Univerzita Karlova
1. lékařská fakulta

Autoreferát disertační práce

Development of rhomboid protease inhibitors as tools for cell biology

Vývoj inhibitorů proteas z rodiny rhomboidů jako nástrojů pro studium jejich biologických funkcí

Mgr. Anežka Tichá

2019

Doktorské studijní programy v biomedicíně
Univerzita Karlova a Akademie věd České republiky

Obor: Biochemie a patobiochemie

Předseda oborové rady: Prof. MUDr. Stanislav Štípek, DrSc.,

Školicí pracoviště: Ústav organické chemie a biochemie AV ČR

Školitel: Ing. Kvido Stříšovský, PhD.

Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

TABLE OF CONTENTS

ABSTRACT

Rhomboids are intramembrane serine proteases that belong to the evolutionarily widespread rhomboid superfamily. Rhomboids developed a slightly different catalytic mechanism compared to classical serine proteases; they utilise a catalytic dyad (Ser/His) instead of the common triad (Ser/His/Asp), and the rhomboid active site is buried in the membrane. This, coupled with their hydrophobicity, makes them quite difficult to study. Therefore, even though they are known to be involved in several important biological processes it is still not clear how exactly most of them are involved in the regulation of or in the pathologies of diseases related to these processes (such as malaria, Parkinson's disease or cancer). Our understanding is hindered by the lack of tools for their characterisation both *in vitro* and *in vivo*. In my thesis I present new fluorogenic substrates based on the LacYTM2 sequence, which is hydrolysed by several different rhomboid proteases. Using Förster resonance energy transfer (FRET)-based methods, these substrates are suitable for continuous monitoring of rhomboid activity *in vitro*. Modifications in the P5-P1 residues can improve selectivity for a specific rhomboid, the choice of FRET pair of fluorophores that absorbes light of longer wavelengths makes them suitable for high throughput screening (HTS).

Selective and potent inhibitors are a valuable tool for studying the molecular mechanisms underlying enzyme function. However, such inhibitors have been lacking for rhomboid proteases. The inhibitors developed in my thesis are non-toxic and easily synthetically accessible and modifiable. The inhibitors based on the N-methylen saccharin or the benzoxazin-4-on scaffold are not potent enough for direct cell biological use, but further derivatisation could lead to improvement in potency. The peptidyl ketoamides are based on the structural understanding of rhomboid specificity and mechanism and they are the most promising class. They combine a substrate-derived peptidyl part with the electrophilic reactive group of the ketoamide, extended by a hydrophobic substituent. The resulting inhibitor scaffold is potent, selective, covalent and reversible. With low nanomolar potency *in vivo*, peptidyl ketoamides are by far the most effective rhomboid inhibitors available. Modifications of the peptidyl part and the C-terminal hydrophobic substituent will enable tuning of inhibitor selectivity to diverse rhomboid proteases.

Key words: intramembrane proteolysis, rhomboid, rhomboid inhibitors, peptidyl ketoamides, fluorogenic substrates

ABSTRAKT

Rhomboidy jsou serinové membránové proteasy, které patří do evolučně rozšířené rodiny rhomboidních proteinů. Rhomboidy si ve srovnání s klasickými serinovými proteasami vyvinuly odlišný katalytický mechanismus; místo běžně triády (Ser/His/Asp) k proteolýze využívají pouze katalytickou dyádu (Ser/His) a jejich aktivní místo se nachází uvnitř lipidové membrány, což společně s obecně vysokou hydrofobicitou membránových proteinů komplikuje jejich studium. Přestože je známé jejich zapojení v mnoha důležitých biologických procesech, stále u většiny rhomboidů není jasný konkrétní mechanismus, kterým přispívají k regulaci těchto procesů nebo k patologickým projevům souvisejících onemocnění (jako např. malárie, Parkinsonova choroba nebo rakovina). Pochopení těchto dějů je ztíženo nedostatkem nástrojů k jejich studiu jak *in vitro*, tak *in vivo*. Ve své práci uvádím nové fluorogenní substráty založené na Försterově rezonančním přenosu energie (FRET) a odvozené od sekvence LacYTM2, které umožňují široké využití pro kontinuální sledování aktivity mnoha rhomboidů *in vitro*. Modifikacemi v P5-P1 pozicích je možné zvýšit specifitu substrátu k cílovému rhomboidu, zatímco výběr FRET páru fluoroforů absorbujícího v červené oblasti viditelného světla umožňuje jejich využití pro rychlé testování knihoven molekul.

Selektivní a účinné inhibitory jsou cenným nástrojem ke studiu molekulárních mechanismů enzymů, avšak pro rhomboidy zatím žádné takové inhibitory nejsou známy. Inhibitory vyvinuté v rámci této práce jsou netoxické, jednoduše synteticky dostupné a modifikovatelné. Inhibitory odvozené od N-methylen saccharinu nebo benzoxazin-4-onu sice nejsou dostatečně aktivní pro biologické aplikace, ale další modifikace by mohly vést ke zvýšení účinnosti. Peptidyl ketoamidy jsou inhibitory založené na mechanismu interakce rhomboidu se substrátem a jsou zatím nejslibnější skupinou inhibitorů rhomboidů. Spojují peptidovou část odvozenou od substrátu s ketoamidovou elektrofilní reaktivní skupinou rozšířenou hydrofobním substituentem. Výsledné inhibitory jsou účinné, selektivní, kovalentní a reversibilní. Vzhledem k jejich aktivitě *in vivo* při nízkých nanomolárních koncentracích jsou peptidyl ketoamidy v současnosti zdaleka nejefektivnějšími inhibitory rhomboidů. Optimalizace peptidové části a C-koncového hydrofobního substituentu umožní návrh selektivních inhibitorů dalších členů této rodiny.

Klíčová slova: intramembránová proteolýza, rhomboid, inhbitory rhomboidů, peptidyl ketoamidy, fluorogenní substráty

1 INTRODUCTION

The rhomboid proteins make up a superfamily that consists both of the active proteases as well as the catalytically inactive proteins, pseudoproteases. They occur in all kingdoms of life and are probably the most widely distributed membrane proteins in nature. Rhomboids were first discovered in the fruit fly *Drosophila melanogaster*, where mutations in *rhomboid* locus resulted in pointed head skeleton phenotype in embryos (*1*). The encoded protein (named Rhomboid 1) was shown to be important in early embryonal differentiation (*2*) through activation of the epidermal growth factor receptor/mitogen-activated protein kinases (EGFR/MAPK) pathway (*3*) although the exact mechanism was not clear. Later it was shown that Rhomboid 1 is actually a novel intramembrane serine protease that activates EGFR by shedding the membrane-bound TGFα-like growth factor Spitz. Thus, Rhomboid 1 plays a critical role in the regulation of the EGFR signalling pathway in *Drosophila* (*4, 5*). Several rhomboid-like proteins, including both the proteases and pseudoproteases, are known to play important roles in diseases such as colon cancer, Parkinson's disease, malaria, or inflammatory diseases (reviewed in (*6*)), but in many cases the underlying mechanisms are not known. The emerging possibility that rhomboid-like proteins could be novel drug targets elevates the general interest in their research.

The rhomboid superfamily consists of numerous paralogue groups with low overall sequence similarity (*7*), but all superfamily members share a typical rhomboid core formed by six transmembrane helices (*4, 8, 9*) and conserved sequence features. This transmembrane core is sometimes extended by an extra N- or C-terminal transmembrane helix or by an extramembrane domain (*10*). The structures substantiate the serine-histidine catalytic dyad (*11*) formed by Ser201 and His254 (in *E. coli* GlpG). The catalytic serine lies on the N-terminus of TMD4 which lies about 10 Å below the membrane surface and is encircled by the remaining five TMDs and the amphipatic structure of the L1 loop, which is half-immersed in the membrane. His254 is located on TMD6 below the level of the membrane. The catalytic dyad lies at the bottom of an aqueous internal cavity open to the bulk solvent.

