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Dissertation theses

Structure, Function and Inhibition of Human Carbonic Anhydrases

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Abstrakt:

Lidské karbonické anhydrasy jsou zinkové metaloenzymy hrající klíčovou roli v řadě fyziologických a patofyziologických procesů. CA IX je transmembránový isoenzym asociovaný s řadou lidských nádorů. V práci jsou prezentovány vysledky exprese, purifikace a krystalizace CA IX spolu se studiem struktury isoenzymu CA II v komplexu s novou třídou nízkomolekulárních inhibitorů. Dále jsou předmětem této práce strukturní studie Fab fragmentu monoklonální protilátky M75 a jeho komplexu s epitopovým peptidem nacházejícím se v unikátní proteoglykanu podobné doméně CA IX.

Klíčová slova: nádory, karbonická anhydrasa, inhibitory karbonických anhydras, krystalová struktura Fab, rozpoznávání antigenu protilátkou

Abstract:

Human carbonic anhydrases are zinc metalloenzymes playing a key role in several physiological and pathophysiological processes. CA IX is a tumor associated transmembrane isozyme representing a valuable therapeutic target. Results concerning expression, purification, and crystallization of CA IX as well as structural studies of CA II in complex with novel class of small molecular inhibitors are presented. Furthermore, structural studies of Fab fragment of monoclonal antibody M75 and its complex with epitope peptide derived from unique proteoglycan-like domain of CA IX are part of this work.

Keywords: cancer, carbonic anhydrase, carbonic anhydrase inhibitors, Fab crystal structure, antigen-antibody recognition

1 Introduction

Human carbonic anhydrases (CA, EC 4.2.1.1) are zinc metalloenzymes playing a key role in several physiological and patho-physiological processes. To date, fifteen human CA isozymes, displaying differences in activity, subcellular localization, and tissue expression profiles have been identified.

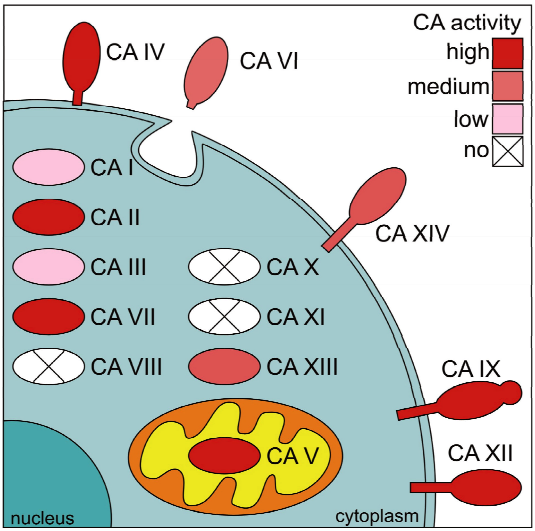

Figure 1 Enzymatic activity, subcellular localization and domain composition of human CAs. *CA IX is the only carbonic anhydrase with an N-terminal proteoglycan-like sequence, engaged in cell-to-cell adhesion. Figure was adapted from Pastorekova & Zavada, 2004.*

Human CA IX isozyme, discovered by team led by Dr. Jan Závada (Pastorekova *et al.*, 1992), is a special member of the family. Under normal conditions, its physiological expression is limited to a very narrow range of tissues. However, this membrane-bound isozyme is highly overexpressed on the cell surface of a variety of solid malignant tumors derived from kidney, cervix, uteri, colon, lung, oesophagus and breast (Zavada *et al.*, 1993). Interesting feature of this isozyme is the presence of N-terminal

proteoglycan-like (PG) domain preceding the catalytic CA domain (Opavsky *et al.*, 1996). The overexpression of CA IX is induced by hypoxia, thus CA IX is used as a marker of tumor hypoxia and also as a prognostic factor for several human cancers (Tunuguntla & Jorda, 2008). For these reasons, CA IX serves as a valuable target for diagnostics and became a target for antitumor therapy (Winum *et al.*, 2008).

