benzenesulfonamides

Binding of 16 compounds synthesized by dr. Jurgis Sūdžius, Department of Organic Chemistry, Faculty of Chemistry, Vilnius University, to human carbonic anhydrase isoforms I, II, VII, and XIII was measured by ITC and TSA methods [2*]. The general formula of tested compounds is presented in figure 9, while the substitutes R, R^1, R^2, and linker length according to number of CH_2 groups n are listed in table 2.

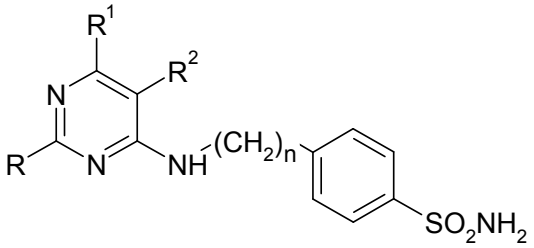

Figure 9. The general structure of 4-[*N*-(Substituted 4-pyrimidinyl)amino]benzenesulfonamides.

The binding affinities expressed as dissociation constants K_d, determined by ITC and TSA methods, are presented in table 3. The strongest binding was observed for compounds **12** and **13**. Unfortunately, they appeared to have some stability issues. It was determined that in DMSO these compounds undergo nucleophilic substitution during which Cl is substituted by O. For example, compound **12** converts to compound **14**. Compound **14** is less efficient binder but has some selectivity towards CA XIII. All analysed compounds (except for compound **7**) of this series are of comparable or better affinity towards tested CA isoforms as compared to the standard inhibitor acetazolamide (AZM). Compounds **8** and **9** are quite unique by their ability to bind CA I with higher affinity than CA II. Most common non-selective inhibitors usually bind stronger to CA II than to CA I.

Crystallographic analysis of carbonic anhydrase II complexed with ligands was carried out in dr. Saulius Gražulis group (Department of Protein–Nucleic Acid Interactions, lead by Prof. Virginijus Šikšnys, Institute of Biotechnology, Vilnius University). Compounds with different linker lengths were analysed (Fig. 10).

The benzene ring with the sulfonamide group is oriented in the same way in all protein-ligand complexes. The position of this ring is fixed by Val121, Thr200

Table 2. The linker length and R group substitutions of 4-[*N*-(substituted 4-pyrimidinyl) amino]benzenesulfonamides presented in figure 9.

Compound	Linker length n	R	R^1	R^2
1	0	SMe	Cl	CHO
2	1	SMe	Cl	CHO
3	2	SMe	Cl	CHO
4	0	SMe	Cl	CN
5	1	SMe	Cl	CN
6	2	SMe	Cl	CN
7	0	SMe	$NHCH_2Ph$	CN
8	0	H	$NHCH_2Ph$	NO_2
9	1	H	$NHCH_2Ph$	NO_2
10	2	H	$NHCH_2Ph$	NO_2
11	0	H	Cl	NO_2
12	1	H	Cl	NO_2
13	2	H	Cl	NO_2
14	1	H	O	NO_2
15	1	H	OMe	NO_2
16	2	H	OMe	NO_2

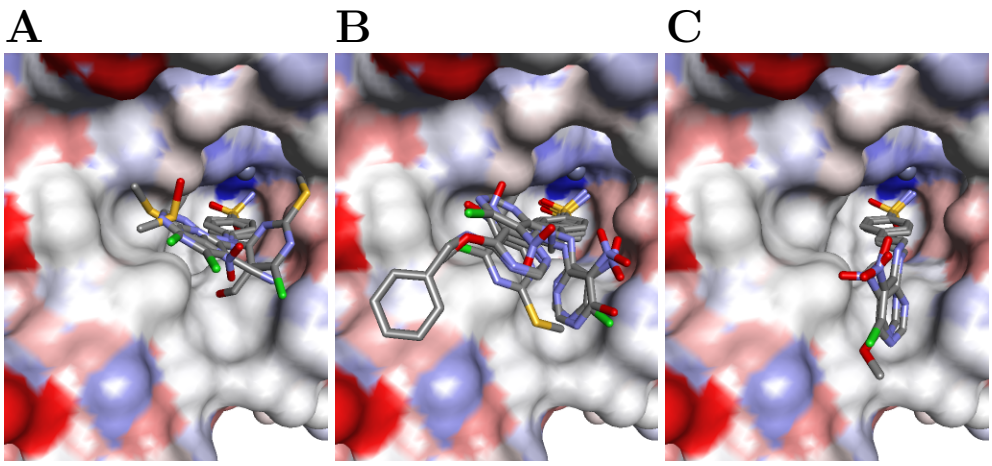

Figure 10. Orientation of 4-[*N*-(substituted 4-pyrimidinyl)amino]benzenesulfonamides in CA II active center: **A** – compounds with shortest linker (n = 0) between aromatic rings **1** (PDB ID 3MHO), **4** and **11** (3M40); **B** – compounds with intermediate length linker (n = 1) – **2** (3M5E), **12**, **14** (3MHI), **15** (3MHL) and **9** (3MHM); **C** – compounds with longest linker (n = 2) – **13** and **16** (3M3X).

and Leu198. Orientation of the other parts of ligand molecule is quite different. Even compounds with the same linker length exhibit variable orientation of the pyrimidine ring. When substitute R^1 is $NHCH_2Ph$, this ring is located outside of the active center.

Table 3. Binding affinities of compounds 4-[*N*-(substituted 4-pyrimidinyl)amino]benzenesulfonamides to human carbonic anhydrase isoforms. The K_ds (in µM) were determined by ITC and TSA methods in phosphate buffer, pH 7.0, 50 mM NaCl, at 37 ℃.