Despite extensive research effort, substrate binding is still poorly understood. According to our current knowledge it probably happens in two steps (*12-14*). There is presumably another interaction site in addition to the active site, the so called "exosite", where substrate binds in the first instance and this is called "interrogation" (*13*) or substrate "docking" (*12*). In the second step the substrate part presenting the recognition motif unwinds and bind to the

rhomboid active site (*12, 15*). Again, the final proof will require structural analysis of rhomboid-substrate complex.

In general, the investigation of the cell biology of rhomboid proteases would greatly benefit from specific and potent rhomboid inhibitors. As rhomboid activity is necessary for several pathological states such as invasion of malaria parasite into the host cell, such rhomboid inhibitors could be also evaluated for therapeutic use. Although there were several attempts to identify rhomboid inhibitors, prior to the current study, only few rhomboid inhibitors were described, none of them fulfilling the above mentioned requirements. They were useful for better understanding of rhomboid-substrate interaction and mechanism (*16, 17*), but unsuitable for cell biology due to low potency, low selectivity and high toxicity, etc. The current rhomboid inhibitors can be conceptually divided into two groups; i) small non-peptidic, usually heterocyclic compounds and ii) substrate derived inhibitors that combine a peptidyl part with a reactive electrophilic group. However, none of the rhomboid inhibitor groups known so far achieve all the desired properties needed for inhibitors suitable for cell-biological assays.

2 AIMS OF STUDY

The initial aim of my project was the development of a new and better substrate and assay platform. As fluorescence intensity remains the most easily accessible and accurate readout, the substrate was to be based on FRET. The goal of this project was to design new fluorogenic rhomboid substrates that would be recognised by several rhomboids and could be used in detergent micelles as well as in proteoliposomes. To be suitable for HTS, the substrate needed to utilise red-shifted variants of fluorophores.

However, the main focus of my thesis is development of potent and selective rhomboid inhibitors. Prior to the start of this work, no rhomboid inhibitors were available that would meet all the requirements for inhibitors suitable for cell biological assays. The main objective was to deliver a general strategy for rhomboid inhibitor design based on known rhomboid-substrate interactions. The second objective was the characterisation of novel classes of small-molecule rhomboid inhibitors.

3   METHODS

This chapter summarises the methods I have used in the publications presented. The work was carried out at the Institute of Organic Chemistry and Biochemistry of the CAS. As part of these projects we established collaborations with several research groups. First part of my thesis are projects that originated in and were led by our lab (Publication 1 and 2). Crystal structures of GlpG in complex with peptidyl α-ketoamides were solved partly in collaboration with Kutti R. Vinothkumar (Richard Henderson's group at Medical Research Council Laboratory of Molecular Biology, Cambridge, UK), the selectivity of inhibitors against a panel of rhomboids was tested in the laboratory of Steven Verhelst (University of Leuven, Leuven, Belgium) and the selectivity against human hydrolases using the EnPlex method in the lab of Daniel A. Bachovchin (Memorial Sloan Kettering Cancer Center, New York, USA). The second part of my thesis is the characterisation of the compounds developed by Sascha Weggen's group (Heinrich Heine University Düsseldorf, Düsseldorf, Germany). They synthesised all the saccharine and benzoxazinone inhibitors, did the docking studies and preliminary activity testing.

This is a brief list of the methods used in this thesis, detailed descriptions are within the publications presented below.

Molecular biology methods:

Transformation of plasmid into *E. coli* cells; expression of recombinant proteins in *E. coli*; isolation and purification of proteins of interest.

Characterisation of substrates and inhibitors:

Measurement of peptide solubility in different conditions; acquisition of fluorescent spectra; measurement of reaction rates and enzyme activity using i) a continuous fluorescence-based assay (following an increase in fluorescence intensity upon fluorescent substrate cleavage), and ii) an end-point assay (following the difference in the amount of substrate hydrolysed after the equivalent time under different conditions); sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE); immunoblot analysis (detection by chemiluminescence or fluorescence); characterisation of substrate and inhibitor properties (K_m, k_{cat}, IC_{50}, K_i, reversibility and modality of inhibition); inhibition of endogenous GlpG in *E. coli*.

4   RESULTS

During my PhD studies I co-authored six publications (three as a first author) describing tools to manipulate rhomboid activity, all of them in peer-reviewed journals. Here I present and discuss four of these; my contribution to *Yang et al.* is rather small and the final paper is a review of the different approaches to study rhomboid proteins that we were asked to write after publishing the paper about peptidyl ketoamides. Our work on peptidyl ketoamides also resulted in an international patent. All of the presented work is the result of joint efforts of several people and my contribution to the publications is summarised at the end of each chapter (in the thesis).

4.1   Publication 1: Sensitive versatile fluorogenic transmembrane peptide substrates for rhomboid intramembrane proteases

<u>Anežka Tichá</u>, Stancho Stanchev, Jan Škerle, Jakub Begán, Marek Ingr, Kateřina Švehlová, Lucie Polovinkin, Martin Růžička, Lucie Bednárová, Romana Hadravová, Edita Poláchová, Petra Rampírová, Jana Březinová, Václav Kašička, Pavel Majer, Kvido Stříšovský

Previous assays to determine rhomboid protease activity suffer from several disadvantages. Briefly, these are as follows: gel-based readout is unsuitable for HTS (*8, 18*), and substrates have low affinity towards different rhomboids (*19*), their use is limited to liposomes (*13*), or the presence of large fluorescent domains constrains their photochemical variability (*20*).

We have chosen LacYTM2 as we know it is a substrate for a range of different rhomboids. Almost the entire TMD is required for efficient cleavage by GlpG, so we did not truncate the peptide and we introduced Glu-Edans at the P5 and Lys-Dabcyl at the P4' sites, positions where mutations do not affect recognition by rhomboid (*12, 21*). The resultant peptide is a new rhomboid fluorogenic substrate, KSp35. We have characterised the peptide in terms of its secondary structure, fluorescence spectra, cleavage site, pH optimum and solubility. For KSp35 to be soluble, the presence of detergent in buffer was required. Importantly, the cleavage rate also strongly depended on detergent concentration, which might have been neglected in most reports to date.

We have also shown that KSp35 is hydrolysed by a panel of rhomboids and that it can be used in liposomes. Its affinity towards different rhomboids can be changed by mutation of the recognition motif. Mutations in P5-P1 (RVRHA) to amino acids preferred by GlpG (*21*)