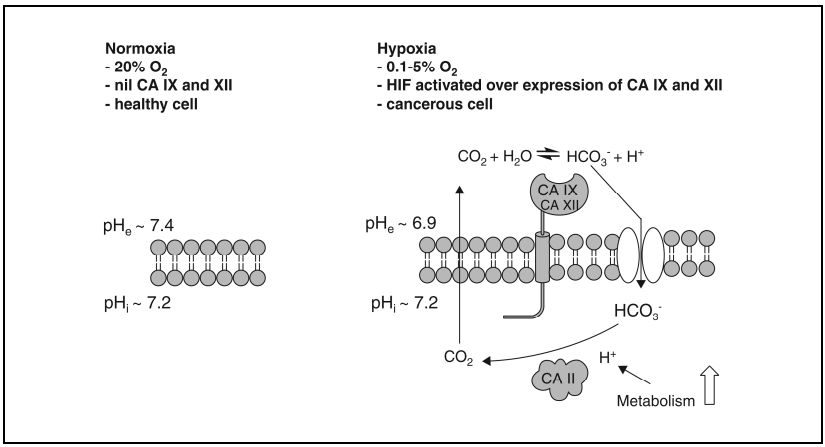

Figure 2 Prosurvival role of carbonic anhydrases IX and XII in hypoxic tumors. *Membrane bound extracellular carbonic anhydrases CA IX and CA XII help tumor cells in maintaining normal intracellular pH. Figure was adapted from Poulsen, 2010.*

Due to the presence of the unique PG domain, human CA IX lends itself to a two-pronged "attack", one arm targeting the PG moiety and the other the active site at the catalytic domain CA. First, the inhibition of the enzymatic activity, crucial for hypoxic tumor growth and progression (Poulsen, 2010) was studied. Structure based drug design requires detailed structural analysis of the active site, so that small molecular inhibitors, that would specifically inhibit the CA IX isozyme, can be designed, with minimal effect on other members of the CA family. The prerequisite for these studies was a sufficient supply of recombinant CA IX protein of crystallization grade. Results concerning expression, purification and crystallization trials of various CA IX constructs are presented.

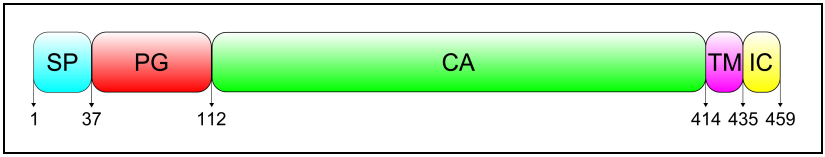

Figure 3 Schematic representation of CA IX domain composition. *The N-terminal proteoglycan-like (PG) sequence is unique for CA IX isozyme, it is followed by carbonic anhydrase catalytic domain (CA), short transmembrane segment (TM) and intracellular tail (IC).*

Complementary to these efforts were structural studies of CA II, abundant, physiologically important and easily available isozyme, which was chosen as a representative model of other family members. The obtained structures of CA II, free and complexed with a novel class of inhibitors based on isoquinoline scaffold, allowed discerning the fine details of the inhibitor binding mode to the active site, thus providing clues for design of inhibitors selective for CA IX.

As for the PG domain, an excellent tool for diagnostics and possibly targeted therapy is a specific mouse monoclonal antibody M75 (Zavada *et al.*, 2000). This antibody recognizes a linear epitope GDEELP localized in the PG domain of CA IX. Crystal structure of M75 Fab fragment complexed with this epitope peptide was determined in the present work with the aim to better understand the antibody-antigen interactions and to obtain structural information that would help in future attempts to humanize the antibody and/or its recombinant fragments.

Overall, the results of this work significantly improve our understanding of antigen-antibody recognition as well as structure of human CA isozymes, and should lead to improved design of both macromolecular (single chain Fv antibody fragments) as well as small molecular compounds selective for clinically important CAs, namely the tumor associated isozyme CA IX.

2 Aims of the thesis

The presented work is a part of an ongoing project focusing on study of human carbonic anhydrase isozymes and their role in human physiology and pathophysiology. The study of CA IX as a cancer related isozyme of the CA family started shortly after its discovery by Dr. Jan Závada and his co-workers and is still in progress. Development of novel compounds with high inhibitory potency and selectivity to human CA isozymes, namely to CA IX represents an attractive strategy to obtain pharmacological tools, while avoiding possible side effects and improving therapeutic safety. The aims of this thesis follow two lines of studies focused on (1) obtaining structural information on CA isozymes which would be utilizable in structure-based drug design and (2) understanding the recognition of CA IX by specific antibodies.

