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Structure–activity relationship, kinetic mechanism, and selectivity for a new class of ubiquitin C-terminal hydrolase-L1 (UCH-L1) inhibitors

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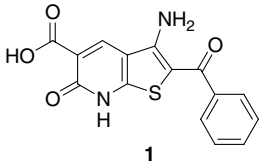
Abstract—3-Amino-2-keto-7*H*-thieno[2,3-*b*]pyridin-6-one derivatives were discovered as moderately potent inhibitors of ubiquitin C-terminal hydrolase-L1 (UCH-L1) utilizing an assay that measures hydrolysis of the fluorogenic substrate Ub-AMC. SAR studies revealed that both the carboxylate at the 5-position and the 6-pyridone ring were critical for inhibitory activity. Furthermore, activity was dependent on the nature of the ketone substituent at the 2-position, with 4-Me-Ph and 2-naphthyl being best. Kinetic mechanism studies revealed that these compounds were uncompetitive inhibitors of UCH-L1, binding only to the Michaelis-complex and not to free enzyme. The active compounds were selective for UCH-L1, exhibiting neither inhibition of other cysteine hydrolases (e.g., UCH-L3, papain, isopeptidase T, caspase-3, and tissue transglutaminase) nor cytotoxicity in N2A cells.

Deubiquitinating enzymes (DUBs) comprise a large family of enzymes that specifically cleave ubiquitin-derived substrates of general structure Ub^{1-72}-Leu^{73}-Arg^{74}-Gly^{75}-Gly^{76}-X, where X can be any number of leaving groups ranging from small thiols and amines to ubiquitin (Ub) and other proteins.[1–4] While at least five families of DUBs[1] have been identified,[4] the two largest subsets and best characterized are the ubiquitin C-terminal hydrolases (UCHs) and the ubiquitin proteases (UBPs). UBPs are generally high molecular weight enzymes (~100 kDa) that cleave substrates where X is a protein or another molecule of Ub. These enzymes are thought to have domains adjacent to the primary Ub-substrate binding site that can recognize and bind these proteinaceous leaving groups. Perhaps the most thoroughly studied UBP is isopeptidase T,[5–7] which cleaves substrates where X is Ub. In contrast, UCHs are lower molecular weight enzymes (~30 kDa) that hydrolyze Ub derivatives where X is a thiol or an amine and are thought not to have a binding domain for protein leaving groups.

Both UBPs and UCHs are cysteine hydrolases and catalyze the hydrolysis of amide bonds of their substrates according to a mechanism in which substrate and enzyme combine to form a non-covalent Michaelis-complex, from within which the sulfhydryl of the active-site cysteine attacks the carbonyl carbon of the amide bond of the substrate to generate an acyl-enzyme intermediate and liberate the first product. Hydrolysis of the acyl-enzyme produces the reaction's second product Ub and regenerates free enzyme.

Our interest in these enzymes originates in the potential involvement of UCH-L1 in Parkinson's disease[8–10] and cancer.[11–13] In the course of screening for UCH-L1 inhibitors, we discovered that the 3-amino-2-keto-7*H*-thieno[2,3-*b*]pyridin-6-one derivative **1** was a moderately potent inhibitor ($K_{i\,app}$ = 2.8 μM). Herein, we report an initial structure–activity relationship (SAR), kinetic mechanism, and selectivity studies for this class of UCH-L1 inhibitors.