increased its catalytic efficiency about 23-times and had a striking effect on selectivity as the mutated substrate was cleaved only by GlpG (**Figure 1**). This suggests an easy route to increased substrate selectivity. The KSp35 peptide can also be turned into a red-shifted variant with the Tamra-QXL610 fluorescence pair which makes it suitable for HTS (the resultant substrate is KSp76 as the WT peptide, and KSp64 as the RVRHA mutant). From previous work, it is known that red-shifted fluorophores are suitable for characterisation of inhibitors that absorb in the UV region, such as isocoumarins (*22*). We based our method on conditions similar to those previously published (*19, 21, 22*) using KSp76 and obtained a comparable IC_{50} for all inhibitors.
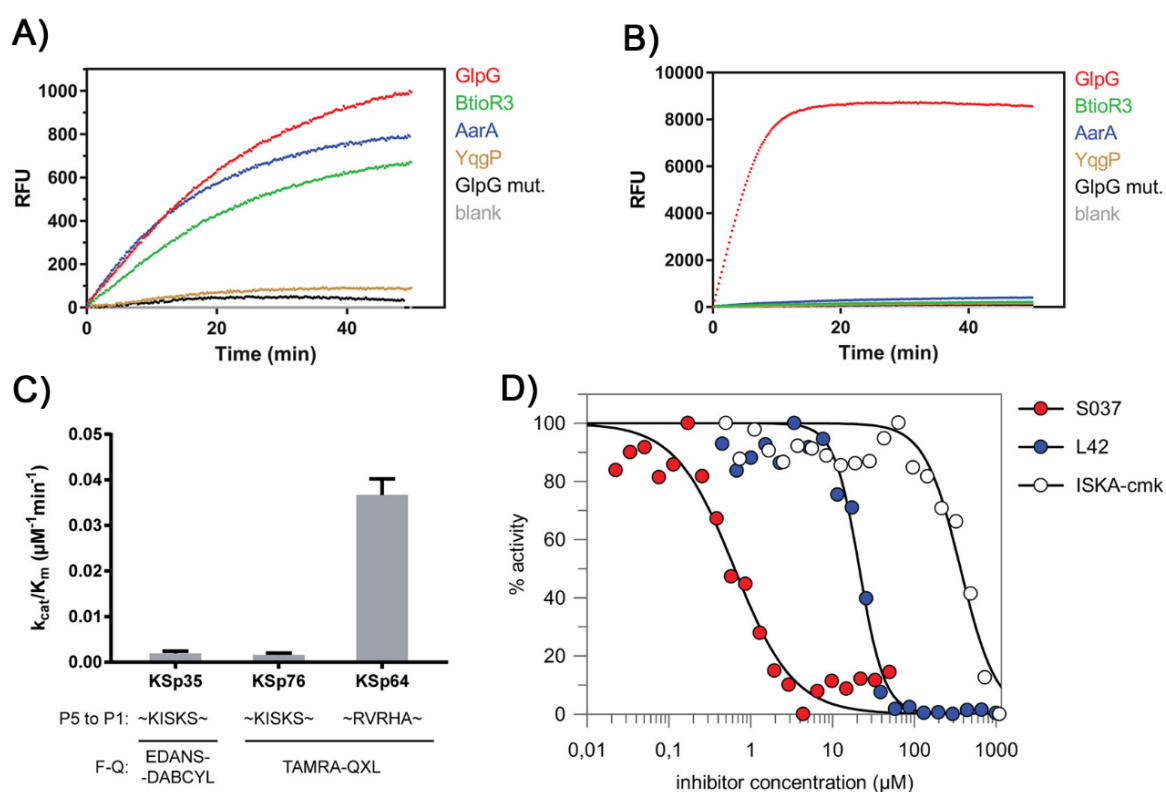

Figure 1: **The recognition motif (P5-P1) is important for substrate turnover and specificity**
A) Cleavage of KSp76, the red-shifted variant of KSp35 with wild type sequence in the P5-P1 region. KSp76 is cleaved by GlpG, BtioR3 and AarA but not by YqgP and an inactive GlpG mutant (S201A/H254A). **B)** Cleavage of KSp64, the red-shifted variant of KSp35 with the P5-P1 sequence optimised for GlpG cleavage (RVRHA). In contrast to KSp76, KSp64 is cleaved by GlpG only, thus the mutation drastically improved specificity towards GlpG. Moreover, the catalytic rate is higher than for KSp76 cleavage. In both cases 10 μM substrate was cleaved by 0.4 μM enzyme (0.04 μM in case of BtioR3). **C)** Catalytic efficiency (k_{cat}/K_m) for the hydrolysis of KSp35, KSp76 and KSp64 by GlpG. Substrates (0.5-20 μM) were incubated with 0.4 μM GlpG in the presence of 0.5 % DDM. Initial reaction rates were plotted against substrate concentration to obtain k_{cat}/K_m. **D)** Inhibition of GlpG by three previously published inhibitors, using KSp76 as a substrate. Inhibitors were pre-incubated with GlpG for 60 min. All experiments were performed in 20 mM HEPES pH 7.4, 150 mM NaCl, 0.05 % (w/v) DDM and 10 % (v/v) DMSO at 37 ºC unless noted otherwise. The readout for reaction progress was an increase in fluorescence (KSp35: λ_{ex} = 335 nm, λ_{em} = 493 nm; KSp76 and KSp64: λ_{ex} = 553 nm, λ_{em} = 583 nm).

4.2 Publication 2: General and modular strategy for designing potent, selective, and pharmacologically compliant inhibitors of rhomboid proteases

<u>Anežka Tichá</u>, Stancho Stanchev, Kutti R. Vinothkumar, David C. Mikles, Petr Pachl, Jakub Began, Jan Škerle, Kateřina Švehlová, Minh T.N. Nguyen, Steven H.L. Verhelst, Darren C. Johnson, Daniel A. Bachovchin, Martin Lepšík, Pavel Majer and Kvido Stříšovský

Potent and selective rhomboid protease inhibitors are highly desirable to enable detailed characterisation of rhomboid function as well as for their potential therapeutic use. However, none of the rhomboid inhibitor groups known so far achieve all the desired properties needed for such inhibitors – potency, specificity and non-toxicity. Therefore, based on our knowledge of substrate-rhomboid interactions, we delivered a general strategy for rhomboid inhibitor design, demonstrating the principle using GlpG protease. Based on the knowledge that interactions with both the recognition motif (*12, 23*) and the TMD are important (*24*) we divided the design into three parts. To most efficiently mimic the substrate, we designed inhibitors that have a peptidyl part to mimic the recognition motif, then a reactive electrophilic group to interact with the catalytic serine (the so-called "warhead"), and thirdly a prime site hydrophobic substituen. The mutations introduced into the fluorogenic substrate in Publication 1 (*24*) proved to be also effective in chloromethyl ketone inhibitors. Thus, we used the RVRHA sequence to form the peptidyl part of the new inhibitor (**Figure 2**). Next, we screened several warheads and out of the six we tested we chose the α-ketoamide group. Other substituents can be attached to it through the ketoamide nitrogen (*25, 26*) and the α-ketoamide group is already used as a pharmacophore (*27*). These features might thus be useful for improving potency and non-toxicity in inhibitors. The final step in the design was optimisation of the prime site hydrophobic substituent. We tested a panel of 11 different substituents and the most potent one identified was the longest, phenylbutyl (**Figure 2**).

We determined the kinetic constants and the mechanism of inhibition for the three most potent compounds (9, 10 and 11). The K_i of the best inhibitor (cpd11) was ~50 nM. All of them are reversible non-competitive inhibitors that covalently bind to GlpG in a substrate-like manner occupying the S4-S2' sites, and efficiently inhibit endogenous rhomboids in living cells with an IC_{50} value of 2.7±0.1 nM for the best compound. We also tested their selectivity and ability to discriminate between several rhomboids using established methods (*28*), as well as their ability to inhibit other human enzymes using the EnPlex assay that includes about