To obtain structural information for structure-based drug design, following specific aims were proposed:

- Production of recombinant CA IX in *E. coli* or eukaryotic expression system. Development of purification protocol to obtain active CA IX in amount and quality suitable for crystallization trials and enzymatic activity assay.
- Preparation and crystallization of complexes of selected small molecule inhibitors with human carbonic anhydrase isozymes CA II and CA IX. Diffraction measurements with crystals from successful crystallization trials. Structure determination and analysis.

To understand the recognition of CA IX by specific antibodies the following specific aim was proposed:

- Determination of crystal structure of fragment of specific antibody recognizing proteoglycan–like domain of CA IX by X-ray crystallography.

3 Material and Methods

3.1 Molecular cloning – construction of CA IX expression plasmids

Standard procedures of molecular cloning (plasmid DNA isolation, vector and insert preparation, ligation of DNA fragments and bacterial transformation) as given in Sambrook manual (Sambrook & Russell, 2001) were used. Reaction conditions for digests with restriction endonucleases, ligation reaction, site-directed mutagenesis, PCR etc. were according to instructions of enzyme and/or kit suppliers.

3.2 Expression of CA IX constructs in E. coli

To achieve expression of CA IX in *E. coli* various cultivation conditions in combination with several host strains were tested. Cultivation temperature, time, and concentration of inducer were the main variables.

3.3 Expression of CA IX constructs in S2 cells

Schneider S2 cells were used for eukaryotic heterologous expression of rCA IX constructs. This cell line, derived from a primary culture of late stage *Drosophila melanogaster* embryos (Schneider, 1972), was supplied as a part of the *Drosophila* Expression System (Invitrogen).

3.4 Purification of recombinant CA IX

The purification protocol was adapted according to the nature of the expressed recombinant construct and to the presence of affinity tags. Mainly established methods including ion exchange chromatography, size exclusion chromatography, and several affinity chromatography techniques were used.

3.5 Carbonic anhydrase activity assay

The procedure of assaying carbonic anhydrase activity, which was utilized in this work was based on Maren's endpoint colorimetric technique (Maren & Ellison, 1967) with some modifications detailed in (Brion *et al.*, 1988). The principle of this method is based on rate of acidification of solution monitored by change in acid-base indicator (phenol red) color.

3.6 Preparation of M75 Fab for crystallization

Limited papain proteolysis of M75 IgG was performed. The course of reaction was monitored by gel filtration on Superdex 200 HR, and the reaction was stopped after 3 h by addition of iodoacetamide (final concentration 5.4 mM). Undigested IgG as well as Fc fragments were removed using Protein A Sepharose.

3.7 Protein crystallography

Crystallization trials were performed with the sparse matrix method (Jancarik & Kim, 1991) as well as with other crystallization screens designed on the basis of results of structural genomics consortia (e.g. JCSG+ screen, Qiagen). Vapor diffusion method of protein crystallization was used for screening and optimization of crystallization conditions at temperature of 18 ºC. Several macro- and micro-seeding techniques were used for improving crystal quality (Thaller *et al.*, 1981).

The testing of crystals for diffraction and data collection on sufficiently large crystals were performed using an in-house diffractometer (Nonius FR 591) connected to 345 mm MarResearch image plate detector at 120 K. Diffraction data collection using synchrotron radiation was performed at 100 K using the X12 EMBL beamline at DESY, Hamburg, Germany. Programs from the CCP4 suite of programs were used for diffraction data processing (*The CCP4 suite: programs for protein*

crystallography, 1994). Crystal structures were determined by the difference-Fourier method or, in cases where crystals were not isomorphous, by molecular replacement using the program Molrep (Vagin & Teplyakov, 2000). Model refinement was carried out using the program REFMAC 5.2 (Murshudov *et al.*, 1997) from the CCP4 package (The CCP4 suite: programs for protein crystallography, 1994), interspersed with manual adjustments using Coot (Emsley & Cowtan, 2004). The final steps included TLS refinement (Winn *et al.*, 2001). The quality of the final models was validated with Molprobity (Lovell *et al.*, 2003). All figures showing structural representations were prepared with the program PyMOL (DeLano, 2002).

Atomic coordinates and experimental structure factors have been deposited with the Protein Data Bank with the codes 2HKH and 2HKF for anti-CA IX antibody fragments and 3IGP for CA II + inhibitor complex.