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Keywords: Ubiquitin C-terminal hydrolase-L1; UCH-L1; Inhibitor; Kinetic mechanism; Selectivity.
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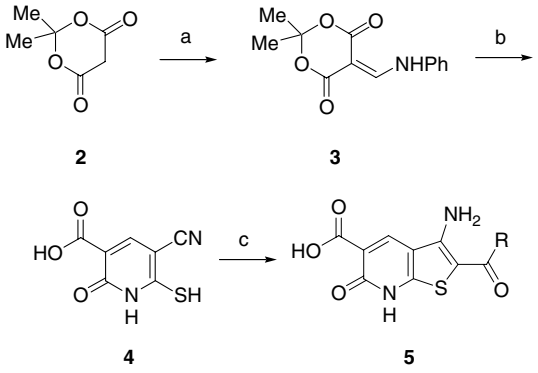
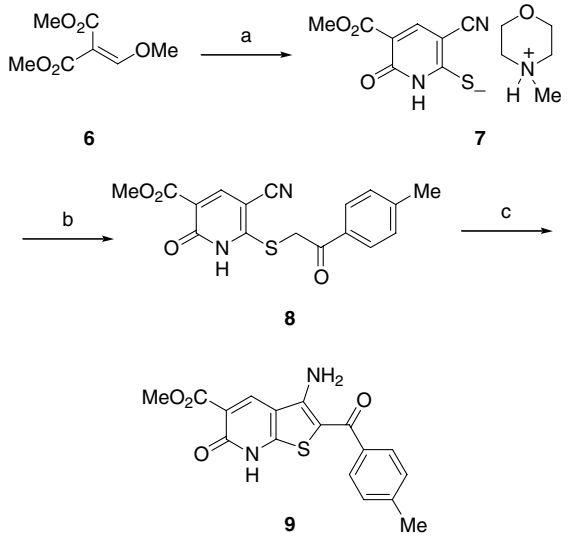
Most of the 3-amino-2-keto-7*H*-thieno[2,3-*b*]pyridin-6-one derivatives were prepared according to the general procedure outlined in Scheme 1.[14] Meldrum's acid, **2**, was treated with triethyl orthoformate and aniline to give derivative **3**. Cyclization of **3** in the presence of cyanothioacetamide under basic conditions gave the 1-pyridone **4**. Alkylation with an α-bromoketone under basic conditions followed by acidification with HCl gave **5**.

Initial attempts to prepare the methyl ester of 3-amino-2-keto-7*H*-thieno[2,3-*b*]pyridin-6-one derivatives by esterification (e.g., with methanol or diazomethane) or by alkylation (e.g., with MeI) of the corresponding carboxylic acids failed to yield the desired products. Instead an alternative synthetic procedure was utilized as depicted in Scheme 2. The dimethyl malonate derivative **6** was allowed to react with cyanothioacetamide in the presence of *N*-methylmorpholine in ethanol to give **7**.[15] This material was alkylated with α-bromo-4-methylacetophenone to give **8**. Finally, treatment with KOH in DMF gave the methyl ester **9**.



Scheme 1. Reagents and conditions: (a) $HC(OEt)_3$, $PhNH_2$, Δ until solution formed then allowed to cool (85%); (b) $NCCH_2C(S)NH_2$, KOH, EtOH, rt, 16 h (69%); (c) $RC(O)CH_2Br$, KOH, EtOH, rt, 16 h, then HCl (34–84%).

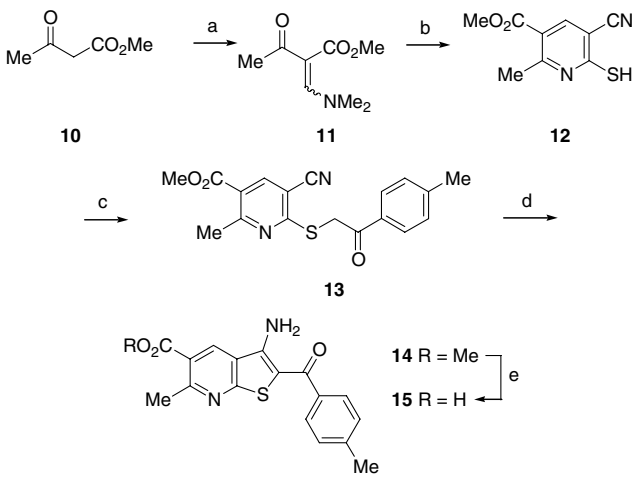


Scheme 2. Reagents and conditions: (a) $NCCH_2CSNH_2$, *N*-methylmorpholine, EtOH, rt, 30 min (45%); (b) *p*-Me–$C_6H_4C(O)CH_2Br$, MeOH, Δ, 1 h (79%); (c) KOH, DMF, rt, 2 h (31%).

