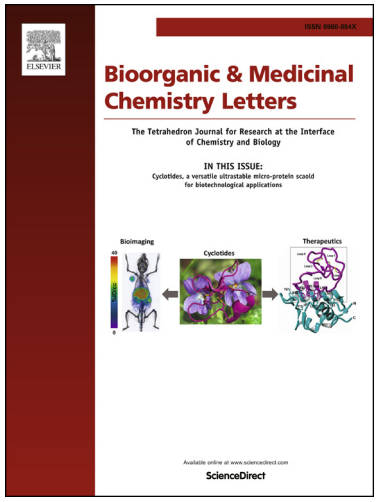
Accepted Manuscript



Benzoxazin-4-ones as novel, easily accessible inhibitors for rhomboid proteases

Jian Yang, Marta Barniol-Xicota, Minh T.N. Nguyen, Anezka Ticha, Kvido Strisovsky, Steven H.L. Verhelst

PII: S0960-894X(17)31230-1
DOI: https://doi.org/10.1016/j.bmcl.2017.12.056
Reference: BMCL 25512

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 14 November 2017
Revised Date: 21 December 2017
Accepted Date: 23 December 2017

Please cite this article as: Yang, J., Barniol-Xicota, M., Nguyen, M.T.N., Ticha, A., Strisovsky, K., Verhelst, S.H.L., Benzoxazin-4-ones as novel, easily accessible inhibitors for rhomboid proteases, *Bioorganic & Medicinal Chemistry Letters* (2017), doi: https://doi.org/10.1016/j.bmcl.2017.12.056

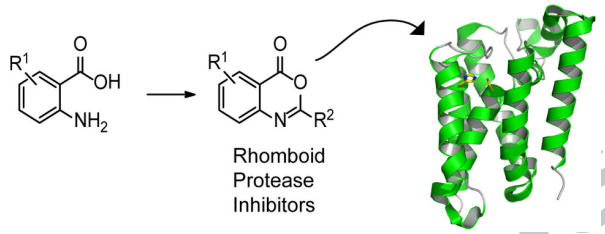
Graphical Abstract

To create your abstract, type over the instructions in the template box below.
Fonts or abstract dimensions should not be changed or altered.

Benzoxazin-4-ones as novel, easily accessible inhibitors for rhomboid proteases

Leave this area blank for abstract info.

Jian Yang[a], Marta Barniol-Xicota[a], Minh T. N. Nguyen[b], Anezka Ticha[c], Kvido Strisovsky[c] and Steven H. L. Verhelst[a,]



Bioorganic & Medicinal Chemistry Letters
journal homepage: www.elsevier.com

Benzoxazin-4-ones as novel, easily accessible inhibitors for rhomboid proteases

Jian Yang[a], Marta Barniol-Xicota[a], Minh T. N. Nguyen[b], Anezka Ticha[c], Kvido Strisovsky[c] and Steven H. L. Verhelst[a,b,*]

[a] *KU Leuven – University of Leuven, Laboratory of Chemical Biology, Department of Cellular & Molecular Medicine, Herestraat 49 Box 802, 3000 Leuven, Belgium*
[b] *Leibniz Institute for Analytical Sciences ISAS, Otto-Hahn-Str. 6b, 44227 Dortmund, Germany*
[c] *Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo n. 2, Prague 166 10, Czech Republic*

ARTICLE INFO

Article history:
Received
Revised
Accepted
Available online

Keywords:
Rhomboid proteases
Intramembrane proteases
Benzoxazinones
Inhibitors
Activity-based protein profiling

ABSTRACT

Rhomboid proteases form one of the most widespread intramembrane protease families. They have been implicated in variety of human diseases. The currently reported rhomboid inhibitors display some selectivity, but their construction involves multistep synthesis protocols. Here, we report benzoxazin-4-ones as novel inhibitors of rhomboid proteases with a covalent, but slow reversible inhibition mechanism. Benzoxazin-4-ones can be synthesized from anthranilic acid derivatives in a one-step synthesis, making them easily accessible. We demonstrate that an alkoxy substituent at the 2-position is crucial for potency and results in low micromolar inhibitors of rhomboid proteases. Hence, we expect that these compounds will allow rapid synthesis and optimization of inhibitors of rhomboids from different organisms.

