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Discovery and optimization of a series of small-molecule allosteric inhibitors of MALT1 protease

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ABSTRACT

We describe a series of potent and highly selective small-molecule MALT1 inhibitors, optimized from a High-Throughput Screening hit. Advanced analogues such as compound **40** show high potency (IC_{50}: 0.01 μM) in a biochemical assay measuring MALT1 enzymatic activity, as well as in cellular assays: Jurkat T cell activation (0.05 μM) and IL6/10 secretion (IC_{50}: 0.10/0.06 μM) in the TMD8 B-cell lymphoma line. Compound **40** also inhibited cleavage of the MALT1 substrate RelB (IC_{50}: 0.10 μM). Mechanistic enzymology results suggest that these compounds bind to the known allosteric site of the protease.

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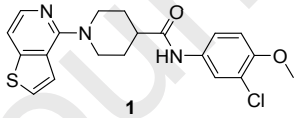
The proliferation and survival of T- and B-lymphocytes downstream of antigen recognition.[1-4] Mouse associated lymphoid tissue lymphoma translocation protein 1 (MALT1) serves a role in the activation of NF-kB. Stimulation of T- or B- cell receptors leads to recruitment of MALT1 into the CARD11(CARMA1)-BCL10-MALT1 (CBM) signalosome complex,[5] where it serves both scaffolding and proteolytic roles. In its scaffolding role, MALT1 binds to TRAF and TAK1, leading to activation of the IκB kinase complex and ultimately activation of the transcription factor NF-κB and cytokine secretion. MALT1 also contains a paracaspase (cysteine protease) domain. Upon activation, the protease cleaves a number of protein substrates that are part of a feedback loop controlling termination of the NF-κB response.[6-7]

MALT1 protease inhibitors may find application in the treatment of B-cell lymphomas driven by constitutive NF-κB activation. Protease function of the paracaspase is also required for optimal activation of T- and B- cells, suggesting that inhibitors may also be useful in the treatment of autoimmune diseases. MALT1 protease activity can be inhibited by small-molecules acting at the active site, with most reports describing peptide-based protease inhibitors that contain a reactive warhead.[8-10] Allosteric inhibitors of MALT1 protease have also been disclosed.[11-13] Small-molecule inhibitors[14-15]of MALT1 protease have demonstrated efficacy in preclinical models of ABC-DLBCL, further supporting a pharmaceutical role of inhibitors on this protease.[16-19] In this work we describe the discovery and optimization of a series of allosteric small-molecule inhibitors of MALT1 protease.[20]

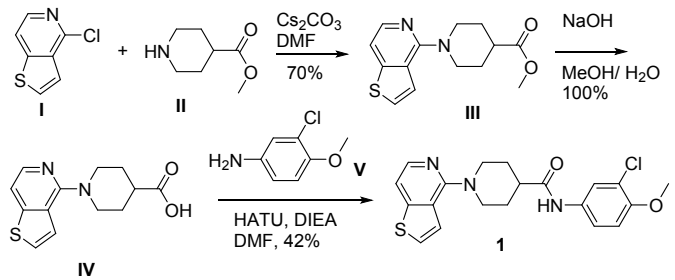
High-Throughput Screening was conducted on a library of ~560K compounds from the Janssen collection using an enzymatic biochemical assay to find MALT1 protease inhibitors.[21] We identified compound **1**, a molecule that lacks an obvious cysteine-reaction warhead but nevertheless shows sub-micro molar activity against MALT1 (Figure 1). Because NF-κB signaling regulates the secretion of multiple cytokines, including IL6 and IL10, secretion of IL6 and IL10 by TMD8 ABC-DLBCL cells was measured using a mesoscale assay.[22] Compound **1** shows single digit micro molar levels of inhibition of on IL6 and IL10 secretion. Compound **1** also blocks cleavage of the MALT1 substrate, RelB. Encouraged by these promising findings we started lead optimization of these class of compounds.