4 Results and discussion

4.1 Targeting active site of human carbonic anhydrases

The results of the structural analyses presented in this thesis have become part of a publication Gitto *at al.*, 2009. Solved and analyzed crystal structures of CA II + DT1 and CA II + DT2 complexes revealed valuable structural information about interactions of 3,4-dihydroisoquinoline-2(1H)-sulfonamides with components of active site of CA II protein and they uncovered some unique features which can be used in future structure-based drug design effort.

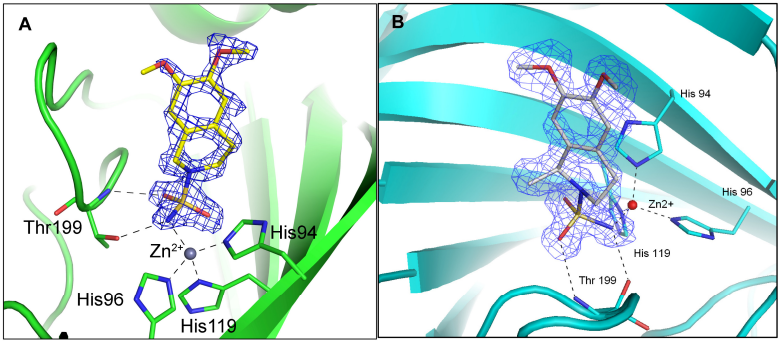

Figure 4 Binding of DT1 and DT2 inhibitor on CA II. *Detail of the CA II active site with the inhibitor DT1 (A) and DT2 (B) Protein is represented in green (panel A) and cyan (panel B) with residues forming polar contacts (gray dashed lines) with inhibitor highlighted in sticks. Also three histidine residues coordinating zinc ion are shown. In panel (A) DT1 inhibitor is represented as a stick model (with carbon and oxygen atoms colored yellow and red, respectively). The 2Fo - Fc electron density maps are contoured at 1.5σ. In panel (B) the DT2 inhibitor represented as a stick model (with carbon and oxygen atoms colored gray and red, respectively). The 2Fo - Fc electron density maps are contoured at 0.8σ.*

The position of the sulfonamide group in the active site is highly similar to all other structures deposited in PDB and proves the importance of tetrahedral coordination of Zn^{2+} cation by four nitrogen atoms with a lone

pair. Positions of the nitrogen atoms, Nε His 94, Nε His 96 and Nδ His 119 form a ligand field which dictates the position of coordinated Zn^{2+} cation and consecutively determine the placing of basic amino group of sulfonamide, to complete the tetrahedral coordination of central zinc ion.

Isoquinoline moiety of inhibitor molecule in the crystal structure of CA II + DT1 complex is stabilized by numerous van der Waals interactions in the active site cavity formed mainly by N-terminal part of CA II, whereas the substituted isoquinoline moiety the substituted isoquinoline moiety in CA II + DT2 complex is rotated around the sulfur – nitrogen covalent bond, and ring system is stabilized by van der Waals interactions with the amino acid residues coming from the central part seven stranded β-sheet on the opposite side of the cavity. Large distance difference, more than 4 Å, between methoxy oxygen atoms of the conjugated ring for the two placements of isoquinoline moiety in the solved structures (see Figure 5) indicated, that more voluminous substituent might be a better fit for the active site cavity. These findings allowed us to design a novel compound with optimized shape of the substituent; this project, however, is out of scope of this thesis. The binding of such a lead compound has already been experimentally proved and the structural studies are in progress.

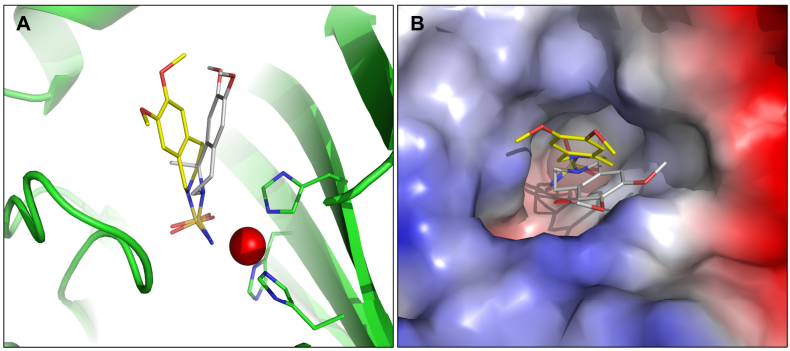

Figure 5 Comparison of DT1 and DT2 binding mode in CA II active site. *Superposistion of the complex structures is based on the best fit for Cα atoms of CA II residues from Gly6 until C-terminus. DT1 inhibitor is represented with yellow carbon atoms, while DT2 carbon atoms are colored gray. In panel (A) protein is represented by ribbon and histidine residues coordinating Zn ion (red sphere) are shown as sticks. Panel (B)shows a top view into the active site where protein is represented by its van der Waals radii colored by the electrostatic potential from negative (red) to positive (blue).*