100 human serine hydrolases (*29*). Our inhibitors showed only partial selectivity between tested rhomboids, but they do not inhibit most of the human hydrolyses tested.
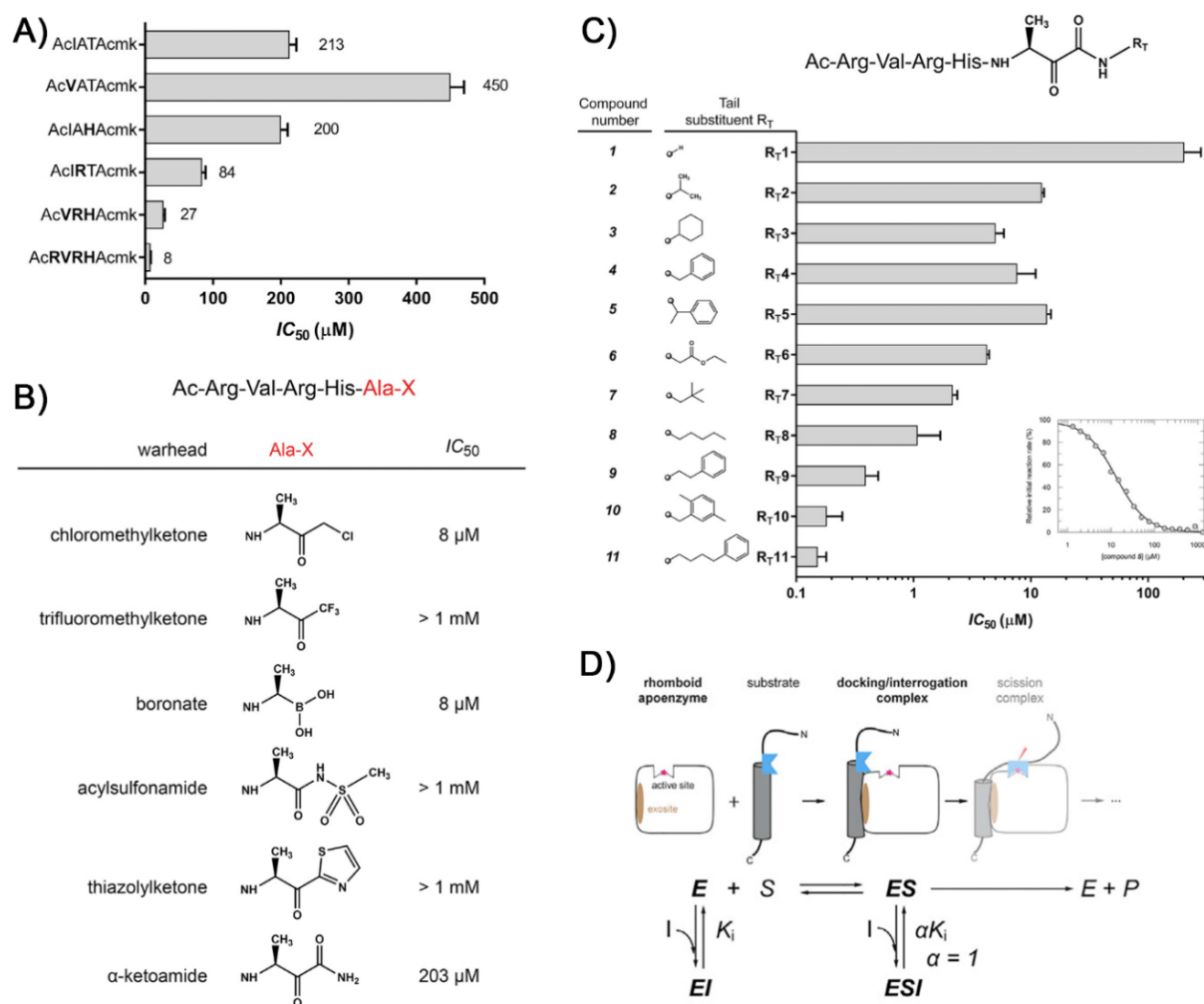

Figure 2: **Design of novel rhomboid inhibitors**
A) Mutations of the parent compound AcIATA-cmk have an additive effect on inhibitor potency. **B)** Screen of several warheads. A-ketoamide group was chosen as it showed the highest potency out of those that can be extended to the prime site. **C)** Hydrophobic prime site substituents have a dramatic effect on inhibitor potency, the optimal substituent improved IC_{50} 1000-fold. The enzyme concentration in the assay was 0.4 μM, so we reached the lower limit of the assay. All experiments were performed in 20 mM HEPES pH 7.4, 150 mM NaCl, 10 % (v/v) DMSO and 0.05 % (w/v) DDM at 37 ºC. GlpG was pre-incubated with each inhibitor for 60 min and the reaction was started by addition of 10 μM KSp35. The readout for initial reaction rates was an increase in fluorescence ($\lambda_{ex} = 335$ nm, $\lambda_{em} = 493$ nm) during the first few minutes of measurement (below 5 % substrate conversion). IC_{50} values were determined from the dependence of initial rate on inhibitor concentration (the inset in C)). The IC_{50} values shown here are means±SD of best fits of 2-3 measurements. **D)** α-ketoamides act as non-competitive inhibitors. As such, they can bind to both free enzyme and enzyme-substrate complex (in this case the docking/interrogation complex), without affecting substrate affinity towards GlpG ($\alpha = 1$).

4.3 Publication 3: Discovery and biological evaluation of potent and selective N-methylene saccharin-derived inhibitors for rhomboid intramembrane proteases

Parul Goel, Thorsten Jumpertz, David C. Mikles, Anežka Tichá, Minh T. N. Nguyen, Steven Verhelst, Martin Hubalek, Darren C. Johnson, Daniel A. Bachovchin, Isabella Ogorek, Claus U. Pietrzik, Kvido Stříšovský, Boris Schmidt, and Sascha Weggen

An alternative approach for rhomboid inhibitor identification was chosen by Sascha Weggen's group (Heinrich Heine University Düsseldorf, Düsseldorf, Germany), a project on which we were invited to collaborate. They took N-methylene-substituted saccharin (1,2-benzisothiazol-3-one 1,1-dioxide) as a starting template for inhibitor development. Saccharins are known to i) act as suicide-inhibitors (*30, 31*), ii) be easily modifiable and iii) have safe toxicological profile (*32*). The inhibitor design strategy was based on a computer-aided approach, which screened the candidate molecules before chemical synthesis and again during the *in vitro* screening.

N-acylsaccharins are known inhibitors of human leukocyte elastase (HLE) and chymotrypsin (*33*). The Weggen study functionalised saccharin inhibitors of a "next generation" (*34*) by attaching a range of different leaving groups (LG) to the saccharin scaffold through the nitrogen of the heterocyclic ring. The best analogues inhibited GlpG with an IC_{50} of 200 nM *in vitro* and a similar effectivity *in vivo* (in *E. coli*).

Mechanistic studies of classical serine proteases show that release of the acidic leaving group is followed by the formation of an irreversible covalent crosslink to the active site serine and histidine (*30*). Crosslinking of the catalytic residues of GlpG causes a characteristic shift in mobility on SDS-PAGE electrophoresis (*21, 22*) which was not observed in the presence of saccharin inhibitor. Complexes of saccharin inhibitors with different GlpG mutants were analysed by mass spectrometry to determine which mutations prevent the formation of the characteristic adduct. However, no conclusive results were obtained from this experiment. Coupled to the fact that inhibition of GlpG by the saccharin inhibitor under study was slowly reversible, these data suggest that the mechanism of rhomboid inhibition by this class of inhibitors differs from the mechanism of inhibition of classical serine proteases.

The selectivity of the best compounds was assessed using the assays discussed in the section on Publication 2 (*28, 29*). The saccharins under study had no inhibitory activity towards eukaryotic rhomboids and inhibited only few human serine hydrolases (out of 71). These results

suggest that further optimisation of the saccharin scaffold could possibly lead to effective rhomboid inhibitors.

4.4 Publication 4: Discovery and validation of 2-styryl substituted benzoxazin-4-ones as a novel scaffold for rhomboid protease inhibitors

Parul Goel, Thorsten Jumpertz, <u>Anežka Tichá</u>, Isabella Ogorek, David C. Mikles, Martin Hubalek, Claus U. Pietrzik, Kvido Stříšovský, Boris Schmidt, Sascha Weggen

This project was a follow up to the previous publication, again in collaboration with Sascha Weggen. An approach similar to that used for saccharin inhibitor identification was used for optimisation of 2-styryl substituted benzoxazi-4-ones. These are known inhibitors of HLE, chymotrypsin and cathepsin G (*30, 35-38*). The benzoxazinon scaffold consists of two fused aromatic rings which provide opportunities for modifications and improved binding to the target enzyme.

Thirteen 2-alkyl and 2-aryl benzoxazinone derivatives were tested *in silico* and *in vitro*. Docking studies of the most favourable molecule (2-styryl substituted benzoxazinon scaffold, compound 3) showed that the heterocyclic ring is oriented towards the S1 subsite whereas the substituent points towards the S2' subsite. The inhibitor also interacts with the adjacent His254 and Phe245. *In vitro*, compound 3 could inhibit Gurken hydrolysis by GlpG, thus confirming the results from the docking studies; compound 3 is the most potent analogue ($IC_{50} \sim 5$ µM). Surprisingly, compound 3 did not inhibit chymotrypsin whereas 50 % inhibition of HLE and trypsin was achieved with 50 µM inhibitor and 10 µM for cathepsin G, therefore showing at least some selectivity towards GlpG. Dilution experiments confirmed the expected reversible mechanism.