Compound	CA I		CA II		CA VII		CA XIII	
	K_d^{TSA}	K_d^{ITC}	K_d^{TSA}	K_d^{ITC}	K_d^{TSA}	K_d^{ITC}	K_d^{TSA}	K_d^{ITC}
1	1.0	2.8	0.17	0.32	4.0	ND	ND	ND
2	0.0071	0.083	0.024	0.043	0.10	0.10	0.028	0.13
3	0.11	0.48	0.11	0.35	1.0	0.77	0.33	0.19
4	1.4	20	0.071	0.22	0.83	1.7	0.095	0.12
5	0.10	ND	0.17	ND	0.10	ND	0.14	ND
6	0.33	ND	0.42	ND	0.10	ND	0.10	ND
7	100	ND	100	ND	3300	ND	100	ND
8	0.071	ND	0.17	ND	10	ND	0.50	ND
9	0.025	ND	0.10	ND	4.2	ND	0.33	ND
10	0.63	ND	0.016	ND	1.4	ND	0.50	ND
11	0.13	0.26	0.091	0.17	0.13	0.78	0.002	0.014
12*	0.013	0.099	0.0002	0.043	0.005	0.40	0.0001	0.01
13*	0.063	0.39	0.17	0.22	0.0017	0.41	0.0002	0.39
14	0.17	ND	0.20	ND	0.25	ND	0.020	ND
15	0.014	0.11	0.050	0.056	0.83	0.83	0.067	0.24
16	0.067	0.28	0.071	0.15	0.13	0.44	0.13	0.23
AZM	1.4	0.78	0.017	0.018	0.053	0.082	0.020	0.027

** marked compounds have stability issues when dissolved in DMSO*

Indapamide-like benzenesulfonamides

Benzensulfonamides substituted at positions 2-, 2,4- and 3,4 bind carbonic anhydrases with lower affinities as compared to 4-substituted derivatives [13]. However this feature could be employed in generating novel ligands selective towards one or another CA isoform. According to literature data [14, 15], indapamide is selective to CA VII, therefore indapamide-like compounds could also possess this property. Cl at position 2- of the benzene ring decreases the pK_a of the sulfonamide group.

15 new compounds (Fig. 11) synthesized by dr. Edita Čapkauskaitė at the Department of Biothermodynamics and Drug Design, Institute of Biotechnology, Vilnius University, were tested as CA ligands by measuring binding using TSA method to isoforms CA I, II, VII, and XIII. Selected compounds were also measured by ITC (table 4) [1*].

S-alkylated imidazo-derivatives **28**–**31** bound stronger (K_ds – 0.02–3.1 µM) than *N*-alkylated benzimidazoles **17**–**27** (K_ds – 0.3–40 µM). Compound **28** is best ligand for CA VII, and compounds **29**–**31** bind CA II most effectively. Indapamide did not show any selectivity towards CA VII by testing its binding by TSA and ITC contrary to literature data where inhibition data is presented [14].

Crystallographic analysis, performed by dr. Saulius Gražulis group, of these compounds in complexes with CA II showed that the benzene ring with the sulfonamide group is oriented in the same way as in all other tested compounds

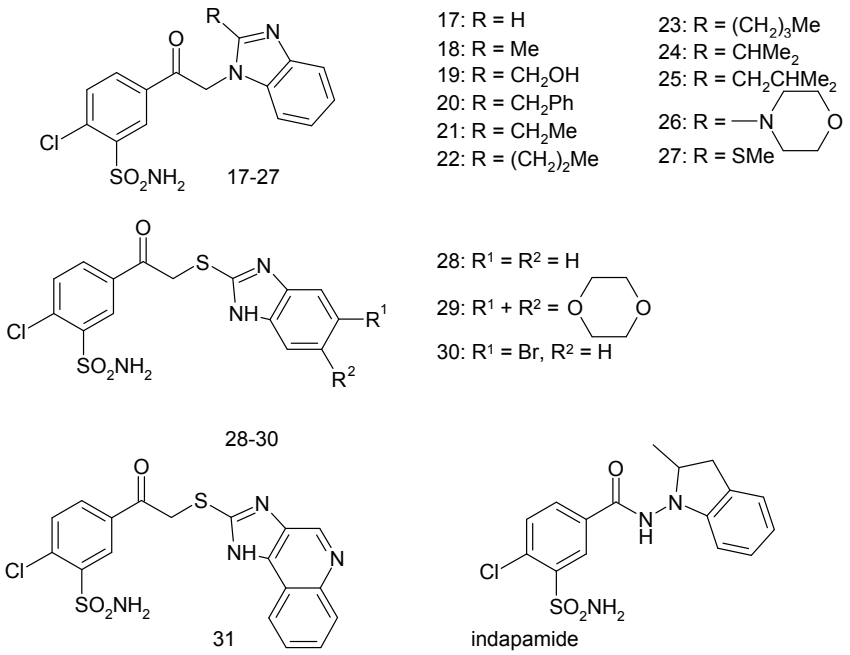

Figure 11. Structural formulas of indapamide-like CA ligands.

Table 4. Dissociation constants in μM, determined by TSA and ITC (in brackets) methods, at 37 ℃, in 50 mM phosphate buffer, pH 7.0, with 50 mM NaCl.

Compound	CA I	CA II	CA VII	CA XIII
17	11	1.6	1.0	0.7
18	10	2.0	2.5	0.4
19	7.1	1.0	0.4	0.3
20	8.3	1.6	3.3	1.0
21	7.1	0.6	1.7	0.4
22	10	0.8	2.5	1.4
23	10	0.5	3.3	0.4
24	4.5	0.7	2.0	0.4
25	3.3	0.4	1.0	2.9
26	40	4.2	10	12.5
27	5.0	1.0	1.7	1.1
28	1.0 (1.3)	0.1 (0.1)	0.03 (0.1)	0.2 (0.1)
29	3.1 (2.2)	0.02 (0.18)	0.1 (1.1)	0.1 (0.3)
30	1.3 (1.4)	0.06	0.1	0.1 (0.2)
31	1.3	0.03	0.1	0.1
Indapamide	10	0.3 (0.2)	0.3 (1.8)	0.1 (0.2)

(Fig. 12).