1

MALT1 IC_{50} 0.69 µM
IL6/IL10 secretion IC_{50} 1.70/1.95 µM
RelB cleavage IC_{50} 1.55 µM

Figure 1. Structure and properties of hit compound **1**

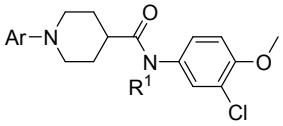


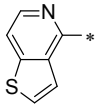
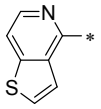
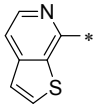
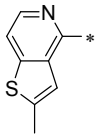
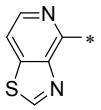
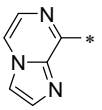
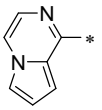
Scheme 1. Synthesis of compound **1**

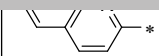
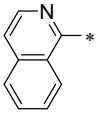
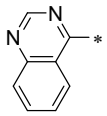
Scheme 1 illustrates the synthesis of 1-heteroarylpiperidine-4-carboxamides using compound **1** as a representative example. First, 4-chlorothieno[3,2-c]pyridine (**I**) was treated with methyl piperidine-4-carboxylate (**II**) and cesium carbonate in DMF to give methyl 1-(thieno[3,2-c]pyridin-4-yl)piperidine-4-carboxylate (**III**) in 70% yield. Next, compound **III** was hydrolyzed to 1-(thieno[3,2-c]pyridin-4-yl)piperidine-4-carboxylic acid (**IV**) using sodium hydroxide in methanol. Compound **IV** was subsequently reacted with 3-chloro-4-methoxyaniline (**V**), HATU, and DIEA in DMF to give final compound **1** in 42% yield. All compounds in the SAR Tables 1-5 were prepared using a similar procedure.

We began structure-activity relationship (SAR) studies by investigating bicyclic replacements for 4-thieno[3,2-c]pyridine (Table 1, see supplementary data for assay conditions). In our study, the most potent N-aryl group is 4-thieno[3,2-c]pyridine (**1**), with an IC_{50} of 0.69 µM. Among the isomeric thieno-fused analogues, 7-thieno[2,3-c]pyridine (**3**) is 2-fold less potent than compound **1** and 2-methyl-4-thieno[3,2-c]pyridine (**4**) is 25-fold less potent than **1**. N-methylation of the secondary amide (**2**) led to complete loss of activity. Although 1-pyrrolo[2,1-c]pyrazine (**7**) was almost equipotent to compound **1**, other heteroaryls attached to the piperidine at the carbon adjacent to the ring juncture such as 4-thiazolo[4,5-c]pyridine (**5**), 8-imidazo[2,1-c]pyrazine (**6**), 1-isoquinoline (**9**), and 4-quinazoline (**10**) are all less potent. Interestingly, when the piperidine attachment is shifted by one atom, as in 2-quinoline (**8**), MALT1 protease inhibitory activity is completely abrogated.

Table 1. SAR for fused bicyclic heteroaryls

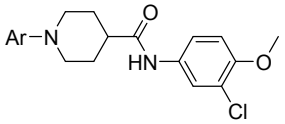


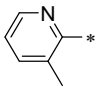
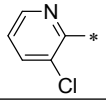
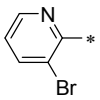
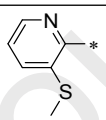
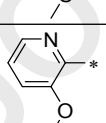
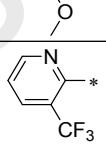
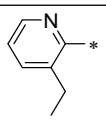
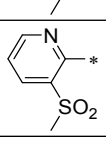
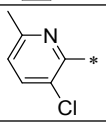
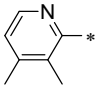
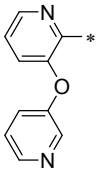
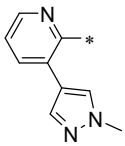
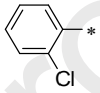
Cpd #	Ar	R^1	MALT1 IC_{50} (µM)
1		H	0.69
2		CH_3	>30
3		H	1.3
4		H	17.8
5		H	12.9
6		H	20.4
7		H	1.1