An important result from the structural studies which will have consequences for design of future experiments is the finding, that absolute configuration of an enantiomer bound in the active site is R. This suggests that only one enantiomer represents an active compound and this discovery will have large impact to selectivity studies of this series of molecules. All activity measurements until now were carried out with mixtures of both enantiomers and the values of activity and selectivity were thus affected by the ratio of the active and non-active enantiomers. This subject should thus be addressed in future research.

In conclusion, the structural study of series of isoquinoline sulfonamides complexes with CA II brought new insight to design of sulfonamide derivatives, especially usage of more voluminous substituents. From the structural point of view, molecules DT1 and DT2 (better expressed: mixture of R and S enantiomers of DT2) served as excellent molecular tools for detailed mapping of active site cavity. These results will be extensively exploited for rational design of molecules which could be highly selective inhibitors for various CA isozymes.

4.2 Structural studies of CA IX

Efforts to produce recombinant human CA IX of crystallization quality in prokaryotic or eukaryotic expression systems, which comprised in total 19 different gene constructs, resulted either in too low yields or in proteins unable to grow as crystals needed for X-ray analysis. Illustrative examples of crystals (Figure 6) are shown below.

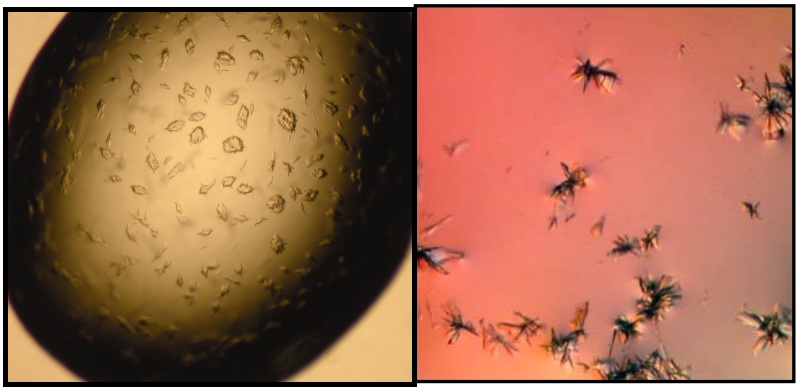

Figure 6 Examples of crystallization hits obtained with recombinant CA IX constructs. *Left panel 10 mg/ml PG+CA (p661) in 0.2 M Magnesium acetate; 0.1 M Sodium Cacodylate pH 6.5; 20 % PEG 8000; Right panel 8 mg/ml CA (p759) (1 M ammonium sulfate; 100 mM HEPES pH 7.5).*

From discussions with colleagues working in the CA field we have learned that our experience was not unique and that others encountered similar pitfalls, e.g. difficulties with affinity chromatography on sulfonamide agarose. Other groups have developed protocols for carbonic anhydrase purification by using metal affinity resins of their own, giving better results after one step purification (Banerjee *et al.*, 2004).

After more than a decade of unsuccessful efforts by many teams, including big pharmaceutical companies, by the end of year 2009 a paper announcing the first 3D structure of CA IX was published (Alterio *et al.*, 2009). Based on the exact sequence of the CA domain (N- and C- termini)

in their construct, including the Cys(41)Ser mutation, which finally led to well crystallizable CA IX in the Alterio *et al* paper, we have also designed such a CA construct and we plan to use it in structure determination experiments.

We also are still interested in the role and structure of the PG domain, so we have not given up yet on crystallization of PG+CA construct either.

4.3 Structure of mouse mAb M75 in complex with epitope peptide

Crystal structure analyses presented here were one part of a wider study which combined various techniques to characterize recognition binding of antibody M75 to its epitope in CA IX. The results of structural studies presented in this thesis have become part of a publication Kral, Mader *et al.*, 2008.