In compounds **17** and **29**–**31**, the oxygen atom of the linker's carbonile group makes hydrogen bonds directly or via water molecule to Asn67, Thr200, Gln92. Position of the heterocyclic ring is stabilized by His64 *in* conformation. Compound **31**'s S atom in the linker makes van der Waals contact to Asn62 (Fig. 12). Position of heterocyclic ring is similar in compounds **29**–**31** – it makes van der

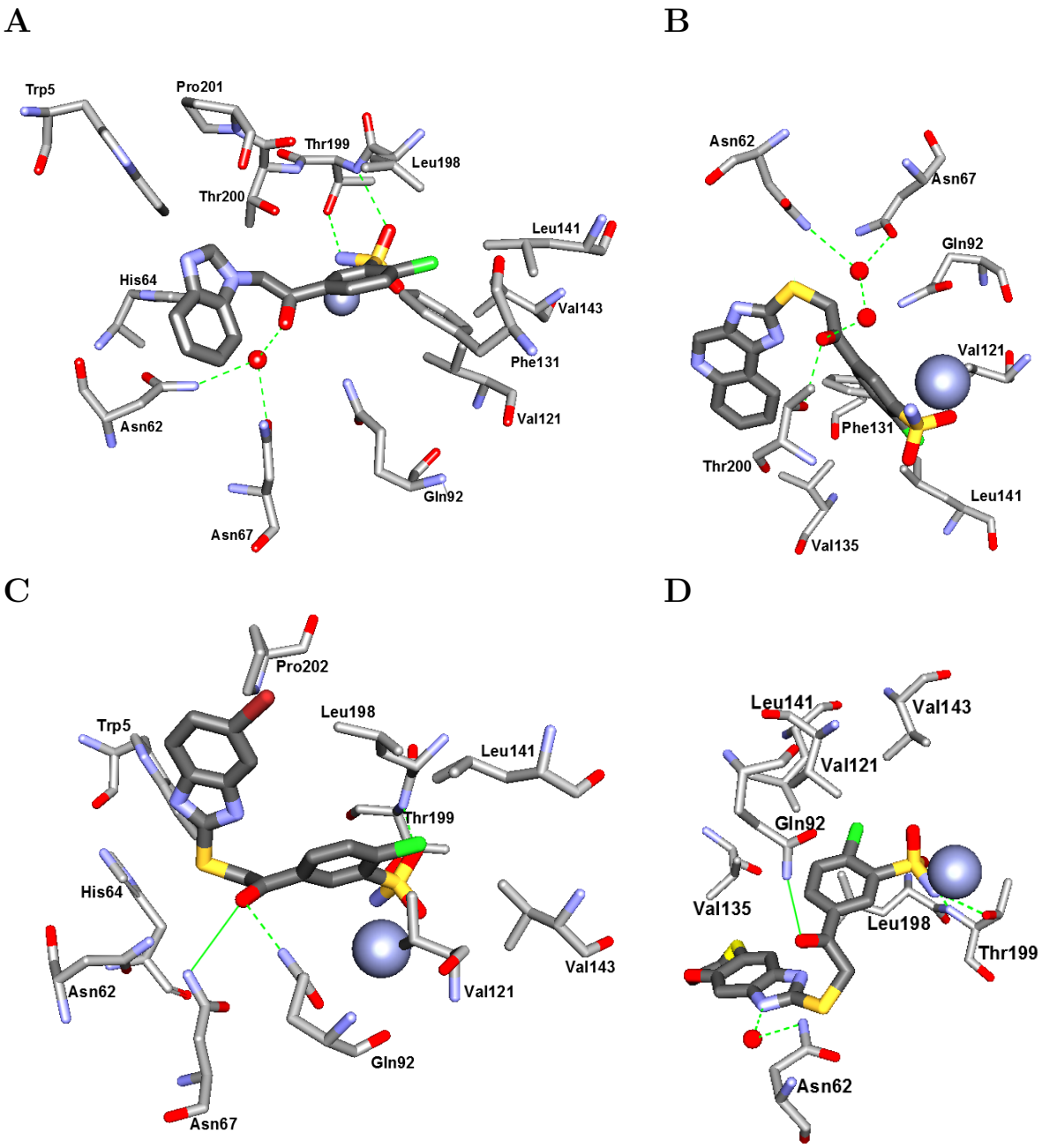

Figure 12. Crystallographic analysis of indapamide-like compounds in complexes with CA II: **A** – compound **17** (PDB ID 3M98), **B** – **31** (3MYQ), **C** – **29** (3M67), **D** – **30** (3M96).

Waals contacts with protein amino acids that form a hydrophobic cavity (Phe131, Val135, Pro202, Leu198 and Thr200). Compound **17**'s heterocyclic ring is surrounded by Asn67, Asn62, His64, Trp5 and Pro201 side chains. Compound **17** binds weaker (K_d = 1.6 µM) to CA II than compounds **29**–**31** (K_ds 20–60 nM).

Benzimidazo[1,2-*c*][1,2,3]-thiadiazole-7-sulfonamides

Benzimidazo[1,2-*c*][1,2,3]-thiadiazole-7-sulfonamides were synthesized by dr. Virginija Dudutienė at the Department of Biothermodynamics and Drug Design, Institute of Biotechnology, Vilnius University [8*]. The main feature of this class of

Table 5. Dissociation and inhibition constants (in µM) of ligand binding to CA I, CA II, and CA IX monomeric form (CA IX^M) and dimeric form (CA IX^D).

Compound	CA I			CA II			CA IX^M	CA IX^D	CA IX	
	K_d^{TSA}	K_d^{ITC}	K_i	K_d^{TSA}	K_d^{ITC}	K_i	K_d^{TSA}	K_d^{TSA}	K_d^{ITC}	K_i
32	0.82	0.84	0.66	0.19	0.41	0.033	0.85	0.22	0.22	1.00
33	0.17	0.39	0.21	0.07	0.18	0.009	3.0	0.27	0.59	0.21
34	0.02	ND	ND	0.038	ND	ND	0.89	0.044	ND	ND
35	0.13	0.40	0.15	0.05	0.054	0.057	1.5	0.17	0.63	0.51
36	2.0	2.6	2.1	0.20	0.22	0.46	1.4	0.35	0.26	0.72
37	0.13	0.27	ND	0.25	0.59	ND	1.5	0.64	0.12	ND
38	0.12	0.18	ND	0.33	0.09	ND	2.4	0.33	ND	ND
39	17	ND	ND	7.9	ND	ND	12	16	ND	ND
40	2.1	ND	ND	0.06	0.56	ND	1.7	0.33	ND	ND
AZM	1.4	0.78	0.25	0.017	0.018	0.012	0.12	0.11	ND	0.025

compounds is a rigid three-ring structure. The Zn^{2+} binding sulfonamide group is attached to the benzene ring. Substitutions are made in the thiadiazole ring (R^1) and the benzene ring (R) (Fig. 13).