5 DISCUSSION

In my thesis I focused on introducing new methodology to study rhomboid proteases. Proteins from the rhomboid superfamily, both the catalytically active proteases as well as the pseudoproteases, were several times shown to be involved in many important biological processes. New tools that I present in this thesis might help to better understand the biology of rhomboid proteases. Novel fluorescent substrates for rhomboids enable continuous measurement of reaction progress and provide a robust method for the assessment of rhomboid

activity. Therefore, they were subsequently used for characterisation of newly developed inhibitors.

We based the fluorogenic substrates on the peptide sequence of the artificial rhomboid substrate LacYTM2 (*8*). This, out of four different substrates (TatA from *P. stuartii*, Gurken and Spitz from *D. melanogaster* and *E. coli* LacYTM2), is best tolerated by the panel of rhomboids we tested (*E. coli* GlpG, *B. subtilis* YqgP, *P. stuartii* AarA and BtioR3 - rhomboid 3 from *B. thetaiotaomicron*). In order to keep the substrate as small as possible and to better understand substrate-rhomboid interactions, we prepared a series of C-terminally truncated versions of LacYTM2 and demonstrated that essentially the entire substrate TMD is required for cleavage to be unaffected. At the time we published this work, there was a general agreement that the recognition motif alters the catalytic turnover of the substrate (*12, 13*), but no research to assess the importance of the substrate TMD had been published at that stage. Following the publication of our study a computational characterisation of rhomboid-substrate interaction was published showing high interaction energies for some of the residues deep in the substrate TMD, suggesting the importance of the substrate TMD for substrate-enzyme interaction (*15*).

One of the main goals of this work was to prepare a substrate useful for HTS. Screened libraries often contain molecules that absorb in the UV region (*39*), which disqualifies KSp35 with its Edans-Dabcyl FRET pair that absorbs light at 300-400 nm. We therefore synthesised KSp76, a substrate that contains the same peptide sequence as KSp35 but possesses a different FRET pair, Tamra-QXL610 (connected through Lys in P5 and Cys in P4', respectively). These changes did not affect the catalytic efficiency of its cleavage or its selectivity towards different rhomboids. Crucially, it absorbs in the red region of the visible spectrum (550-700 nm). Out of the rhomboids investigated (GlpG, BtioR3, AarA, YqgP and GlpG S201A/H254A as a negative control) KSp35 and KSp76 were not cleaved by the inactive GlpG mutant (as expected) and YqgP. This is not surprising either as YqgP's catalytic activity strongly depends on the presence of lipids (*18*). However, mutation of residues in the recognition motif (P5-P1) of the substrate to amino acids strongly preferred by GlpG (*21*), altered the specificity. Therefore the substrate with the RVRHA sequence in the P5-P1 site is cleaved by GlpG only. This optimisation improved the catalytic efficiency (k_{cat}/K_m) about 23-fold, confirming the previously reported importance of the recognition motif for substrate turnover (*12, 13*). To test the suitability of our substrates for HTS we used KSp76 to test rhomboid inhibition by several inhibitors published previously – isocoumarin S037 (*22*), β-lactam L42 (*19*) and ISKA-cmk (*21*). Our measurements agree with published IC_{50} values (under comparable conditions). In summary, we presented here a series of novel fluorogenic substrates with proposed easy-to-implement modifications to

increase specificity towards the targeted rhomboid. The choice of a suitable FRET pair enables their use in the HTS. The newly established fluorescence substrates were subsequently exploited in the development of new rhomboid inhibitors.

We have discovered peptidyl α-ketoamides as new potent and selective rhomboid inhibitors. The crystal structure of GlpG in complex with a peptidyl ketoamide showed a similar binding mode to that previously reported for CMKs (*21*) and peptidyl aldehydes (*14*). However, we added to known data by demonstrating that peptidyl ketoamides bind to the S4-S2' GlpG subsites and that the P5 residue (Arg in the peptidyl part) does not dramatically affect the inhibitory potency. In contrast, even though the P5 and P6 residues were not visible in the crystal structure of the GlpG-RKVRMA-CHO complex, *in vitro* analysis shows that their presence improves the inhibitory potency 5-fold (*14*). The discrepancy might be explained by looking at the structures and potencies of the aldehyde and ketoamide inhibitors. The long version of the aldehyde inhibitor (RKVRMA-CHO) has a K_i of about 20 µM whereas ketoamide compound 9 (for which we tested the effect of shortening the peptidyl part) has a K_i of about 0.2 µM, and is therefore a 100-fold more potent inhibitor. We have demonstrated that the prime side substituent has a large impact on inhibitor potency and this effect might outweigh the contribution of the peptidyl part. This could explain the apparently conflicting data.

Rhomboids are expected to share a similar mechanism (*7, 40*), hence our approach towards inhibitor design could therefore serve as a general strategy for delivery of potent rhomboid inhibitors optimised for a protease of interest. In case of GlpG we started from the analysis based on mutations in the P5-P1 positions of a rhomboid substrate TatA (*21*). There are several other approaches that can address the specificity of a protease, but they often comprise of complex labelling strategies which impedes their convenient use (*41*). In contrast, multiplex substrate profiling utilises a library of synthetic peptides coupled with mass spectrometry. It is an easy and rapid method to map sequence preferences on both the prime and non-prime sides (*42*) and could be used to identify further rhomboid sequence requirements as suggested by a recent work (*43*).

The second part of the inhibitor responsible for its potency and selectivity is the hydrophobic tail substituent. By varying the chemical residue attached to the ketoamide nitrogen we were able to improve the inhibitor potency by over three orders of magnitude. The crystal structure showed that the inhibitor binds from the active site up to the S2' subsite of GlpG. Unfortunately, we were not able to crystallise the complex of GlpG with our best compound (cpd11) but comparison of GlpG complexes with other ketoamides (cpd9 and 10) and previously reported β-lactam L29 (*44*) and isocoumarin S016 (*22*) shows that there is still

space for improvement of the hydrophobic substituent (**Figure 3**). The C-terminal tail binds to the S2' cavity which is absent in the native rhomboid, but forms upon ligand binding (*44*). Additionally, the introduction of a longer substituent, potentially of a branched and hydrophobic nature, to the prime side might increase the potency of the inhibitor because, as we show in Publication 1, the membrane part of the substrate is also important for substrate turnover. Other potential approaches to further improve ketoamide inhibitors is based on deeper understanding of the rhomboid catalytic mechanism. Specifically, the S1 cavity in GlpG was shown to encompass a water retention site which explains the strong preference for small amino acids in P1 (especially Ala) (*12, 21, 23*). Thus, bigger residues might possibly fit into the S1 cavity but interfere with water transport to the active site and result in a more effective and rhomboid-selective inhibitor.

The ketoamide warhead is already in use as a pharmacophore in treatment of hepatitis C infection (*27*). The family of drugs does not cause any unwanted side effects even though they comprise of an electrophilic warhead that covalently modifies the target protein. As these licensed drugs are also peptidyl ketoamides, we assume that our compounds behave similarly, and as such are suitable for cell biological assays.
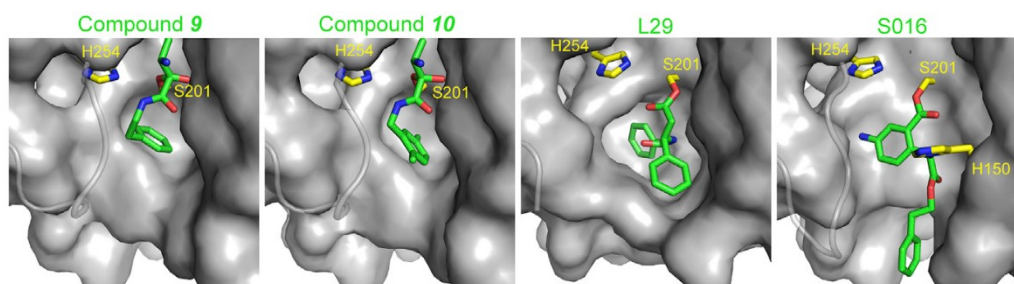

Figure 3: **Comparison of the binding modes of different rhomboid inhibitors**
All of the illustrated inhibitors bind to the S2' cavity, but each of them occupies a slightly different space. This suggests a direction for the future design of N-terminal hydrophobic substituents of peptidyl ketoamides. This is a comparison of crystal structures PDB: 5MT6, 5MTF, 3ZMI and 3ZEB. The rhomboid surface is depicted in grey, the catalytic dyad in yellow and inhibitors in green.