			
9		H	2.3
10		H	4.0

Because fused heteroaryls did not improve potency, we turned our attention to substituted mono(hetero)aryls (Table 2). Replacing 4-thieno[3,2-c]pyridine with mono-substituted pyridines, we found that 3-methyl (**11**), 3-chloro (**12**), 3-bromo (**13**), 3-methylthio (**14**), 3-methoxy (**15**), 3-trifluoromethyl (**16**), and 3-ethyl (**17**) each have equal or improved potency compared to **1**, with 3-bromo (**14**) being more than 10-fold better. By contrast, 3-methylsulfonyl (**18**) is 9-fold less potent than compound **1**. Unsubstituted pyridine (**19**) is 5-fold less potent than **1** and disubstituted 5-chloro-2-methyl-6-pyridine (**20**) is equally potent, but 3,4-dimethyl-2-pyridine (**21**) is 25-fold less potent. Larger substituents, such as in compounds **22** and **23** give reduced potency. 2-Chlorophenyl (**24**) is 3-fold more potent than compound **1** but 2-fold less potent than the corresponding pyridine (**13**).

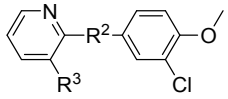
Table 2. SAR for mono(hetero)aryls

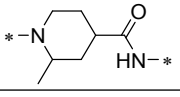


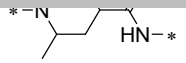
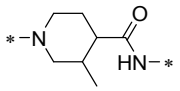
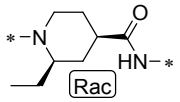
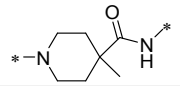
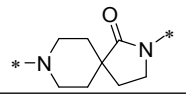
Cpd #	Ar	MALT1 IC_{50} (μM)
11		0.10
12		0.10
13		0.05
14		0.98
15		0.35
16		0.41
17		0.83
18		6.03
19		
20		0.55
21		17.8
22		4.27
23		13.5
24		0.23

Based on its balanced properties including acceptable MALT1 potency, reasonable molecular weight (359.9), and good ligand efficiency (0.37), compound **12** was selected for pharmacokinetic (PK) study in mice. Although the compound is bioavailable (F = 35%), it shows high clearance (70 mL/min/mg) and a very short half-life (0.25 h) after IV dosing. *In vitro* ADME studies -show compound **12** to be highly unstable in mouse liver hepatocyte and microsomal preparations (Cl_{int}: 132 μL/min/mg), Stability in mouse plasma is also poor, due to amide bond cleavage (~20% remaining after 4 h). Metabolite ID studies in mouse and human liver microsomes indicated that the major metabolic liability is amide hydrolysis. In an attempt to solve this issue, we explored SAR around piperidine substitutions that might stabilize the amide bond against enzymatic hydrolysis through steric effects (Table 4). In term of MALT1 activity, we found that 2-methyl substitution leads to either a 2-fold potency improvement (compare **25** and **12**) or equal potency (compare **26** and **14**). On the other hand, incorporation of 3-methyl (**27**) or 2-ethyl (**28**) reduces potency by 2-fold. 4-Methyl substitution (**29**) and spirocyclization of the amide (**30**) results in significant loss of potency or complete inactivity. Unfortunately, plasma stability for the equipotent compound **25** (15% remaining at 7 h) was disappointing. Compound **28** (12% remaining at 7 h) is also poorly stable in mouse plasma. Only the 2 fold weaker 2-methyl compound **27** is stable in mouse plasma (100% remaining at 7 h).[23]