Rhomboid proteases are amongst the most widespread intramembrane protease (IMP) families, and have been found in virtually all sequenced organisms.[1, 2] They utilize a serine-histidine dyad to cleave their substrates, which are also membrane proteins, in a transmembrane helix (TM) or a juxtamembrane region. Rhomboid proteases were originally discovered in *Drosophila melanogaster*.[3] In this organism, Rhomboid-1 is a vital player in the epidermal growth factor receptor pathway. Various biological roles in other organisms have been discovered since then, such as the mediation of quorum sensing by processing of a component of the twin arginine translocase in the bacterium *Providencia stuartii*[4] and ER associated degradation of membrane proteins.[5] More medically relevant roles of rhomboid proteases include cleavage of adhesins that help apicomplexan parasites invade host cells,[6, 7] and processing of Pink-1, a protein involved in mitophagy and linked to Parkinson's disease.[8-10] Potent and selective inhibitors would be attractive research tools to examine these processes and assess the drugability of rhomboids.

After protocols had been established for the expression and purification of rhomboid proteases in detergent micelles,[11, 12] several different types of inhibitors were discovered,[13, 14] all of which contain an electrophile that reacts with the serine nucleophile in the active site. These inhibitors include 4-chloro-isocoumarins,[15, 16] fluorophosphonates,[17, 18] β-lactams,[19, 20] β-lactones,[21] and peptidyl chloromethyl ketones,[22] aldehydes[23] and ketoamides (Figure 1A).[24]

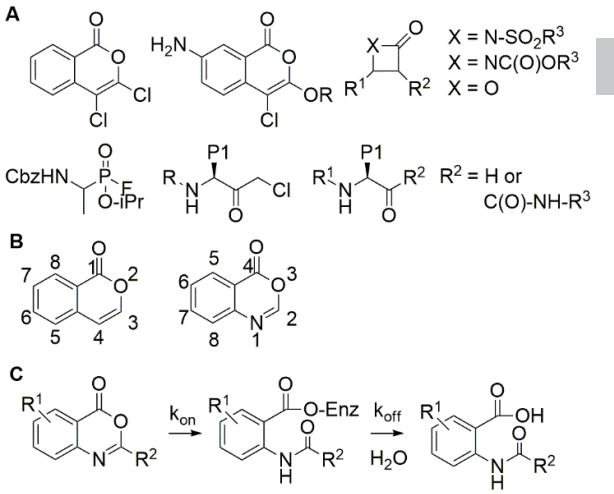
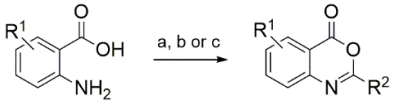


Figure 1. (a) Overview of known rhomboid inhibitors. The general serine protease inhibitor 3,4-dichloroisocoumarin (top left), the 4-chloro-isocoumarin scaffold with a 7-amino group (top middle), β-lactams and β-lactones (top right), the fluorophosphonate CAPF (bottom left), peptide chloromethyl ketones (bottom middle) and peptide aldehydes (bottom right). (b) Comparison of the isocoumarin and benzoxazin-4-one scaffolds. (c) Mechanism of the inactivation of serine proteases by benzoxazinones and their reactivation upon hydrolyss of the inhibitor acyl-enzyme complex.