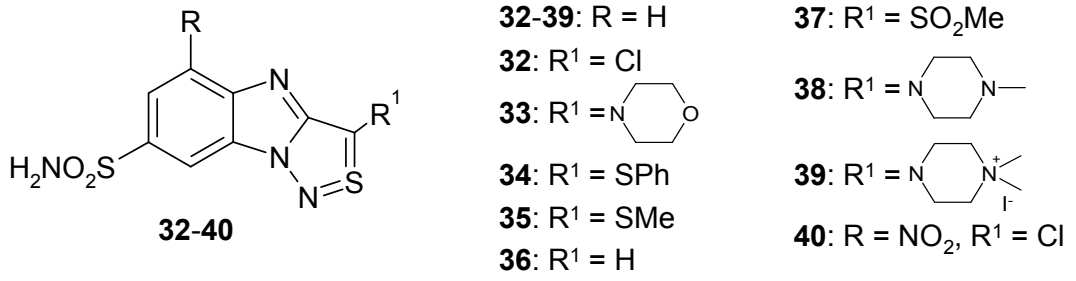

Figure 13. Structures of benzimidazo[1,2-*c*][1,2,3]-thiadiazole-7-sulfonamides.

Binding of these compounds to human CA I, II and IX was tested by TSA and ITC. Inhibition of CO_2 hydration was measured stop-flow technique by the Prof. Claudiu Supuran group in the Department of Chemistry, Laboratory of Bioinorganic Chemistry, Universitá degli Studi di Firenze, Italy [3*]. Results are presented in table 5 as dissociation and inhibition constants.

Most compounds were strongest binders of CA II (K_ds 0.04–7.9 µM). Using the TSA method, it was determined that dimeric CA IX form binds ligands stronger than the monomeric form. ITC and inhibition measurements were performed without the separation of oligomeric forms.

Crystallographic analysis revealed that in addition to the classical interaction of the lfonamide group with the CA, the rest of ligand molecule mainly interacts to protein by van der Waals contacts or through water molecules. Compound **40**, due to the substitution in the benzene ring has an altered position in comparison to other compounds, and makes one additional hydrogen bond between

thiadiazole N atom and Thr200 OH group. Compound **33** was observed in two alternative conformations (Fig. 14).

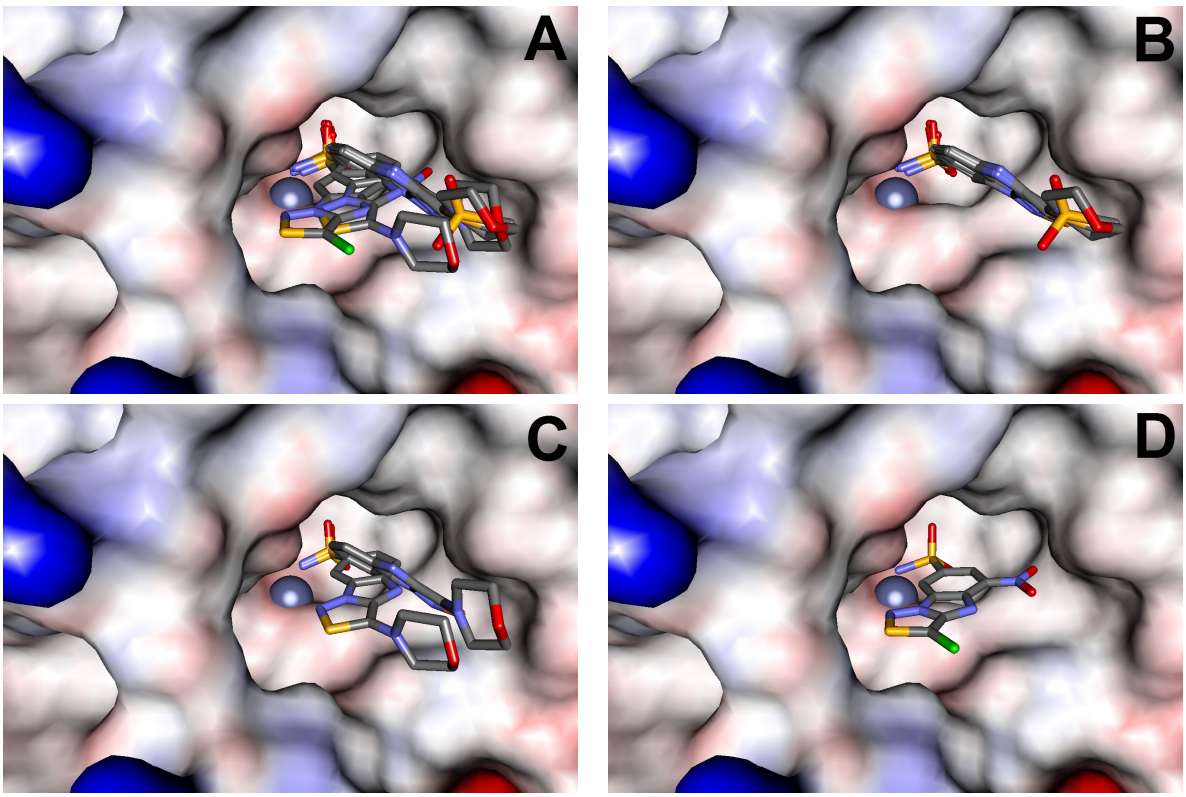

Figure 14. Position of benzimidazo[1,2-*c*][1,2,3]-thiadiazole-7-sulfonamides in CA II active center: **A** – compounds **33**, **35** (PDB ID 3HLJ), **37** and **40**, **B** – dominating position of heterocyclic system, **C** – alternative binding modes of compound **33**, **D** – compound **40** has different orientation in CA II active center.

Thermodynamic analysis of sulfonamide inhibitor binding to CA XIII

Three ligands (Fig. 15), bearing a sulfonamide group with different pK_a values were selected for this analysis [9*]: trifluoromethanesulfonamide (TFMSA) (pK_a 5.9–6.3 [16, 17]), ethoxzolamide (EZA) (pK_a 8.0–8.1 [18, 19]) and metolazone (MTZ) (pK_a 9.6 [20]).

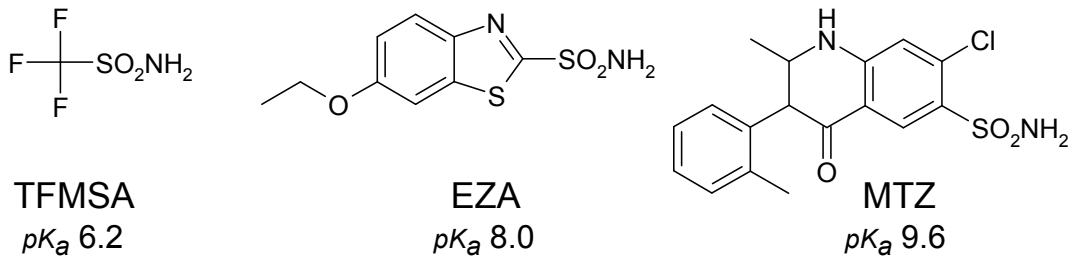

Figure 15. Ligands used for thermodynamic analysis of CA XIII binding to RSO_2NH_2.