Table 3. SAR for substituted piperidines

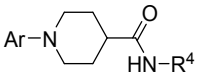


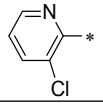
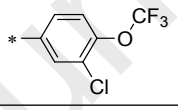
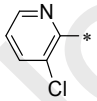
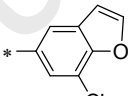
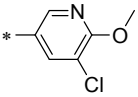
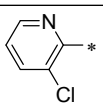
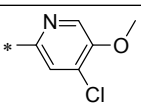
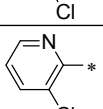
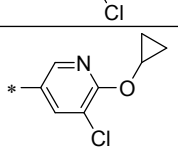
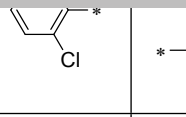
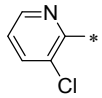
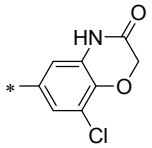
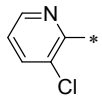
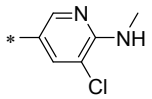
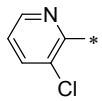
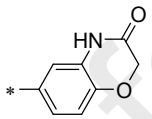
Cpd #	R^3	R^2	MALT1 IC_{50} (μM)
25	CH_3		0.05

			
27	CH_3		0.23
28	CH_3		0.29
29	CH_3		>30
30	CH_3		11.2

Next, we expanded our SAR investigation to the RHS amide substituent, limiting substitution on the LHS to mono-(hetero)aryls, specifically 3-halogen-substituted pyridin-2-yl. Combination of 3-chloropyridin-2-yl on the LHS and 3-chloro-2-trifluoromethoxy-phenyl on RHS led to compound **31**, which is more than 10-fold less potent than its direct methoxy analog **12**. The cyclized benzofuran analog **32** maintains the same potency as compound **12**, while replacing 3-chloropyridin-2-yl with 3-bromopyridin-2-yl **33** results in a 3-fold potency increase. Continuing with the lower molecular weight 3-chloropyridin-2-yl group as the LHS substituent, additional RHS exploration led to several compounds more potent than **12**: 3-chloro-2-cyclopropoxy-5-pyridine (**35**), ~3-fold more potent; 8-chloro-2H-6-benzo[b][1,4]oxazin-3(4H)-one (**37**), 5-fold more potent; 4-chloro-5-methoxy-2-pyridine (**34**) and 3-chloro-2-(pyridin-3-yloxy)-5-pyridine (**36**), both equipotent. However, substitution of MeO by MeNH gave **38**, 10-fold less potent than **13**, while removing a chloro from compound **37** results in more than 25 fold potency loss (compound **39**)

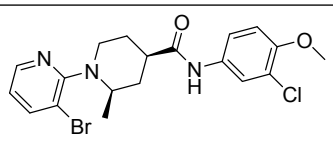
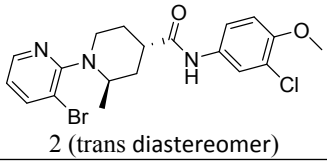
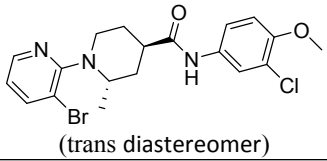
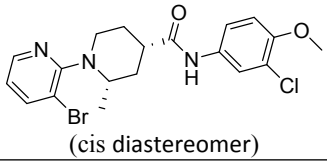
Table 4. SAR for RHS



Cpd #	Ar	R^4	MALT1 IC_{50} (μM)
31			1.74
32			0.10
33			0.03
34			0.13
35			0.04
			
37			0.02
38			1.0
39			0.54

Compound **26** was first separated into cis and trans isomers by column chromatography on silica gel and then separated into 4 enantiomerically pure diastereomers using chiral chromatography (Table 5)[24]. In the MALT1 biochemical assay, the cis diastereomer **40** is greater than10-fold more potent than the other 3 diastereomers (**41-43**). Unfortunately, *in vitro* ADME testing showed compound **40** still highly unstable in mouse liver hepatocytes (Cl_{int}: 31 μL/min/mg), mouse microsomal preparation (Cl_{int}: 262 μL/min/mg) and mouse plasma (40% remaining after 4 h).

