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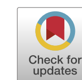
Discovery and validation of 2-styryl substituted benzoxazin-4-ones as a novel scaffold for rhomboid protease inhibitors



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ABSTRACT

Rhomboids are intramembrane serine proteases with diverse physiological functions in organisms ranging from archaea to humans. Crystal structure analysis has provided a detailed understanding of the catalytic mechanism, and rhomboids have been implicated in various disease contexts. Unfortunately, the design of specific rhomboid inhibitors has lagged behind, and previously described small molecule inhibitors displayed insufficient potency and/or selectivity. Using a computer-aided approach, we focused on the discovery of novel scaffolds with reduced liabilities and the possibility for broad structural variations. Docking studies with the *E. coli* rhomboid GlpG indicated that 2-styryl substituted benzoxazinones might comprise novel rhomboid inhibitors. Protease *in vitro* assays confirmed activity of 2-styryl substituted benzoxazinones against GlpG but not against the soluble serine protease α-chymotrypsin. Furthermore, mass spectrometry analysis demonstrated covalent modification of the catalytic residue Ser201, corroborating the predicted mechanism of inhibition and the formation of an acyl enzyme intermediate. In conclusion, 2-styryl substituted benzoxazinones are a novel rhomboid inhibitor scaffold with ample opportunity for optimization.

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Results and discussion

Rhomboids are intramembrane serine proteases present in prokaryotic, archaeal and eukaryotic organisms.[1] In 2001, the first rhomboid was discovered in *Drosophila* and shown to perform a critical proteolysis step in EGF-receptor signaling.[2,3] Since then, rhomboids have been implicated in a wide range of biological processes including bacterial quorum sensing[4], mitochondrial dynamics and integrity[5,6], and protein quality control.[7] In addition, rhomboids have been identified as putative drug targets in the context of multiple diseases[8] such as cancer[9], diabetes[10,11], parasitic diseases[12,13], and Parkinson's disease.[5] The crystal structures of rhomboids from *E. coli* and *H. influenzae* have been solved and revealed that rhomboids are serine-histidine dyad proteases composed of 6 core transmembrane helices, which form a V-shaped cavity and expose the active site to a partially hydrophilic environment.[14,15] These structures together with numerous biochemical studies have provided a detailed understanding of the catalytic mechanism of rhomboid proteases[16], but this has not yet translated into the development of potent, selective and drug-like inhibitors.[17] Through different strategies, from the testing of candidate molecules to rational synthesis to the screening of small molecule libraries, isocoumarins[3,18,19], fluorophosphonates[20], β-lactams[21], and β-lactones[22] were found to be effective against rhomboids, but these inhibitors generally displayed low potency and/or insufficient selectivity.[18,20,21] Effectively, inherent liabilities as exemplified by the high reactivity of isocoumarins likely preclude or limit further development of these compound classes.[23,24]

Accordingly, using a computer-aided candidate approach, we focused on the discovery of novel scaffolds with reduced liabilities and the possibility for broad structural variations. One scaffold we selected was 2-substituted derivatives of 4*H*-3,1-benzoxazin-4-ones, which were previously used as heterocyclic acylating agents against serine proteases such as HLE, α-chymotrypsin, and cathepsin G.[23,25–28] The mechanism of inhibition involves the formation of an O-acyl enzyme intermediate. The nucleophilic serine reacts with the C-4 carbonyl of the benzoxazinone, which results in opening of the heterocyclic ring and formation of the O-acyl enzyme

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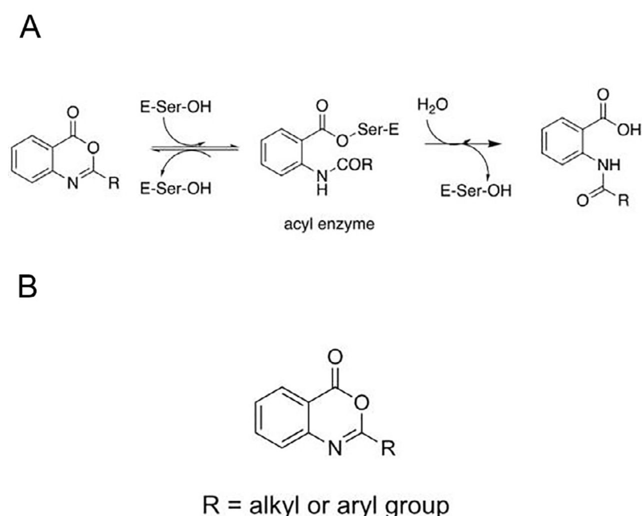
intermediate (Fig. 1A).[23] The enzyme selectivity and potency of acylating agents is promoted by fast acylation and slow deacylation, which is dependent on the substitution of the aromatic ring and the C-2 position in case of benzoxazinones.[25,29,30] A major advantage of benzoxazin-4-ones is that the core structure consists of two fused aromatic rings, which allows extensive structural modifications and optimization with respect to the target enzyme. For initial docking studies into the rhomboid active site, we assembled a molecular database of thirteen 2-alkyl or 2-aryl substituted benzoxazinones (Fig. 1B).