Linked reactions

During sulfonamide ligand binding to carbonic anhydrase at least four different reactions take place (Fig. 16): 1) binding of deprotonated sulfonamide ligand to carbonic anhydrase when water molecule is coordinated to Zn^{2+} ion in its active center; 2) protonation of Zn^{2+} coordinated hydroxide ion; 3) deprotonation of ligand sulfonamide group; 4) compensating protonation or deprotonation of buffer.

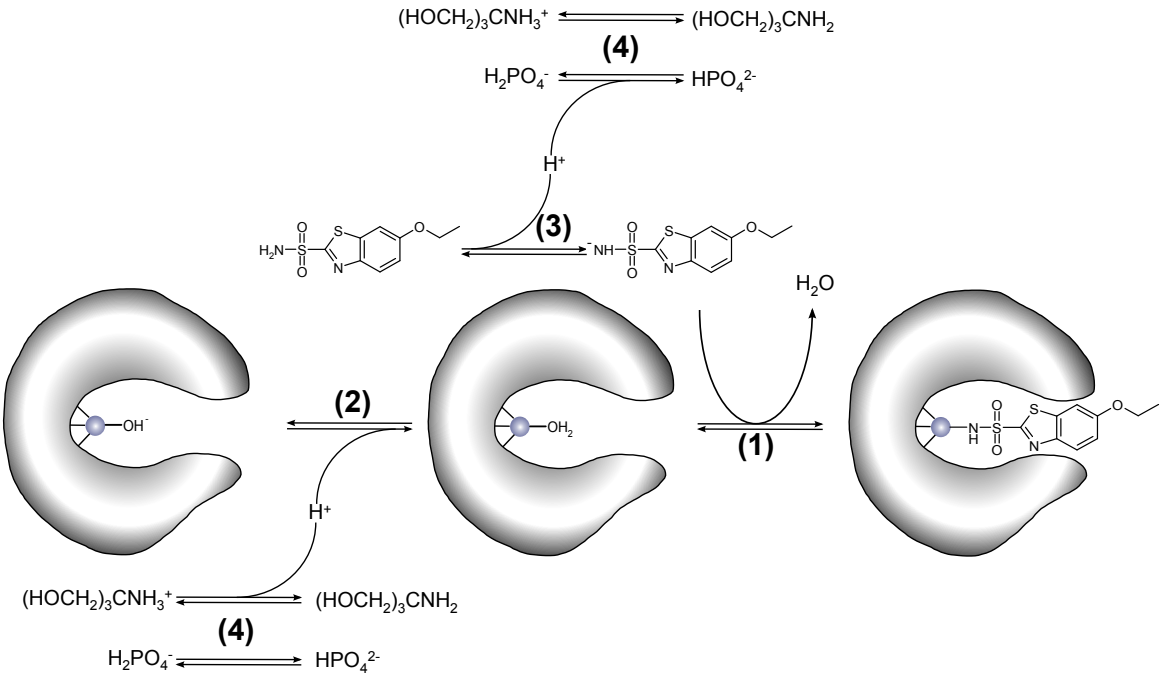

Figure 16. Linked reactions that take place when sulfonamide ligand binds to carbonic anhydrase XIII. Reactions denoted by numbers are listed in the text.

ITC or other biophysical methods measure the sum of all these reactions. However, for the QSAR analysis, only the intrinsic parameters (K_b, ΔH) are relevant.

Determination of the intrinsic binding constant

Intrinsic binding constant K_b depends on observed binding constant K_{b-obs} and the fractions of active reacting species (deprotonated sulfonamide and protonated CA):

$$K_b = \frac{K_{b-steb}}{f_{\mathrm{RSO_2NH^-}} f_{\mathrm{CAZnH_2O}}} \tag{9}$$

The fractions of active species depend on pH of the system and pK_a values:

$$f_{\mathrm{RSO_2NH^-}} = \frac{10^{pH-pK_{a-\mathrm{RSO_2NH_2}}}}{1+10^{pH-pK_{a-\mathrm{RSO_2NH_2}}}}, f_{\mathrm{CAZnH_2O}} = 1-\frac{10^{pH-pK_{a-\mathrm{CAZnH_2O}}}}{1+10^{pH-pK_{a-\mathrm{CAZnH_2O}}}} \tag{10}$$

When sulfonamide pK_a value is low (as in case of TFMSA), then $K_{b-obs} \approx K_b$, when this pK_a is higher, then $K_{b-obs} < K_b$ (Fig. pav:DH-obs).