Table 5. SAR of four diastereomers of compound **26**

Cpd #	Structures	MALT1 IC_{50} (μM)
40	 (cis diastereomer)	0.01
41	 2 (trans diastereomer)	0.18
42	 (trans diastereomer)	0.15
43	 (cis diastereomer)	17.4

The cellular activities of selected MALT1 inhibitors were evaluated. Inhibition of secretion of both IL6 and IL10 was tested in TMD8 cells, and inhibition of cleavage of a MALT1 substrate, RelB, was examined in OCI-Ly3 cells. Additionally, selected compounds were also tested for inhibition of PMA/ionomycin-stimulated IL2 production in Jurkat T cells to assess the effects of MALT1 inhibitors on T cell activation. The tested compounds show potencies for inhibiting IL6 and IL10 secretion and IL2

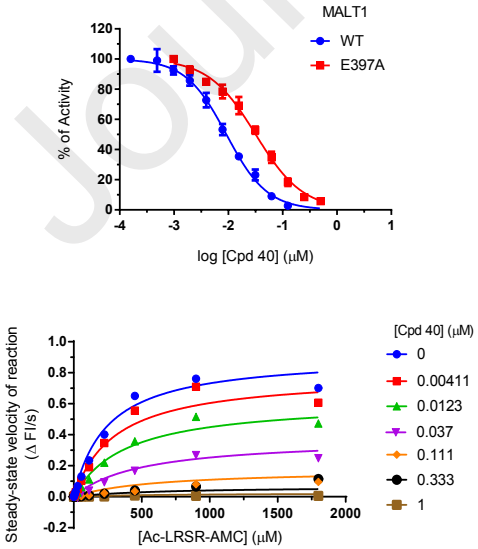
proc); RelB in the range of 0.07 – 1.55 µM (Table 6). inhibition mechanism of compound **40** (bottom figure)

Table 6. *In vitro* activity for selected compounds

Cpd #	**1**	**11**	**25**	**26**	**40**
MALT1 IC_{50} (µM)	0.69	0.10	0.05	0.05	0.01
MALT1 E397A IC_{50} (µM)	12.0	0.46	0.32	0.21	0.06
IL6/IL10, TMD8 cells IC_{50} (µM)	1.70/ 1.95	1.05/ 0.76	0.50/ 0.41	0.25/ 0.30	0.10/ 0.06
IL2, Jurkat PMA IC_{50} (µM)	2.34	1.02	0.05	0.02	0.05
Rel B, OCI-Ly3 cells, IC_{50} (µM)	1.55	0.60	0.20	0.11	0.07

To address any potential off target protease activities, compound **40** was tested against a panel of 63 enzymes (Reaction Biology), revealing no activity at 10 µM for any protease. Compound **40** also shown no activity against kinases (DiscoverX, <50% inhibition of 109 kinases at 1 µM) and did not broadly inhibit proliferation of cancer cells (Oncolead, IC_{50} >1 µM across a panel of 91 tumor cell lines).

It was reported MALT1 has two binding sites for inhibitors: the paracaspase active site and allosteric binding site.[11] In order to understand why our compounds are so selective, we first evaluated their ability to inhibit E397A mutant MALT1 protein[25] (glutamic acid 397 forms a key part of the allosteric binding pocket[8]), in order to confirm activity similar to that seen with known allosteric site inhibitors such as phenothiazine dervatives.[11] In our MALT1-E397A assay, inhibitory potency of compound **40** was reduced by about 4-fold (Figure 2). Second, a detailed kinetic study[26] indicated that the behavior of compound **40** best fit to a non-competitive inhibition mechanism (Figure 2). Data analysis yielded the binding affinity (K_i) for the free enzyme to be 9.6 ± 2.3 nM. Affinity for the enzyme-substrate complex was 2.8-fold weaker than that for the free enzyme. Third, an assay measuring fluorescence of an allosteric site tryptophan residue (Trp580)[11] showed fluorescence quenching reaching saturation with an increasing amount of the compound **40** (data not shown). All these findings suggest that our inhibitors bind to an allosteric pocket at the interface of the caspase catalytic domain and the Ig3 domain.