A derivative containing a thieno[2,3-*b*]pyridine in place of the 7*H*-thieno[2,3-*b*]pyridin-6-one was prepared according to the procedure outlined in Scheme 3. Methyl acetoacetate, **10**, was converted to **11** with dimethylformamide dimethyl acetal. Cyclization in the presence of cyanothioacetamide gave pyridine **12**.[16] Again, alkylation with α-bromo-4-methylacetophenone followed by base cyclization utilizing sodium methoxide gave methyl ester **14**. Ester hydrolysis followed by acidification gave pyridine-5-carboxylate **15**.

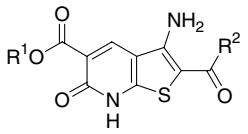
The prepared compounds were evaluated for UCH-L1 inhibitory activity utilizing an assay in which hydrolysis of the fluorogenic substrate Ub-AMC, the 7-amido-4-methylcoumarin C-terminus derivative of ubiquitin, is measured in the presence of different compound concentrations.[7] Ki_{app} values were calculated using a four-parameter fit from the dependence of the initial velocity values on inhibitor concentration.

Esterification of the carboxylic acid in the 5-position of the 3-amino-2-keto-7*H*-thieno[2,3-*b*]pyridin-6-one ring (**9**) or replacing the 6-pyridinone portion with a pyridine (**15**) resulted in complete loss in UCH-L1 inhibitory activity (Table 1). Furthermore, the inhibitory activity was dependent on the nature of the ketone substituent at the 2-position. Substituents at the *ortho*-position (**17**, **20**, and **24**) or electron-donating substituents at the *para*-position of the aromatic ketone (**19**) were detrimental to activity. Introduction of alkyl (**16**) or electron-withdrawing groups at the *para*-position of the aromatic ketone (**23**) improved activity. Also, introduction of a 2-naphthyl ketone (**26**) resulted in improved inhibitory activity. Heterocyclic ketones (**27** and **28**) or aliphatic ketones (**29** and **30**) with the exception of cyclohexyl ketone (**31**) were detrimental to activity. Replacement of



Scheme 3. Reagents and conditions: (a) $Me_2NC(OMe)_2$, DMF, rt, 16 h; (b) $NCCH_2C(S)NH_2$, NaH, DMF, rt, 16 h (30% yield over two steps); (c) *p*-Me–$C_6H_4C(O)CH_2Br$, NaOMe (2 equiv), MeOH, rt, 16 h (62%); (d) NaOMe, DMF, MeOH, rt, 16 h (82%); (e) LiOH, THF/H_2O (1:1), Δ, 1 h then HCl (58%).

Table 1. 7*H*-Thieno[2,3-*b*]pyridin-6-one derivatives prepared for structure–activity relationship study and $K_{i\,app}$ values for UCH-L1 inhibition



Compound[a]	R^1	R^2	$K_{i\,app}$[b] (μM)
1	H	Ph	2.8
9	Me	4-Me-Ph	>30
16	H	4-Me-Ph	0.91
17	H	2-Me-Ph	10
18	H	4-*t*-Bu-Ph	1.4
19	H	4-OMe-Ph	30
20	H	2-OMe-Ph	5.3
21	H	4-CF_3-Ph	3.6
22	H	2-CF_3-Ph	30
23	H	4-Cl-Ph	1.2
24	H	2-Cl-Ph	11
25	H	1-Naphthyl	1.5
26	H	2-Naphthyl	0.74
27	H	4-Pyridyl	30
28	H	2-Thienyl	20
29	H	Me	>30
30	H	*t*-Bu	20
31	H	*c*-Hexyl	2.0
32	H	NH_2	>30
33	H	*N*-Piperidinyl	>30

[a] HCl salt.
[b] Standard deviation <10%.

the ketone with a primary (**32**) or tertiary amide (**33**) was also not tolerated.

The kinetic mechanism of inhibition for these compounds was elucidated in experiments in which we determined V_{max} and V_{max}/K_m values for the UCH-L1-catalyzed hydrolysis of Ub-AMC at various fixed concentrations of inhibitor. An example is shown in Figure 1 for inhibition by **1**, where we observed that while V_{max} titrates with a K_i value of 2.8 μM, values of V_{max}/K_m were independent of inhibitor concentration. Such behavior is diagnostic of uncompetitive inhibition where inhibitor binds not to free enzyme but to some form of the enzyme that is complexed with substrate. For cysteine and serine hydrolases, the form of the enzyme can be either the Michaelis-complex or the acyl-enzyme. To probe this, we conducted transient-state kinetic experiments and found that the acyl-enzyme does not accumulate in the steady-state; that is, acylation of the active-site cysteine is rate-limiting (data not shown). This indicates that **1** must bind to the Michaelis-complex. However, we cannot exclude the possibility that the inhibitor also stays bound to the acyl-enzyme and perhaps even to forms of the enzyme with product bound.