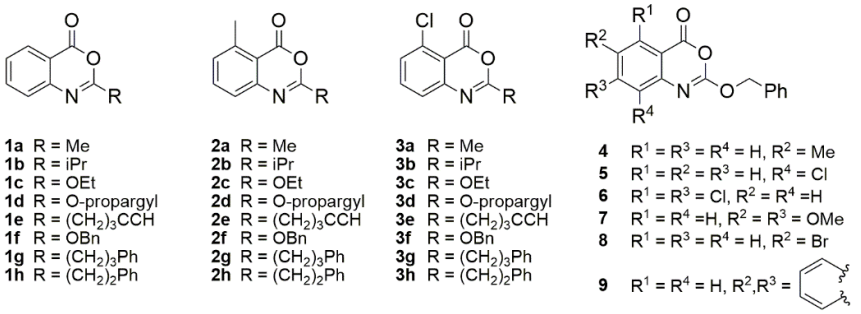
Whereas 3,4-dichloroisocoumarin (DCI) and the fluorophosphonate-based activity-based probe FP-Rh are pan-inhibitors of rhomboid proteases, β-lactones and other chloro-isocoumarins have shown some degree of selectivity.[25] During the course of this work, ketoamides were reported as a scaffold with selectivity against the E. coli protease GlpG over other, soluble serine proteases.[24]

Synthetically, most of these rhomboid inhibitors are not very easily accessible and require multistep organic synthesis. We therefore decided to seek for an inhibitor scaffold that would be easier to synthesize. Benzoxazin-4-ones (Figure 1B) are heterocyclic, mechanism-based inhibitors of soluble serine proteases.[26] Attack on the carbonyl by the active site serine leads to acylation of the serine side chain, similar as with isocoumarins (Figure 1C). Usually, the ester bond between protease and inhibitor hydrolyses over time, and the potency of benzoxazin-4-ones in part depends on the rate of deacylation (Figure 1C).[26] Conveniently, benzoxazin-4-ones can be made in one synthetic step from substituted anthranilic acids and acid anhydrides, acid chlorides or chloroformates. The two fused aromatic ring structures in this class of inhibitors offer the possibility of broad chemical variation and optimization for a particular target protease. Since the first report of benzoxazin-4-ones as serine protease inhibitors,[27] various publications have followed, for example on inhibition of human leukocyte elastase,[28, 29] cathepsin G,[30] chymase,[31] and the complement system C1r serine protease.[32] We hypothesized that benzoxazinones may also inhibit rhomboid proteases, because of the structural analogy to 4-chloro-isocoumarins (Figure 1B). From medicinal chemistry point-of-view, it is of interest to note that benzoxazinones with similar substitution pattern as 4-chloro-isocoumarins have a slightly lower ClogP (Supplemental Figure 1). Rhomboid inhibitors of the 4-chloro-isocoumarin class carry small and large hydrophobic groups at the 3-position, which corresponds to the 2-position in the benzoxazin-4-one scaffold. We therefore selected a range of chloroformates and activated carboxylic acids to yield these type of substituents and increase the likelihood of obtaining potent rhomboid inhibitors.

We also chose a number of commercially available anthranilic acid derivatives to introduce variety at the aryl ring. For soluble serine proteases, the substituents at the aryl ring influence the rate of deacylation. It was reported that steric bulk at the 5-position slows down the deacylation step for human leukocyte elastase.[28] A 5-methyl group slows down this step for cathepsin G.[30] For HSV-1 protease, a 5-chloro substituent generally led to lower IC_{50} values.[33] We therefore chose the anthranilic acid precursors to include 5-chloro and 5-methyl groups, but we also selected a variety of other substituents to explore the influence on the potency against rhomboid proteases.



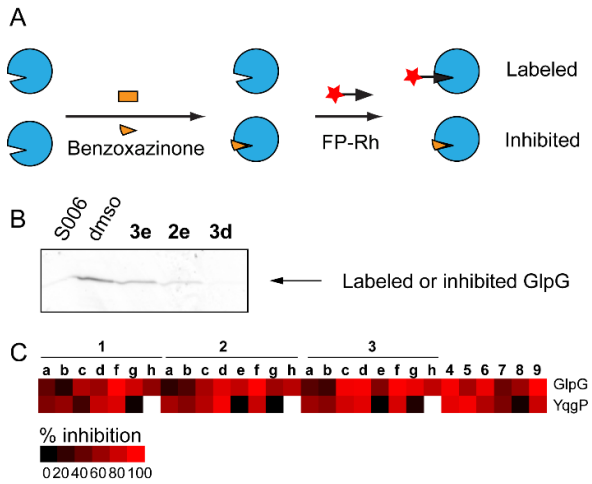
Scheme 1. Synthesis of benzoxazin-4-ones from anthranilic acids. *Reagents and* conditions: (a) Acetic anhydride, 130 °C, 86-87%. (b) Acid chloride, triethylamine, dichloromethane, 0 °C to room temperature, 13-98%. (c) Chloroformate, pyridine, room temperature, 6-57%.