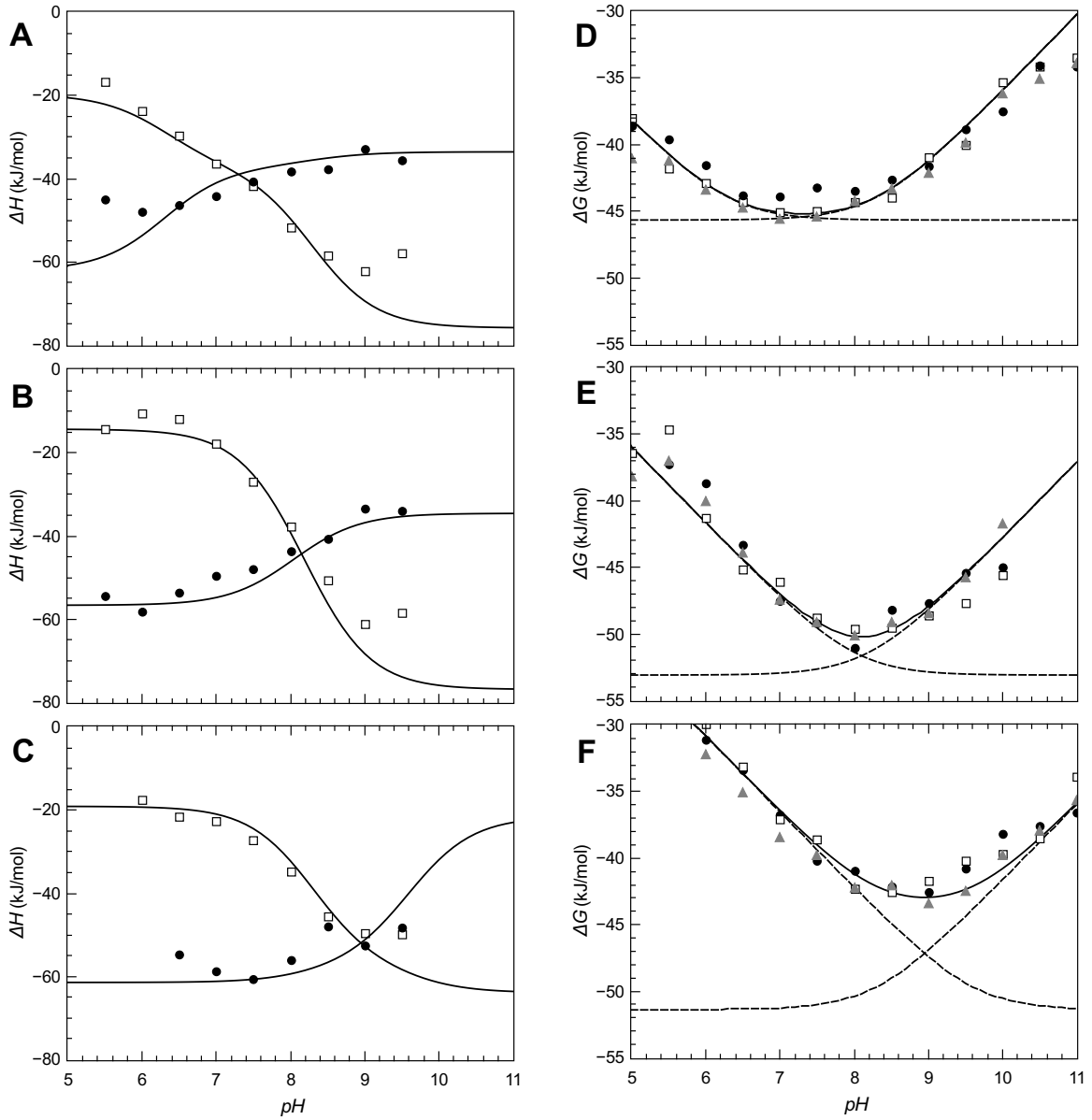

Figure 17. Observed thermodynamic parameters in different buffers as a function of pH. **A**, **B**, **C** – observed enthalpies and **D**, **E**, **F** – observed Gibbs free energies. Reactions carried out using ITC in tris are marked by (●), ITC in phosphate – by (□), and by TSA in universal buffer mix – with (▲). **A** and **D** are for CA XIII binding to TFMSA, **B** and **E** – CA XIII–EZA, **C** and **F** – CA XIII–MTZ.

Dissection of the observed binding enthalpy

The observed binding enthalpy ΔH_{obs}, measured by ITC, is equal to the sum of heat effects of all reactions, displayed in Fig. 16:

$$\Delta H_{obs} = \Delta H_b + n_{sulf}\Delta H_{\mathrm{RSO_2NH_2}} + n_{CA}\Delta H_{\mathrm{CAZnH_2O}} + n_{buf}\Delta H_{buf} \qquad (11)$$

where ΔH_b is the intrinsic binding enthalpy; $\Delta H_{\mathrm{RSO_2NH_2}}$ – the enthalpy of ligand sulfonamide group protonation; $n_{sulf} = f_{\mathrm{RSO_2NH^-}} - 1$ – the number of protons that are released upon ligand deprotonation; $\Delta H_{\mathrm{CAZnH_2O}}$ – the pro-

tonation enthalpy of hydroxide ion that is coordinated to zinc ion in CA active center; $n_{CA} = 1 - f_{\mathrm{CAZnH_2O}}$ – the number of protons that are used for Zn^{2+} coordinated OH^- protonation; ΔH_{buf} – the buffer protonation enthalpy; $n_{buf} = -(f_{\mathrm{RSO_2NH_2}} + f_{\mathrm{CAZnH_2O}})$ – the number of protons that are accepted or released by the buffer.

Determination of ligand pK_a and $\Delta H_{\mathrm{RSO_2NH_2}}$. The pK_a value of the ligand sulfonamide group can be determined by the potentiometric titration with acid or alkali. In this study, the literature values for the pK_a were used. However, $\Delta H_{\mathrm{RSO_2NH_2}}$ was measured by performing the same experiment using ITC (Fig. 18).

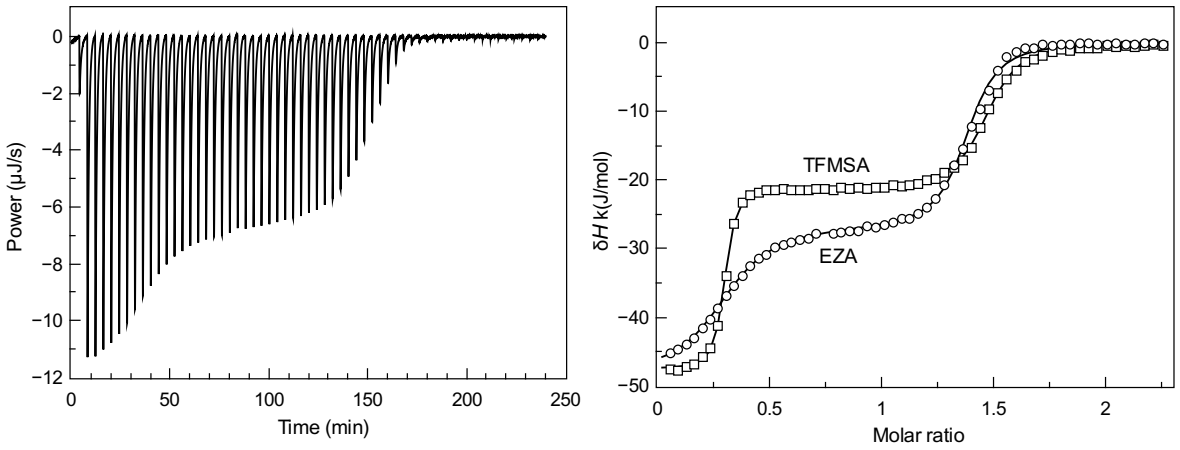

Figure 18. Determination of ligand sulfonamide group protonation enthalpy by ITC: **A** – EZA titration with HNO_3, in the presence of 1.5 eqv. NaOH, raw data, **B** – EZA (○) and TFMSA (□) titration with HNO_3, in the presence of 1.5 eqv. NaOH, integrated data.