Apart from peptidyl ketoamide inhibitors we also tested other classes of molecules potentially active against rhomboids, namely next-generation saccharin inhibitors and benzoxazin-4-on derived inhibitors. Both scaffolds offer a broad range of structural variations. The effect of varying the LG of saccharin inhibitors was initially determined *in silico* with docking studies. Binding of all 50 proposed derivatives to the GlpG active site was simulated based on the structure of GlpG in complex with the phosphonofluoridate inhibitor CAPF. The docking studies revealed that saccharins with the aryl carboxylic acid LG were the most efficient ligands (also confirmed *in vitro*) and that the binding mode of these inhibitors is similar to that of CAPF (*45*). The sulfoxide group interacts with His150 and Gly199 and the LG

interacts with the prime side of the enzyme. The preference for aryl carboxylic acid LG could be explained by the fact that the aryl ring participates in π-π interactions with Phe245. This interaction might also contribute to inhibitor selectivity as the aryl ring fits nicely into a hydrophobic region around the GlpG active site. More detailed studies of the electrostatic interactions between inhibitor and enzyme suggested that small electron-withdrawing substituents on the aryl ring at the *ortho* or *para* positions could increase the inhibitor potency as it would become more favourable for nucleophilic attack by the catalytic Ser. Surprisingly, neither further docking studies nor *in vitro* inhibition assays of GlpG yielded further inhibitors with improved affinity. Briefly, the IC_{50} value of the most potent compound was 0.2 µM which is comparable to the potency of the most efficient β-lactams (*19*) or isocoumarin (*22*). Unlike β-lactams, which inhibit endogenous GlpG with an IC_{50} of 5-10 µM (*19*), the saccharin inhibitors have a similar potency *in vivo* as they do in *in vitro* studies. Their advantage over isocoumarins is their slowly reversible mechanism.

Saccharin-based inhibitors are expected to form a doubly-linked covalent complex with the target enzyme (*30*), although it is not clear which second residue, in addition to Ser201, of GlpG they form a covalent bond with. Therefore, GlpG variations with candidate residues mutated to Ala were prepared and incubated with the saccharin inhibitors. Based on the proposed mechanism, the LG should be released and the complexes formed should result in an identical mass shift of 195 Da. The complex was not formed upon His254 deletion implicating His254 as the second binding partner. On the other hand, the catalytic histidine is needed for proper activation of the catalytic serine (*46*) which may be another explanation for why the complex was not formed. Coupled with the fact that the expected change in the protein mobility using SDS-PAGE (*21, 22*) was not observed, this suggests the possibility that the doubly-linked product is either not formed or not stable, or that another nucleophile (apart from the His254) in closer proximity to the catalytic serine might be involved in the crosslinking. As the saccharins are slowly reversible inhibitors, the hypothesis including another binding partner is favoured.

A similar approach was utilised in the design of benzoxazin-4-one derived inhibitors. Thirteen candidates underwent molecular docking studies, again based on the structure of GlpG in complex with CAPF (*45*) and 2-styryl substituted compound 3 was shown to be the most promising derivative. Benzoxazinones also share a similar orientation with previously reported inhibitors. In this case, the styryl extension points towards the S2' subsite in the enzyme, as observed with previously characterised inhibitors (*44, 45*). *In vitro* experiments confirmed compound 3 as one of the most potent benzoxazinone derivatives, with an IC_{50} of 4 µM.

An electron withdrawing substituent increased the inhibitor activity, similarly to the saccharin inhibitors. The potency of the best benzoxazinone compound is one order of magnitude lower than that of the previously discussed saccharins, as well as of β-lactams (*19*) and isocoumarins (*22*). Benzoxazinones are known inhibitors of HLE, chymotrypsin and cathepsin G (*30, 35-38*). Surprisingly, the two most potent derivatives did not show any inhibitory activity against α-chymotrypsin up to 250 µM concentration. Neither did they inhibit bovine trypsin or human neutrophil elastase at 10 µM, but several inhibitory variants were able to decrease the activity of these enzymes to 50 % at 50 µM concentration. One of the inhibitors significantly reduced the activity of cathepsin G at 10 µM concentration. The specificity profile was not uniform for all variants tested, suggesting that these inhibitors could show some selectivity for GlpG over other serine proteases.

We also collaborated with another group interested in benzoxazine-4-on scaffold-based inhibitors (Steven Verhelst's group, University of Leuven, Leuven, Belgium) and these two papers were published together (*47, 48*). The Verhelst group used a different set of substituents attached to the benzoxazine-4-on core, the most effective of which were the 2-alkoxy substituents. Even though they showed slightly higher activity against GlpG, they lost the selectivity over bovine chymotrypsin and trypsin (*48*). Therefore, it would be interesting to fully understand the relationship between the inhibitor structure and its potency and selectivity for rhomboids, potentially leading to an inhibitor that combines the optimal features from each type. From the mechanical point of view, benzoxazinones bind covalently to the GlpG which was confirmed by a mass spectrometry by formation of a new peak of a mass corresponding to the expected rhomboid-inhibitor adduct. Their reversibility was confirmed by the dilution experiment.

As inhibitor potency is not the only important feature of the inhibitor that determines its suitability in cell biological assays (not to mention its potential therapeutic use), we tested the peptidyl ketoamides and saccharin inhibitors for their selectivity across about 100 human hydrolases, using the EnPlex method (*29*) and their selectivity across a panel of different rhomboid proteases (*28*). Both ketoamides and saccharins displayed preference for bacterial and archaeal rhomboid proteases. We also compared their selectivity in the EnPlex assay with β-lactam L41 (*19*) and isocoumarins S006 and S016 (*22*). Both ketoamides and saccharins have a better selectivity profile than both isocoumarins. What is more, ketoamides inhibited only two out of all the tested hydrolases (dipeptidyl-peptidase 2 and prolylcarboxypeptidase), L41 inhibited just one of them (probable serine carboxypeptidase). Together with the high potency of peptidyl ketoamides and their safe toxicological profile this makes them the most effective

rhomboid inhibitors available to date. Undoubtedly, this scaffold can be further developed to target the rhomboid proteases of medical interest, such as the mitochondrial rhomboid PARL or the Plasmodium rhomboid ROM4.

6 SUMMARY

In my thesis I presented several novel tools to study rhomboid proteases, namely new fluorogenic substrates, and potent and selective rhomboid inhibitors. In general, their main advantage is that they should be readily modifiable to target different rhomboids.

With respect to the fluorogenic substrate, our primary goal was to deliver tools that could be used in continuous rhomboid activity assays, would be well tolerated by many rhomboid proteases and would be suitable for HTS. We have met all of these requirements in the substrate presented in chapter 4.1, and subsequently demonstrated their practical use in identifying new rhomboid inhibitors. The substrates were used in *in vitro* assays to characterise the effects of different substituents on inhibitor activity as well as for the kinetic characterisation of the interaction between the most promising compounds and rhomboids.

Regarding the inhibitor design, I present in this thesis three new classes of rhomboid inhibitors. The peptidyl ketoamides are, to date, the best inhibitors available. Their high potency, selectivity and the fact that the same class of compounds is already used as therapeutics makes them ideal candidates for further drug development. Additional, we have contributed to the improvement of saccharin and benzoxazine-4-on scaffold based inhibitors. Their major advantage is an easy and cheap synthesis route which offers many possibilities for future structure-activity relationship studies that could lead to the development of a potent and selective inhibitor that could be readily synthesised in high yields.