Scheme 1 outlines the synthesis of the benzoxazinones we designed above. An overview of all synthesized compounds is given in Figure 2. Compounds **1a-3a** were made from the corresponding anthranilic acid derivative and acetic anhydride under reflux conditions (Scheme 1). The other benzoxazinones were constructed by reacting the anthranilic acids with acid chlorides or chloroformates at 0 °C to room temperature, using triethylamine or pyridine as a base. All designed benzoxazinones were successfully synthesized, except for compound **1e**. The reason for this is unclear, as the close analogs **2e** and **3e** were both obtained in 86% yield.

Figure 2. Overview of benzoxazin-4-ones used in this study.

Having the benzoxazinones at hand, we performed an inhibition assay against the *E. coli* rhomboid GlpG and the *B. subtilis* rhomboid YqgP by competitive activity-based protein profiling (ABPP). In competitive ABPP, protease samples are first treated with the compound to be tested and then treated with an activity-based probe (ABP) to label the remaining amount of



active protease (Figure 3A). Depending on the tag of the ABP, detection can take place in various manners. Here, we used FP-Rh, an ABP that reacts with the majority of all serine hydrolases[34] by means of its fluorophosphonate reactive group. It is also a suitable pan-rhomboid probe.[25] Hence, GlpG or YqgP were first incubated with the different benzoxazinone analogs for 30 min, subsequently incubated with FP-Rh for an additional 60 min, followed by SDS-PAGE. Whereas labeling of DMSO vehicle treated GlpG gave clear labeling by FP-Rh, the known isocoumarin inhibitor S006, which reacts with the active site serine and an additional histidine,[16] led to almost complete abrogation of the signal (Figure 3B). To our delight, we found that several benzoxazinones also inhibited GlpG and YqgP (Figure 3B and 3C).

Figure 3. Inhibition assay by competitive ABPP. (a) Schematic representation of competitive ABPP for rhomboid proteases. A purified rhomboid protease is treated with a benzoxazinone. Subsequently, the residual active rhomboid is labeled by means of the ABP FP-Rh. (b) Active GlpG is efficiently labeled by FP-Rh and visualized by in-gel fluorescent scanning. Treatment with GlpG inhibitor S006 or different benzoxazinones leads to various degrees of inhibition. (c) The residual activities of GlpG and YqgP treated with the indicated benzoxazinones, as quantified by gel band density and visualized in a heat map representation.

For compounds that displayed lower than 5% residual activity in the initial screen, we determined the apparent IC_{50} value against the two rhomboids and two commercially available soluble serine proteases (Table 1). The potency against the two rhomboids is in the low micromolar to submicromolar range. Compound **2f**, the most potent benzoxazinone for GlpG, displays an apparent IC_{50} of 1.0 ± 0.64 µM. Benzoxazinone **5** turned out to be the most potent compound for YqgP with an apparent IC_{50} of 0.47 ± 0.16 µM. These potencies are comparable with those of the most potent 4-chloroisocoumarins[16] and β-lactams.[19, 20]

Table 1. Apparent IC_{50} values (µM) of selected benzoxazin-4-ones.