The determined $\Delta H_{\mathrm{RSO_2NH_2}}$ values were: EZA -28.8 kJ/mol, TFMSA -22.4 kJ/mol, MTZ -11,0 kJ/mol.

Effect of buffer and pH on the observed enthalpy. When linked protonation reactions are present, the observed binding enthalpy is highly dependent on pH (Fig. 17). This parameter also differs if the same reaction is measured in buffers with different protonation enthalpy, even if the pH is the same (Fig. 19)

Determination of $\mathbf{CAZnH_2O}$ pK_a and $\Delta H_{\mathrm{CAZnH_2O}}$. These parameters cannot be determined by straightforward titration, therefore the method, proposed by Baker and Murphy [21] is used. The binding of the same system is measured in at least two buffers with different protonation enthalpies in a wide pH range (Fig. 17). $CAZnH_2O$ pK_a and $\Delta H_{\mathrm{CAZnH_2O}}$ are obtained by global fitting. The values were: $CAZnH_2O$ $pK_a = 8.3$ and ΔH = -44 kJ/mol. These values, once dissected for this protein, will be used for other ligand binding analyses with only the total binding enthalpy and the ligand protonation parameters required to be measured at a single pH.

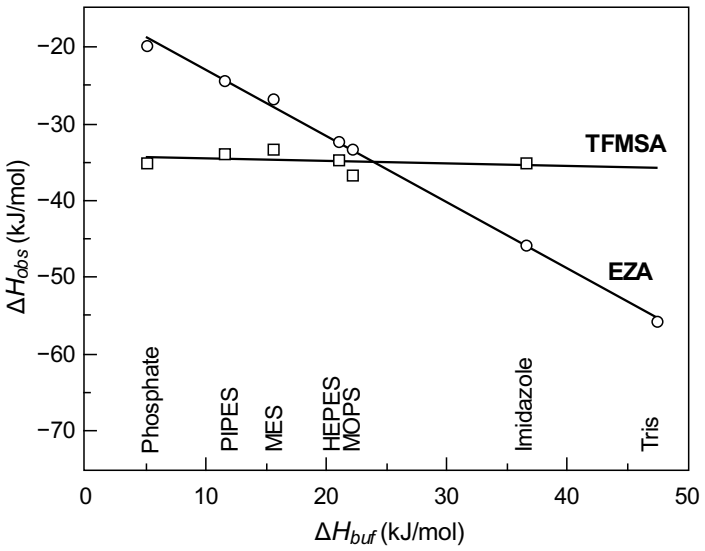

Figure 19. The observed enthalpy of CA XIII binding to EZA and TFMSA in buffers with different protonation enthalpy. At pH 7.0, 50 mM NaCl.

Intrinsic parameters of RSO_2NH_2 binding to CA XIII

Summary of the analysed reactions – CA XIII binding to TFMSA, EZA and MTZ – is presented in table 6. According to the observed binding constants, the strongest binding is between CA XIII and EZA (at pH 7.0 $K_b = 7.6{\times}10^9$ M^{-1}), and the weakest – CA XIII–MTZ (at pH 7.0 $K_b = 3.8{\times}10^6$ M^{-1}). The difference between observed parameters is about 2000 times, and the difference between calculated intrinsic parameters is only about 1.4 times. The main cause is the difference in the sulfonamide group pK_a value.

Table 6. Thermodynamic parameters of CA XIII binding to TFMSA, EZA and MTZ, and pK_a values. The enthalpy is in in kJ/mol, and binding constants – in M^{-1}.

	Compound parameters		Protein parameters		Intrinsic binding parameters	
	pK_a	$\Delta H_{RSO_2NH_2}$	pK_a	ΔH_{CAZnH_2O}	K_b	ΔH_b
TFMSA	6.2	-22.4			1×10^8	-37
EZA	8.0	-28.8	8.3	-44	1.4×10^9	-38
MTZ	9.6	-11.0			1×10^9	-25

Calculated intrinsic binding parameters (K_b and ΔH) are the only parameters that could be used for the correlation with the structural data.

Conclusions

1. The analysed carbonic anhydrase isoforms are stable at pH 5–9. The values of unfolding parameters (ΔH, ΔC_p) of these proteins are comparable to the literature data for proteins of similar size.

2. The recombinant human CA IX exists in solution as a covalently bound dimer.

3. Protein–ligand binding can be modelled both when stabilization and destabilization is observed using the TSA method. Destabilization can be described as ligand binding to the unfolded protein.

4. When the protein concentration exceeds the ligand concentration, the duplication of the protein unfolding transitions is observed. It can be described by a model where the sizes of transitions depend on protein–ligand added proportion.

5. 2-chloro-5-{[(substituted 2-imidazolyl)sulfanyl]acetyl}benzenesulfonamides, 2-chloro-5-[(2-substituted 1-benzimidazolyl)acetyl]benzenesulfonamides, 4-[*N*-(substituted 4-pyrimidinyl)amino]benzenesulfonamides, and benzimidazo[1,2-*c*][1,2,3]-thiadiazole-7-sulfonamides bind to human CA I, II, VII, IX, and XIII with comparable or better affinity as compared to drugs used for carbonic anhydrase inhibition.

6. CA XIII active site Zn^{2+} coordinating hydroxide ion protonation parameters – pK_a and enthalpy of protonation – were determined by applying the thermodynamic additivity principle. These parameters can then be used to calculate intrinsic binding parameters for any sulfonamide ligand binding to CA XIII from its observed binding constant and observed enthalpy of binding.

List of publications

The thesis is based on the following original publications:

[1*] E. Čapkauskaitė, **L. Baranauskienė**, D. Golovenko, E. Manakova, S. Gražulis, S. Tumkevičius, D. Matulis. (2010) Indapamide-like benzenesulfonamides as inhibitors of carbonic anhydrases I, II, VII, and XIII. *Bioorg Med Chem* 18:7357-64.