In summary, a series of potent and highly selective small-molecule MALT1 inhibitors was discovered starting from an HTS hit. Biochemical assay of an allosteric site mutant of MALT1 (E397A), mechanism of inhibition studies, and the Trp580 fluorescence quenching assay suggest that these compounds are allosteric site binders. Advanced leads such as compound **40** show high potency in the biochemical enzyme assay as well as in cellular assays. Compound **40** also inhibited cleavage of MALT1 substrates RelB and BCL10 in OCI-Ly3 cells and was shown to be highly selective for MALT1 versus other proteases and kinases.

ABBREVIATIONS

MALT1: Mouse-associated lymphoid tissue lymphoma translocation protein 1; ABC: activated B-cell; DLBCL: diffuse large B-cell lymphoma; IL: interleukin; TMD8: Tokyo Medical and Dental university 8; HTS: high throughput screening.

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/

References and notes

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20. A related series of compounds was recently published by Schlapbach A, Revesz L, Soldermann CP, Zoller T, Régnier CH, Bornancin F, Radimerski T, Blank J, Schuffenhauer A, Renatus M, Erbel M, Melkko S, Heng R, Simic O, Endres R, Wartmann M, Quancard J, *Bioorg. Med. Chem. Lett.,* 2018, 28, 2153-2158
21. MALT1 protease activity was assessed in an *in vitro* assay using a tetrapeptide substrate and full-length MALT1 protein (Strep-MALT1(1-824)-His) purified from baculovirus-infected insect cells. The tetrapeptide LRSR is coupled to 7-amino-4-methylcoumarin (AMC) and provides a quenched, fluorescent substrate for the MALT1 protease (SM Biochemicals). Cleavage of AMC from the arginine residue results in an increase in coumarin fluorescence measured at 460 nm (excitation 355 nm). The final assay was performed in the presence of 10 nM full-length MALT1 protein. Diluted compounds were pre-incubated with MALT1 enzyme for lded subsequently, and the reaction was then incubated for 4 hours at RT, after which fluorescence was measured.
22. TMD8 cells were treated with diluted compounds for 24 hours at 37°C and 5% CO_2. After 24 hours of incubation, 50 µL of the supernatant was transferred to an MSD plate (V-Plex Proinflammation Panel 1 [human] kit) and incubated for 2 hours at RT followed by a 2-hour incubation with IL-6/10 antibody solution. Plates were read on a SECTOR imager.
23. Note that compounds **25-28** were tested as mixtures of 4 diastereomers;
24. The absolutely configurations are unknow, the steric centers are assigned arbitrarily.
25. Dose dependence inhibition studies were performed in the presence of 300 µM substrate Ac-LRSR-AMC and 5 nM MALT1 enzyme. The steady-state reaction velocity was measured under all conditions and the percent of activity remaining was calculated by comparing to an uninhibited reaction.
26. The steady-state activity of MALT1 was determined as a function of the concentration of substrate, peptide Ac-LRSR-AMC and 4.8 nM enzyme in the presence of 0, 0.00411, 0.0123, 0.037, 0.111, 0.333 and 1 µM of compound **40**.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Graphical Abstract

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Discovery and optimization of a series of sma of MALT1 protease

Tianbao Lu * [a], Peter J. Connolly[a], Ulrike Philippar[b], Weim Barbay[a], Luc Gys[b], Luc Van Nuffel[b], Nigel Austin[b], Mariet Attar[a], Lieven Meerpoel[b], James Edwards[a]