Compound	GlpG	YqgP	Trypsin	Chymotrypsin
1f	2.5 ± 0.34	3.7 ± 1.2	< 0.1	< 0.1
2d	N.D.[a]	2.8 ± 0.59	1.1 ± 0.11	< 0.1
2f	1.0 ± 0.64	15 ± 6.0	< 0.1	< 0.1
3c	2.2 ± 0.47	7.1 ± 1.5	< 0.1	< 0.1
3d	1.7 ± 0.34	6.2 ± 2.6	1.2 ± 0.12	0.41 ± 0.24
3f	1.2 ± 0.45	1.6 ± 0.38	< 0.1	< 0.1
4	N.D.	4.6 ± 1.6	< 0.1	< 0.1
5	63 ± 18	0.47 ± 0.16	< 0.1	< 0.1
9	2.4 ± 1.2	7.3 ± 2.3	< 0.1	< 0.1

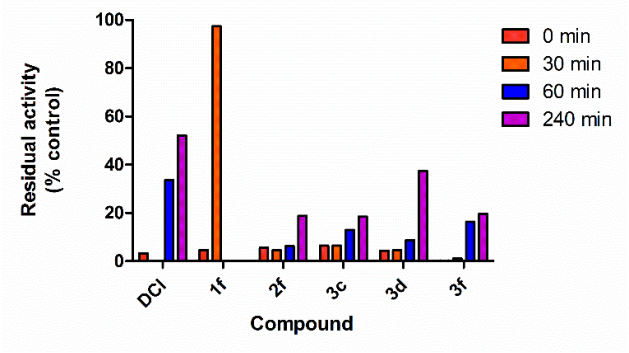
[a]Not determined.

From the series of compounds tested, we were able to derive a basic structure-activity relationship. The oxygen substituent on the 2-position seems to be crucial for the potency, as all compounds that showed low micromolar activity, carried an alkoxy substitutent at the 2-position. Notably, compounds **2f** and **3f**, which both carry an OCH_2Ph substituent at the 2-position, display low micromolar activity, whereas the isosteric compounds **2h** and **3h**, which have a CH_2CH_2Ph group at this position, did not even make the cut-off in the general screen at 100 µM compound concentration (Figure 3C), clearly indicating the requirement for the oxygen atom.

Interestingly, some benzoxazinones are able to discriminate between the different rhomboids. For example, **2f** inhibits GlpG with an IC_{50} value in the low micromolar range, while being only moderately active against YqgP, representing a selectivity of 15 fold. Compound **5** shows an apparent IC_{50} of 0.47 ± 0.16 µM for YqgP and approximately 60 µM for GlpG, corresponding to a selectivity of 130 fold. Since benzoxazinones have been reported as inhibitors of soluble serine proteases, we checked whether the rhomboid hits also inhibit two model serine proteases: bovine trypsin and chymotrypsin. We found that these benzoxazinones displayed higher potencies against these proteases than against rhomboids. Nevertheless, compound **3d** shows inhibition in the same order of magnitude for both rhomboids and soluble proteases, and for compound **2d**, the potency for YqgP and trypsin is comparable. In an accompanying paper (see co-submitted paper by Goel, Weggen and colleagues), benzoxazinones with a 2-styryl substituent show some selectivity against rhomboids, but have lower potency than the here described compounds with a 2-alkoxy substituent. This illustrates that the substituents on the benzoxazinones have an influence on both potency and selectivity, and further derivatization may lead to better properties. To gain further insight into this, the selectivity of benzoxazinones against a broad panel of serine proteases and hydrolases should be explored in future experiments.

For some of the most potent benzoxazinones, we checked whether these were able to inhibit GlpG *in vivo* using *E. coli* cells with a permeabilized outer membrane.[35, 36] Although compounds **3c**, **3d**, **3f** did not show any or only weak (**1f**) inhibition, we found that compound **2f** showed an IC_{50} value of 8.5 ± 0.17 µM (see Supplemental Figure 2), illustrating the capacity of benzoxazinones to inhibit rhomboid proteases in a membrane environment of living cells.