The selectivity of **1** toward UCH-L1 was demonstrated in experiments in which we observed no inhibition of other cysteine hydrolases, including UCH-L3, tissue transglutaminase (TGase 2), papain, and caspase-3

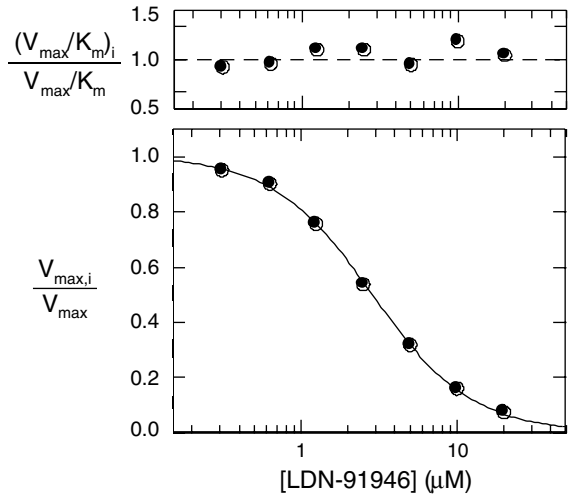


Figure 1. Uncompetitive inhibition of UCH-L1 by **1** (LDN-91946). At seven fixed concentrations of **1** that ranged from 0.3 to 20 μM, we determined the dependence of initial reaction velocity on substrate concentration for the UCH-L1-catalyzed hydrolysis of Ub-AMC. Each of these data sets, comprising no less than six (v_o, [*S*]) pairs, was then fit by non-linear least squares to the Michaelis–Menten equation to provide apparent values of V_{max} and V_{max}/K_m, which are plotted here as a function of **1**. The solid line through the data set for the dependence of $V_{max,i}/V_{max}$ on [**1**] was drawn using a simple binding isotherm and the best fit K_i value of 2.8 ± 0.1 μM.

Table 2. Activity of 7*H*-thieno[2,3-*b*]pyridin-6-one derivatives against other cysteine hydrolases

Compound	Enzyme	Result (μM)
1	UCH-L3	No activity at 20
	TGase 2	No activity at 40
	Papain	No activity at 40
	Caspase-3	No activity at 40
16	UCH-L3	30% inhibition at 20
	Papain	No activity at 40
	Isopeptidase T	No activity at 40
23	Papain	No activity at 40

(Table 2). Compounds **16** and **23** were also inactive against papain at 40 μM. Compound **16** demonstrated very weak activity (30% inhibition at 20 μM) against UCH-L3 and no activity against isopeptidase T at 40 μM. In addition, we observed no cytotoxicity when serum-starved N2A cells were treated with **1** at concentrations as high as 0.1 mM.

In conclusion, a class of 3-amino-2-keto-7*H*-thieno[2,3-*b*]pyridin-6-one derivatives were discovered as moderately potent UCH-L1 inhibitors. A preliminary SAR study revealed that both the carboxylate at the 5-position and the 6-pyridinone ring were necessary for inhibitory activity. Furthermore, inhibitory activity was dependent on the nature of the ketone substituent at the 2-position, with 4-Me-Ph and 2-naphthyl being best. A kinetic mechanism study revealed that these compounds were uncompetitive inhibitors of UCH-L1, binding only to the Michaelis-complex and not to free enzyme. Finally, the active compounds were selective for UCH-L1, exhibiting no inhibition of other cysteine hydrolases (e.g., UCH-L3, papain, isopeptidase T,

caspase-3, and TGase 2) or cytotoxicity in serum-starved N2A cells. The UCH-L1 inhibitors reported herein provide useful tools for investigating the role of UCH-L1 in normal cellular physiology, as well as in pathological conditions, such as Parkinson's disease and some forms of cancer.

Acknowledgments

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