Compd	-R	Compd	-R
1		8	
2	$—CH_3$	9	
3		10	
4		11	
5		12	
6		13	
7			

Fig. 1. (A) Mechanism of inhibition of soluble serine proteases by 2-substituted benzoxazin-4-one derivatives.[30] (B) Molecular database of 2-alkyl or aryl substituted benzoxazin-4-ones assembled for docking studies into the rhomboid active site.

In the docking studies, we focused on the initial interactions between the benzoxazinones and the active site of the rhomboid protease rather than the final reaction product. For preparation of the docking receptor, we used the co-crystal structure of the *E. coli* rhomboid GlpG and the fluorophosphonate inhibitor CAPF (PDB ID: 3UUB), in which the active site Ser201 is covalently bound to CAPF.[31] The molecular modelling experiments were performed in the molecular operating environment software (MOE) with the DOCK module and the MMFF94x force field, and scored by London dG and Affinity dG followed by energy minimization within the enzyme active site cleft.[32,33] The output data was ranked based on the calculated ligand efficiencies (cLE = docking score/number of heavy atoms)[34], which revealed that the 2-styryl substituted compound **3** was the most favorable of all 2-substituted benzoxazinones (cLE = −0.3164). A comparative analysis of the protein/ligand docking results of compound **3** and CAPF indicated that **3** was adequately fitting into the binding pocket of the enzyme and was not exposed to the external environment (Fig. 2A and B). The core heterocyclic ring of **3** was oriented towards the S1 subsite while the 2-styryl extension pointed towards the S2′ subsite of the rhomboid, which had been defined in previous structures of GlpG in complex with different inhibitors.[31,35] Moreover, close interactions of **3** with the neighbouring residues His254 and Phe245 as shown in the ligand interaction map were observed and suggested to further explore the scaffold (Fig. 2C).

To validate the docking results, all derivatives listed in Fig. 1B were synthesized by methods shown in schemes 1 and 2 (Fig 3A).[36] The benzoxazinone derivatives were then evaluated for their inhibitory potency in an established *in vitro* enzyme activity assay with the *E. coli* rhomboid GlpG and the transmembrane domain 2 of the *Drosophila* protein Gurken as a substrate.[21,37–39] Each of the benzoxazinones were pre-incubated with GlpG at a single concentration of 250 μM for 30 min at 37 °C. Subsequently, the Gurken substrate was added, the reaction was continued for another 90 min at 37 °C, and the *N*-terminal Gurken substrate cleavage fragment was visualized by SDS-PAGE and quantified using ImageJ. Only the 2-styryl substituted benzoxazinones **3**, **5** and **11** showed activity at this concentration. For IC_{50} determinations, fluorogenic rhomboid substrates were applied as described previously.[40–42] Consistent with the docking results, compound **3** was a potent rhomboid inhibitor with an IC_{50} value of 4.4 ± 1.6 μM (Fig. 3B). Compound **5** was equally potent (IC_{50} 3.7 ± 1.3 μM) while **11** displayed around 10-fold lower activity (IC_{50} 48 ± 14.1 μM). Among these three compounds with a single substitution at the aromatic ring of the styryl substituent, an electron withdrawing group appeared to increase the potency, which could be further explored in subsequent studies.

Next, we evaluated the active benzoxazinones in a well-established *in vitro* activity assay for the soluble serine protease α-chymotrypsin.[43,44] The compounds were pre-incubated with bovine α-chymotrypsin for 30 min at 25 °C. Subsequently, the substrate *N*-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide was added, which under alkaline conditions is turned over by α-chymotrypsin to p-nitroaniline, a yellow compound that can be detected spectroscopically at 410 nm. 3,4-Dichloroisocoumarin (DCI) was used as a positive control and inhibited α-chymotrypsin with an IC_{50} value of 3.5 μM, comparable to previously reported values.[21] In contrast, the benzoxazinones **3** and **5** did not display any inhibitory activity in the α-chymotrypsin *in vitro* activity assay at the highest concentration of 250 μM (data not shown). In addition, we examined the active benzoxazinones in similar *in vitro* protease activity assays for bovine trypsin, human neutrophil elastase, and human cathepsin G.[45] At a concentration of 10 μM, none of the benzoxazinones inhibited trypsin or neutrophil elastase, while DCI almost completely abolished the activity of both enzymes. At a concentration

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