For soluble proteases, benzoxazin-4-ones are covalent inhibitors with a reversible mechanism: they acylate the active site serine to give an acyl intermediate, which can be hydrolyzed to re-form the active protease species (Figure 1C). To get insight into the mechanism of inhibition of rhomboids by benzoxazinones, we checked whether the covalent *O*-acyl enzyme-inhibitor complex was able to hydrolyze and regain activity. Therefore, we incubated GlpG with compounds DCI, **1f**, **2f, 3c**, **3d** or **3f** at a concentration that gives full inhibition. Then, the excess of inhibitors was removed from the solution by a gel filtration spin column, followed by 0 min, 30 min, 1 h, or 4 h incubation in order to allow hydrolysis of the acyl intermediate. Subsequently, the residually active GlpG was detected by ABPP using the FP-Rh probe as in our previous experiments. As shown in Figure 4, GlpG treated with DCI regained 50% of its activity after 4h of incubation. Only with compound **1f**, GlpG regained activity more rapidly: in 30 min, almost the full activity was restored. For the other benzoxazinones, the hydrolysis of the O-acyl intermediate was much slower and after 4 h, most inhibition was maintained. Interestingly, the only difference between the fast hydrolyzing **1f** and the more stable **2f** and **3f** is the presence of a 5-methyl or 5-chloro-substituent, respectively. It has been suggested that after reaction with the active site serine and ring opening of the benzoxazinone scaffold, the carbonyl group



moves out of the plane of the ring and can be shielded by the substituent in the 5-position against attack by a water molecule.[28] Another possibility is the binding of the 5-substituent in the S1 pocket,[15, 22] yielding a more stable complex and possibly blocking the access of water from the water retention site. Only a structure of GlpG with a bound benzoxazinone may give an answer to this question. All in all, the results shown here indicate that most benzoxazinones are slowly reversible rhomboid inhibitors with an off-rate lower than DCI.

Figure 4. Residual activities of GlpG, respectively, 0, 30, 60 and 240 min after removing the excess of inhibitors from the enzyme-inhibitor complex.

In conclusion, we have shown in this paper that benzoxazin-4-ones are micromolar inhibitors of rhomboid proteases. Importantly, all compounds can be obtained by a one-step synthesis from commercially available and low-cost starting materials. In this way, we have synthesized 29 benzoxazin-4-one structures and tested them against two model rhomboids. The most potent activities of the compounds synthesized here are in the micromolar range and one compound displayed *in vivo* activity. Inspection of the active structures revealed that the substituents have substantial impact on the potency. Specifically, a 2-alkoxy substituent seems critical for activity. We have also shown that the off-rate of the covalent ester intermediate is on the timescale of hours. Because of the easy synthesis, we expect that benzoxazin-4-ones offer the possibility for future optimization of potency and selectivity against rhomboid proteases.

Acknowledgments

We thank Dr. E. Ruijter and Prof. R.V.A. Orru (Vrije Universiteit Amsterdam) for providing some initial benzoxazinones for activity screening. We acknowledge funding by the Chinese Scholarship Council (to JY), H2020 (Marie Curie Fellowship to MBX), the Deutsche Forschungsgemeinschaft, the the Ministerium für Innovation, Wissenschaft und Forschung des Landes Nordrhein-Westfalen, the Senatsverwaltung für Wirtschaft, Technologie und Forschung des Landes Berlin and the Bundesministerium für Bildung und Forschung. KS acknowledges support from EMBO (Installation Grant no. 2329), Ministry of Education, Youth and Sports of the Czech Republic (projects no. LK11206 and LO1302), Marie Curie Career Integration Grant (project no. 304154), and the National Subvention for Development of Research Organisations (RVO: 61388963) to the Institute of Organic Chemistry and Biochemistry.