The rhomboid inhibitors, particularly the peptidyl ketoamides, could also be used as a starting point in the search for potential therapeutic molecules against many diseases. Inhibition of parasite invasion of the host cells through inhibition of *Plasmodium* rhomboids might be a feasible malaria treatment (*49, 50*), mitochondrial rhomboid protein PARL probably cooperates on regulation of mitophagy, therefore inhibition of PARL might stimulate mitophagy and potentially decrease the pathological changes associated with Parkinson's disease (*51, 52*), and regulation of EGFR signalling through RBHDL4 could be beneficial in the treatment of colorectal cancer (*53*). Furthermore it is tempting to speculate that inhibitors of active rhomboids could also be tools for the study of the rhomboid pseudoproteases, whose mechanisms remain largely unclear but like contain a structural equivalent of a rhomboid protease active site cavity (in most cases lacking the catalytic residues though).

7 REFERENCES

1. G. Jurgens, E. Wieschaus, C. Nussleinvolhard, H. Kluding, Mutations Affecting the Pattern of the Larval Cuticle in Drosophila-Melanogaster .2. Zygotic Loci on the 3rd Chromosome. *Roux Arch Dev Biol* **193**, 283-295 (1984).
2. E. Bier, L. Y. Jan, Y. N. Jan, Rhomboid, a gene required for dorsoventral axis establishment and peripheral nervous-system development in *Drosophila melanogaster Genes Dev* **4**, 190-203 (1990).
3. A. Guichard *et al.*, Rhomboid and Star interact synergistically to promote EGFR/MAPK signaling during Drosophila wing vein development. *Development* **126**, 2663-2676 (1999).
4. S. Urban, J. R. Lee, M. Freeman, Drosophila Rhomboid-1 defines a family of putative intramembrane serine proteases. *Cell* **107**, 173-182 (2001).
5. J. R. Lee, S. Urban, C. F. Garvey, M. Freeman, Regulated intracellular ligand transport and proteolysis control EGF signal activation in Drosophila. *Cell* **107**, 161-171 (2001).
6. S. Dusterhoft, U. Kunzel, M. Freeman, Rhomboid proteases in human disease: Mechanisms and future prospects. *Biochimica et Biophysica Acta - Molecular Cell Research* **1864**, 2200-2209 (2017).
7. M. K. Lemberg, M. Freeman, Functional and evolutionary implications of enhanced genomic analysis of rhomboid intramembrane proteases. *Genome Research* **17**, 1634-1646 (2007).
8. S. Maegawa, K. Ito, Y. Akiyama, Proteolytic action of GlpG, a rhomboid protease in the Escherichia coli cytoplasmic membrane. *Biochemistry* **44**, 13543-13552 (2005).
9. D. O. Daley *et al.*, Global topology analysis of the Escherichia coli inner membrane proteome. *Science* **308**, 1321-1323 (2005).
10. E. V. Koonin *et al.*, The rhomboids: a nearly ubiquitous family of intramembrane serine proteases that probably evolved by multiple ancient horizontal gene transfers. *Genome Biology* **4**, R19 (2003).
11. M. K. Lemberg *et al.*, Mechanism of intramembrane proteolysis investigated with purified rhomboid proteases. *Embo J* **24**, 464-472 (2005).
12. K. Strisovsky, H. J. Sharpe, M. Freeman, Sequence-Specific Intramembrane Proteolysis: Identification of a Recognition Motif in Rhomboid Substrates. *Molecular Cell* **36**, 1048-1059 (2009).
13. S. W. Dickey, R. P. Baker, S. Cho, S. Urban, Proteolysis inside the Membrane Is a Rate-Governed Reaction Not Driven by Substrate Affinity. *Cell* **155**, 1270-1281 (2013).
14. S. Cho, S. W. Dickey, S. Urban, Crystal Structures and Inhibition Kinetics Reveal a Two-Stage Catalytic Mechanism with Drug Design Implications for Rhomboid Proteolysis. *Molecular Cell* **61**, 329-340 (2016).
15. M. Shokhen, A. Albeck, How does the exosite of rhomboid protease affect substrate processing and inhibition? *Protein Science* **26**, 2355-2366 (2017).
16. K. R. Vinothkumar *et al.*, The structural basis for catalysis and substrate specificity of a rhomboid protease. *Embo J* **29**, 3797-3809 (2010).
17. Y. Xue, Y. Ha, Catalytic Mechanism of Rhomboid Protease GlpG Probed by 3,4-Dichloroisocoumarin and Diisopropyl Fluorophosphonate. *J Biol Chem* **287**, 3099-3107 (2012).
18. S. Urban, M. S. Wolfe, Reconstitution of intramembrane proteolysis in vitro reveals that pure rhomboid is sufficient for catalysis and specificity. *Proc Natl Acad Sci U S A* **102**, 1883-1888 (2005).
19. O. A. Pierrat *et al.*, Monocyclic beta-Lactams Are Selective, Mechanism-Based Inhibitors of Rhomboid Intramembrane Proteases. *ACS Chem Biol* **6**, 325-335 (2011).
20. E. Arutyunova *et al.*, Allosteric regulation of rhomboid intramembrane proteolysis. *Embo J* **33**, 1869-1881 (2014).
21. S. Zoll *et al.*, Substrate binding and specificity of rhomboid intramembrane protease revealed by substrate-peptide complex structures. *Embo J* **33**, 2408-2421 (2014).
22. O. Vosyka *et al.*, Activity-based probes for rhomboid proteases discovered in a mass spectrometry-based assay. *Proc Natl Acad Sci U S A* **110**, 2472-2477 (2013).
23. Y. Akiyama, S. Maegawa, Sequence features of substrates required for cleavage by GlpG, an Escherichia coli rhomboid protease. *Molecular Microbiology* **64**, 1028-1037 (2007).
24. A. Ticha *et al.*, Sensitive Versatile Fluorogenic Transmembrane Peptide Substrates for Rhomboid Intramembrane Proteases. *J Biol Chem* **292**, 2703-2713 (2017).
25. S. Chatterjee *et al.*, P2-achiral, P'-extended alpha-ketoamide inhibitors of calpain I. *Bioorg Med Chem Lett* **9**, 2371-2374 (1999).
26. Y. Liu *et al.*, Hepatitis C NS3 protease inhibition by peptidyl-alpha-ketoamide inhibitors: kinetic mechanism and structure. *Arch Biochem Biophys* **421**, 207-216 (2004).
27. F. G. Njoroge, K. X. Chen, N. Y. Shih, J. J. Piwinski, Challenges in modern drug discovery: A case study of boceprevir, an HCV protease inhibitor for the treatment of hepatitis C virus infection. *Accounts Chem Res* **41**, 50-59 (2008).

28. E. V. Wolf, A. Zeissler, S. H. Verhelst, Inhibitor Fingerprinting of Rhomboid Proteases by Activity-Based Protein Profiling Reveals Inhibitor Selectivity and Rhomboid Autoprocessing. *ACS Chem Biol* **10**, 2325-2333 (2015).

29. D. A. Bachovchin *et al.*, A high-throughput, multiplexed assay for superfamily-wide profiling of enzyme activity. *Nature Chemical Biology* **10**, 656-663 (2014).

30. J. C. Powers, J. L. Asgian, O. D. Ekici, K. E. James, Irreversible inhibitors of serine, cysteine, and threonine proteases. *Chemical Reviews* **102**, 4639-4750 (2002).

31. B. Walker, J. F. Lynas, Strategies for the inhibition of serine proteases. *Cell Mol Life Sci* **58**, 596-624 (2001).

32. D. J. Hlasta *et al.*, A novel class of cyclic beta-dicarbonyl leaving groups and their use in the design of benzisothiazolone human leukocyte elastase inhibitors. *J Med Chem* **38**, 4687-4692 (1995).

33. M. Zimmerman *et al.*, Inhibition of elastase and other serine proteases by heterocyclic acylating agents. *J Biol Chem* **255**, 9848-9851 (1980).

34. W. C. Groutas *et al.*, Design, synthesis, and in vitro inhibitory activity toward human leukocyte elastase, cathepsin G, and proteinase 3 of saccharin-derived sulfones and congeners. *Bioorg Med Chem* **4**, 1393-1400 (1996).