Figure 7 Structure of Fab M75 antibody fragment in complex with epitope peptide.
Heavy chain is colored blue, light chain red, peptide yellow, the respective CDR loops are colored pale blue for H1, green H2, gray H3, light magenta L1, mauve L2, dark magenta L3

The combination of X-ray crystallography, ITC experiments, and MD simulations allowed us to assess the key structural parameters responsible for Fab M75 antibody epitope recognition and binding.

We observed good correlation between structural elements and thermodynamic parameters of the association of antibody fragment to the epitope. Comparisons of the crystal structures of free Fab M75 and its complex with the epitope peptide reveal major readjustments of CDR-H1 and CDR-H3. In contrast, the overall conformations and positions of CDR-H2 and CDR-L2 remain unaltered, and their positively charged residues may thus present a fixed frame for epitope recognition. Adoption of the altered CDR-H3 conformation in the structure of the complex is accompanied by an obvious local stabilization.

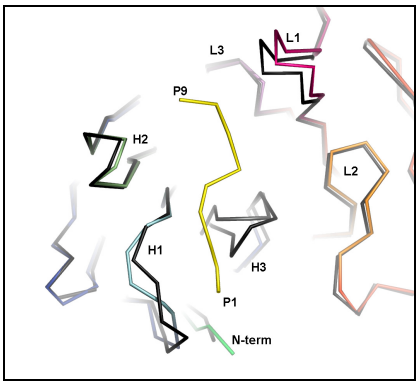

Figure 8 Superimposed Cα traces of the free and complexed Fab M75.
A "top view" into the binding cleft. The peptide and CDRs of the complex are are pale blue for H1, green H2, gray H3, light magenta L1, mauve L2, dark magenta L3; the "free" structure is colored black

By analysis of the two crystal structures, we also gained critical information necessary for further protein engineering of this important antibody. The analysis of interaction of epitope peptide with individual CDRs of M75 suggest that the antigen binding, especially its interaction

with the light chain of the antibody, is not optimal and can be substantially improved.

In conclusion, the structural study performed as part of this PhD thesis helped understanding the details of antigen-antibody recognition and the design of improved antibody fragments of potential therapeutic and diagnostic interest.

5 Conclusions

This work presents a part of an ongoing project focused on isozymes of human carbonic anhydrase (CA) and their roles in human physiology and pathophysiology. Carbonic anhydrase IX (CA IX), a cancer related isozyme of CA, has for long time been a center of attempts of many laboratories, both in academia and in pharmaceutical industry, to develop potent, yet selective CA inhibitors: these would provide promising pharmaceutical tools of improved therapeutic safety and reduced incidence of side effects.

One of major aims of this work was to get detailed structural information on isozymes of human CA as the essential precondition for structure-based drug design. Crystal structures of CA II in complexes with two inhibitors of a novel isoquinoline class have shown their binding modes in the isozyme active site to considerable details: such structural information permits fine-tuning of structures of the CA II inhibitors, and gives at the same time clues for the design of this class inhibitors selective toward CA IX. Inhibitory potency of such designed compounds will be rationalized with crystal structures of their complexes with recombinant CA IX isozyme expressed from a recently developed construct, which apparently, and so far uniquely, provides recombinant CA IX protein amenable to structural studies. All our previous efforts to produce a recombinant human CA IX of protein-crystallization quality (using in total 19 different constructs in both prokaryotic and eukaryotic expression systems) failed because of insufficient yields or because of inadequate crystal growths.

The other proposed main aim was to determine crystal structure of Fab fragment of monoclonal antibody M75 which recognizes the proteoglycan-like (PG) domain of CA IX. Two structures were solved, of

free Fab fragment and of its complex with the epitope peptide. The fact that PG domain is a unique feature of CA IX, absent in other members of the CA family, makes this domain an attractive diagnostic and therapeutic target. The information obtained from the two crystal structures is valuable not only for its contribution to understanding of the antibody-antigen recognition in its own right, but also for the detailed knowledge on the M75 antibody variable domain structure: such insight will be useful in the planned engineering of the antibody to higher affinity, stability and humanization.

6 List of publications

The results presented in this PhD thesis were published in two original papers and as five short communications and abstracts.

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*Vlastimil Král and Pavel Mader contributed equally to this work.

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