References and notes

1. Koonin EV, Makarova KS, Rogozin IB, Davidovic L, Letellier MC, Pellegrini L. The rhomboids: a nearly ubiquitous family of intramembrane serine proteases that probably evolved by multiple ancient horizontal gene transfers. *Genome Biology.* 2003;4(3): R19.
2. Lemberg MK, Freeman M. Functional and evolutionary implications of enhanced genomic analysis of rhomboid intramembrane proteases. *Genome Res.* 2007;17(11): 1634-1646.
3. Urban S, Lee JR, Freeman M. Drosophila Rhomboid-1 defines a family of putative intramembrane serine proteases. *Cell.* 2001;107(2): 173-182.
4. Stevenson LG, Strisovsky K, Clemmer KM, Bhatt S, Freeman M, Rather PN. Rhomboid protease AarA mediates quorum-sensing in Providencia stuartii by activating TatA of the twin-arginine translocase. *Proc Natl Acad Sci U S A.* 2007;104(3): 1003-1008.
5. Fleig L, Bergbold N, Sahasrabudhe P, Geiger B, Kaltak L, Lemberg MK. Ubiquitin-dependent intramembrane rhomboid protease promotes ERAD of membrane proteins. *Mol Cell.* 2012;47(4): 558-569.
6. Baker RP, Wijetilaka R, Urban S. Two Plasmodium rhomboid proteases preferentially cleave different adhesins implicated in all invasive stages of malaria. *PLoS Pathog.* 2006;2(10): e113.
7. O'Donnell RA, Hackett F, Howell SA, et al. Intramembrane proteolysis mediates shedding of a key adhesin during erythrocyte invasion by the malaria parasite. *J Cell Biol.* 2006;174(7): 1023-1033.
8. Jin SM, Lazarou M, Wang C, Kane LA, Narendra DP, Youle RJ. Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. *J Cell Biol.* 2010;191(5): 933-942.
9. Meissner C, Lorenz H, Weihofen A, Selkoe DJ, Lemberg MK. The mitochondrial intramembrane protease PARL cleaves human Pink1 to regulate Pink1 trafficking. *J Neurochem.* 2011;117(5): 856-867.
10. Shi G, Lee JR, Grimes DA, et al. Functional alteration of PARL contributes to mitochondrial dysregulation in Parkinson's disease. *Hum Mol Genet.* 2011;20(10): 1966-1974.
11. Lemberg MK, Menendez J, Misik A, Garcia M, Koth CM, Freeman M. Mechanism of intramembrane proteolysis investigated with purified rhomboid proteases. *EMBO J.* 2005;24(3): 464-472.
12. Urban S, Wolfe MS. Reconstitution of intramembrane proteolysis in vitro reveals that pure rhomboid is sufficient for catalysis and specificity. *Proc Natl Acad Sci USA.* 2005;102(6): 1883-1888.
13. Wolf EV, Verhelst SH. Inhibitors of rhomboid proteases. *Biochimie.* 2016;122: 38-47.
14. Strisovsky K. Rhomboid protease inhibitors: Emerging tools and future therapeutics. *Semin Cell Dev Biol.* 2016;60: 52-62.
15. Vinothkumar KR, Strisovsky K, Andreeva A, Christova Y, Verhelst S, Freeman M. The structural basis for catalysis and substrate specificity of a rhomboid protease. *EMBO J.* 2010;29(22): 3797-3809.
16. Vosyka O, Vinothkumar KR, Wolf EV, Brouwer AJ, Liskamp RM, Verhelst SHL. Activity-based probes for rhomboid proteases discovered in a mass spectrometry-based assay. *Proc Natl Acad Sci U S A.* 2013;110(7): 2472-2477.
17. Xue Y, Ha Y. Catalytic mechanism of rhomboid protease GlpG probed by 3,4-dichloroisocoumarin and diisopropyl fluorophosphonate. *J Biol Chem.* 2012;287(5): 3099-3107.
18. Xue Y, Chowdhury S, Liu X, Akiyama Y, Ellman J, Ha Y. Conformational change in rhomboid protease GlpG induced by inhibitor binding to its S' subsites. *Biochemistry.* 2012;51(18): 3723-3731.
19. Pierrat OA, Strisovsky K, Christova Y, et al. Monocyclic beta-lactams are selective, mechanism-based inhibitors of rhomboid intramembrane proteases. *ACS Chem Biol.* 2011;6(4): 325-335.
20. Vinothkumar KR, Pierrat OA, Large JM, Freeman M. Structure of rhomboid protease in complex with beta-lactam inhibitors defines the S2' cavity. *Structure.* 2013;21(6): 1051-1058.
21. Wolf EV, Zeissler A, Vosyka O, Zeiler E, Sieber S, Verhelst SH. A new class of rhomboid protease inhibitors discovered by activity-based fluorescence polarization. *PLoS One.* 2013;8(8): e72307.
22. Zoll S, Stanchev S, Began J, et al. Substrate binding and specificity of rhomboid intramembrane protease revealed by substrate-peptide complex structures. *Embo J.* 2014;33(20): 2408-2421.
23. Cho S, Dickey SW, Urban S. Crystal Structures and Inhibition Kinetics Reveal a Two-Stage Catalytic Mechanism with Drug Design Implications for Rhomboid Proteolysis. *Mol Cell.* 2016;61(3): 329-340.
24. Tichá A, Stanchev S, Vinothkumar KR, et al. General and Modular Strategy for Designing Potent, Selective, and Pharmacologically Compliant Inhibitors of Rhomboid Proteases. *Cell Chem Biol.* in press.
25. Wolf EV, Zeissler A, Verhelst SH. Inhibitor fingerprinting of rhomboid proteases by activity-based protein profiling reveals inhibitor selectivity and rhomboid autoprocessing. *ACS Chem Biol.* 2015;10(10): 2325-2333.
26. Powers JC, Asgian JL, Ekici OD, James KE. Irreversible inhibitors of serine, cysteine, and threonine proteases. *Chemical Reviews.* 2002;102(12): 4639-4750.
27. Teshima T, Griffin JC, Powers JC. 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.* 1982;257(9): 5085-5091.