[2*] J. Sūdžius, **L. Baranauskienė**, D. Golovenko, J. Matulienė, V. Michailovienė, J. Torresan, J. Jachno, R. Sukackaitė, E. Manakova, S. Gražulis, S. Tumkevičius, D. Matulis. (2010) 4-[N-(substituted 4-pyrimidinyl)amino]-benzenesulfonamides as inhibitors of carbonic anhydrase isozymes I, II, VII, and XIII. *Bioorg Med Chem* 18:7413-21.

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International patent:

[1] D. Matulis, V. Dudutienė, J. Matulienė, **L. Mištinaitė**. Benzimidazo[1,2-*c*][1,2,3]-thiadiazol-7-sulfonamides as inhibitors of carbonic anhydrase and the intermediates for production thereof. EP2054420 ir WO/2008/016288.

Other publications:

[1] E. Čapkauskaitė, A. Zubrienė, **L. Baranauskienė**, G. Tamulaitienė, E. Manakova, V. Kairys, S. Gražulis, S. Tumkevičius, D. Matulis. (2012) Design of [(2-pyrimidinylthio)acetyl]benzenesulfonamides as inhibitors of human carbonic anhydrases. *Eur J Med Chem* 51, 259-270.

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Conference presentations:

[1] 2012 June 26–29, Amsterdam, Netherlands. The 13^{th} Tetrahedron symposium. Synthesis of S-alkylated Benzimidazole and Imidazole Derivatives as Inhibitors of Carbonic Anhydrases. E. Čapkauskaite, J. Gylytė, A. Zubrienė, **L. Baranauskienė**, A. Smirnov, D. Golovenko, E. Manakova, S. Gražulis, S. Tumkevičius, D. Matulis.

[2] 2012 April 11–15, Antalia, Turkey. The 9^{th} International Conference on Carbonic Anhydrases. Design of [(2-Pyrimidinylthio)acetyl]benzene-sulfonamides as inhibitors of human carbonic anhydrases. E. Čapkauskaitė, A. Zubrienė, **L. Baranauskienė**, E. Manakova, G. Tamulaitienė, J. Kazokaitė, V. Kairys, S. Gražulis, S. Tumkevičius, D. Matulis.

[3] 2011 September 7–10. Craiova, Romania. The 1^{st} Central and Eastern European Conference on Thermal Analysis and Calorimetry. Drug Binding Energetics by Titration Calorimetry, Thermal and Pressure Shift Assay. A. Zubrienė, **L. Baranauskienė**, E. Kazlauskas, Z. Toleikis, D. Matulis.

[4] 2011 July 31–August 4, Glasgow, Scotland. 23^{rd} International Congress on Heterocyclic Chemistry. Synthesis of Pyrimidine Derivatives as Inhibitors of Carbonic Anhydrase. E. Čapkauskaitė, A. Zubrienė, **L. Baranauskienė**, G. Tamulaitienė, E. Manakova, S. Gražulis, S. Tumkevičius, D. Matulis.

[5] 2011 June 22–24, Montecatini, Italy. FEBS satellite CA meeting. Inhibitor binding thermodynamics and stability characterization of human carbonic anhydrases. V. Jogaitė, A. Zubrienė, **L. Baranauskienė**, V. Dudutienė, E. Čapkauskaitė, V. Michailovienė, J. Gylytė, H. Šebėka, D. Matulis.

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[10] 2010 November 22–25, Brno, Czechia, COST Action TD0905 Epigenetics – Bench to Bedside. Drug Binding Energetics by Titration Calorimetry, Thermal and Pressure Shift Assay. A. Zubrienė, **L. Baranauskienė**, E. Kazlauskas, Z. Toleikis, R. Chaleckis, V. Michailovienė, V. Petrikaitė, E. Čapkauskaitė, V. Dudutienė, J. Matulienė, D. Matulis.

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Santrauka

Šio darbo objektas – žmogaus karboanhidrazės (CA) – fermentai, katalizuojantys virsmus tarp anglies dioksido ir bikarbonato. Karboanhidrazių slopinimas gali būti taikomas gydyti tokias skirtingas ligas kaip glaukoma, vėžys, nutukimas, epilepsija, osteoporozė ir kt. Šiuo metu yra beveik 30 mažamolekulinių junginių, kurie naudojami kaip vaistai, su padidėjusiu karboanhidrazių aktyvumu susijusioms ligoms gydyti. Daug demesio skiriama siekiant sukurti savitus skirtingų karboanhidrazių izoformų slopiklius, kurie veiktų tik pasirinktą taikinį, tuo būdu tikintis sumažinti nepageidaujamus šalutinius poveikius. Turint omeny didelį karboanhidrazių izoformų skaičių, jų struktūrinį panašumą bei gana platų daugelio izoformų paplitimą įvairiuose audiniuose, tokių junginių sukūrimas yra itin sudėtingas.

Šio darbo metu buvo tiriama rekombinantinių žmogaus karboanhidrazių I, II, VII, IX ir XIII sąveika su sulfonamidinę grupę turinčiais ligandais. Baltymų–ligandų sąveikos bei baltymų stabilumo tyrimams taikyti biofizikiniai metodai – izoterminio titravimo klorimetrija, diferencinio skenavimo kalorimetrija, terminio poslinkio analizė.

Išmatuoti 40-ies naujų cheminių junginių sąveikos su karboanhidrazėmis termodinaminiai parametrai – dauguma tirtų junginių yra efektyvūs tirtų karboanhidrazių izoformų ligandai / slopikliai. Taip pat nustatyti tirtų baltymų stabilumo termodinaminiai parametrai, kurie yra artimi literatūroje nurodomoms vertėms panašaus dydžio baltymams. Nustatyta, kad priešvėžinis taikinys CA IX tirpale yra disulfidiniais ryšiais sutvirtintas dimeras.

Rekombinantinės žmogaus karboanhidrazės panaudotos modeliniais baltymais vystant terminio poslinkio analizės metodą. Praplėstos šio metodo taikymo ribos, įvedant galimybę analizuoti baltymo stabilumą mažinančių ligandų jungimąsi bei paaiškinti tranzicijos dvigubėjimą baltymo denatūracijos kreivėje, kai baltymas stipriai jungiasi su ligandu.

Taikant termodinaminio adityvumo metodą žmogaus CA XIII jungimosi su sulfonamidiniais ligandais analizei, įvertinta susijusių protonizacijos reakcijų įtaka stebimiesiems termodinaminiams parametrams ir apskaičiuoti tikrieji jungimosi parametrai.

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