35. M. Gutschow *et al.*, 2-(diethylamino)thieno1,3oxazin-4-ones as stable inhibitors of human leukocyte elastase. *J Med Chem* **42**, 5437-5447 (1999).

36. M. Gutschow, U. Neumann, Inhibition of cathepsin G by 4H-3,1-benzoxazin-4-ones. *Bioorg Med Chem* **5**, 1935-1942 (1997).

37. A. Krantz *et al.*, Design and synthesis of 4H-3,1-benzoxazin-4-ones as potent alternate substrate inhibitors of human leukocyte elastase. *J Med Chem* **33**, 464-479 (1990).

38. T. Teshima, J. C. Griffin, J. C. Powers, A new class of heterocyclic serine protease inhibitors. Inhibition of human leukocyte elastase, porcine pancreatic elastase, cathepsin G, and bovine chymotrypsin A alpha with substituted benzoxazinones, quinazolines, and anthranilates. *J Biol Chem* **257**, 5085-5091 (1982).

39. A. Simeonov *et al.*, Fluorescence spectroscopic profiling of compound libraries. *J Med Chem* **51**, 2363-2371 (2008).

40. M. K. Lemberg, M. Freeman, Cutting proteins within lipid bilayers: rhomboid structure and mechanism. *Molecular Cell* **28**, 930-940 (2007).

41. S. Mahrus *et al.*, Global sequencing of proteolytic cleavage sites in apoptosis by specific labeling of protein N termini. *Cell* **134**, 866-876 (2008).

42. A. J. O'Donoghue *et al.*, Global identification of peptidase specificity by multiplex substrate profiling. *Nature Methods* **9**, 1095-1100 (2012).

43. J. D. Lapek, Jr. *et al.*, Quantitative Multiplex Substrate Profiling of Peptidases by Mass Spectrometry. *Mol Cell Proteomics*, (2019).

44. K. R. Vinothkumar, O. A. Pierrat, J. M. Large, M. Freeman, Structure of Rhomboid Protease in Complex with beta-Lactam Inhibitors Defines the S2 ' Cavity. *Structure* **21**, 1051-1058 (2013).

45. Y. Xue *et al.*, Conformational Change in Rhomboid Protease GlpG Induced by Inhibitor Binding to Its S ' Subsites. *Biochemistry* **51**, 3723-3731 (2012).

46. Y. Ha, Y. Akiyama, Y. Xue, Structure and Mechanism of Rhomboid Protease. *J Biol Chem* **288**, 15430-15436 (2013).

47. P. Goel *et al.*, Discovery and validation of 2-styryl substituted benzoxazin-4-ones as a novel scaffold for rhomboid protease inhibitors. *Bioorg Med Chem Lett* **28**, 1417-1422 (2018).

48. J. Yang *et al.*, Benzoxazin-4-ones as novel, easily accessible inhibitors for rhomboid proteases. *Bioorg Med Chem Lett* **28**, 1423-1427 (2018).

49. R. P. Baker, R. Wijetilaka, S. Urban, Two Plasmodium rhomboid proteases preferentially cleave different adhesins implicated in all invasive stages of malaria. *PLoS Pathog* **2**, 922-932 (2006).

50. J. W. Lin *et al.*, Loss-of-function analyses defines vital and redundant functions of the Plasmodium rhomboid protease family. *Molecular Microbiology* **88**, 318-338 (2013).

51. C. Meissner, H. Lorenz, B. Hehn, M. K. Lemberg, Intramembrane protease PARL defines a negative regulator of PINK1- and PARK2/Parkin-dependent mitophagy. *Autophagy* **11**, 1484-1498 (2015).

52. E. Y. L. Chan, G. A. McQuibban, The mitochondrial rhomboid protease: Its rise from obscurity to the pinnacle of disease-relevant genes. *Biochimica et Biophysica Acta - Biomembranes* **1828**, 2916-2925 (2013).

53. W. Song *et al.*, Rhomboid domain containing 1 promotes colorectal cancer growth through activation of the EGFR signalling pathway. *Nat Commun* **6**, (2015).

8 LIST OF PUBLICATIONS

Publications related to dissertation project

a) Research articles

1. Yang, J., Barniol-Xicota, M., Nguyen, M. T. N., **Ticha, A.**, Strisovsky, K., Verhelst, S. H. L. (2018): Benzoxazin-4-ones as novel, easily accessible inhibitors for rhomboid proteases. *Bioorganic and Medicinal Chemistry Letters* **28** (8), 1423-1427. **IF(2017) = 2.442**

2. Goel, P., Jumpertz, T., **Ticha, A.**, Ogorek, I., Mikles, D. C., Hubalek, M., Pietrzik, C. U., Strisovsky, K., Schmidt, B., Weggen, S. (2018): Discovery and validation of 2-styryl substituted benzoxazin-4-ones as a novel scaffold for rhomboid protease inhibitors. *Bioorganic and Medicinal Chemistry Letters* **28** (8), 1417-1422. **IF(2017) = 2.442**

3. **Ticha, A.**, Stanchev, S., Vinothkumar, K. R., Mikles, D. C., Pachl, P., Began, J., Skerle, J., Svehlova, K., Nguyen, M. T. N., Verhelst, S. H. L., Johnson, D. C., Bachovchin, D. A., Lepsik, M., Majer, P., Strisovsky, K. (2017): General and Modular Strategy for Designing Potent, Selective, and Pharmacologically Compliant Inhibitors of Rhomboid Proteases. *Cell Chemical Biology* **24** (12), 1523-1536. **IF(2017) = 5.592**

4. **Ticha, A.**, Stanchev, S., Skerle, J., Began, J., Ingr, M., Svehlova, K., Polovinkin, L., Ruzicka, M., Bednarova, L., Hadravova, R., Polachova, E., Rampirova, P., Brezinova, J., Kasicka, V., Majer, P., Strisovsky, K. (2017): Sensitive Versatile Fluorogenic Transmembrane Peptide Substrates for Rhomboid Intramembrane Proteases. *Journal of Biological Chemistry* **292** (7), 2703-2713. **IF(2017) = 4.010**

5. Goel, P., Jumpertz, T., Mikles, D. C., **Ticha, A.**, Nguyen, M. T. N., Verhelst, S., Hubalek, M., Johnson, D. C., Bachovchin, D. A., Ogorek, I., Pietrzik, C. U., Strisovsky, K., Schmidt, B., Weggen, S. (2017): Discovery and Biological Evaluation of Potent and Selective N-Methylene Saccharin-Derived Inhibitors for Rhomboid Intramembrane Proteases. *Biochemistry* **56** (51), 6713-6725. **IF(2017) = 2.997**

b) Review articles

   Ticha, A., Collis, B., Strisovsky, K. (2018): The Rhomboid Superfamily: Structural Mechanisms and Chemical Biology Opportunities. *Trends in Biochemical Sciences* **43** (9), 726-739. **IF(2017) = 15.678**

Other publications

1. Prazienkova, V., **Ticha, A.**, Blechova, M., Spolcova, A., Zelezna, B., Maletinska, L. (2016): Pharmacological characterization of lipidized analogs of prolactin-releasing peptide with a modified C-terminal aromatic ring. *Journal of Physiology and Pharmacology* **67** (1), 121-128.

2. Maletinska, L., Nagelova, V., **Ticha, A.**, Zemenova, J., Pirnik, Z., Holubova, M., Spolcova, A., Mikulaskova, B., Blechova, M., Sykora, D., Lacinova, Z., Haluzik, M., Zelezna, B., Kunes, J. (2015): Novel lipidized analogs of prolactin-releasing peptide have prolonged half-lives and exert anti-obesity effects after peripheral administration. *International Journal of Obesity* **39** (6), 986-993, doi:10.1038/ijo.2015.28.

3. Maletinska, L., **Ticha, A.**, Nagelova, V., Spolcova, A., Blechova, M., Elbert, T., Zelezna, B. (2013): Neuropeptide FF analog RF9 is not an antagonist of NPFF receptor and decreases food intake in mice after its central and peripheral administration. *Brain Research* **1498**, 33-40, doi:10.1016/j.brainres.2012.12.037.