28. Krantz A, Spencer RW, Tam TF, et al. Design and synthesis of 4H-3,1-benzoxazin-4-ones as potent alternate substrate inhibitors of human leukocyte elastase. *J Med Chem.* 1990;33(2): 464-479.
29. Gutschow M, Kuerschner L, Neumann U, et al. 2-(diethylamino)thieno1,3oxazin-4-ones as stable inhibitors of human leukocyte elastase. *J Med Chem.* 1999;42(26): 5437-5447.
30. Gutschow M, Neumann U. Inhibition of cathepsin G by 4H-3,1-benzoxazin-4-ones. *Bioorg Med Chem.* 1997;5(10): 1935-1942.
31. Neumann U, Schechter NM, Gutschow M. Inhibition of human chymase by 2-amino-3,1-benzoxazin-4-ones. *Bioorg Med Chem.* 2001;9(4): 947-954.
32. Hays SJ, Caprathe BW, Gilmore JL, et al. 2-amino-4H-3,1-benzoxazin-4-ones as inhibitors of C1r serine protease. *J Med Chem.* 1998;41(7): 1060-1067.
33. Jarvest RL, Parratt MJ, Debouck CM, et al. Inhibition of HSV-1 protease by benzoxazinones. *Bioorganic & Medicinal Chemistry Letters.* 1996;6(20): 2463.
34. Bachovchin DA, Ji T, Li W, et al. Superfamily-wide portrait of serine hydrolase inhibition achieved by library-versus-library screening. *Proc Natl Acad Sci USA.* 2010;107(49): 20941-20946.
35. Ruiz N, Falcone B, Kahne D, Silhavy TJ. Chemical conditionality: a genetic strategy to probe organelle assembly. *Cell.* 2005;121(2): 307-317.
36. Ticha A, Stanchev S, Skerle J, et al. Sensitive Versatile Fluorogenic Transmembrane Peptide Substrates for Rhomboid Intramembrane Proteases. *J Biol Chem.* 2017;292(7): 2703-2713.

Supplementary Material

Supplementary material that may be helpful in the review process should be prepared and provided as a separate electronic file. That file can then be transformed into PDF format and submitted along with the manuscript and graphic files to the appropriate editorial office.