

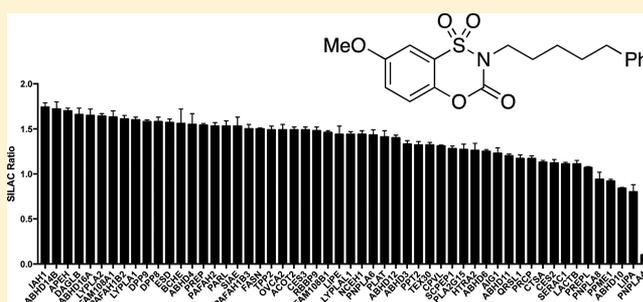
Design of Benzoxathiazin-3-one 1,1-Dioxides as a New Class of Irreversible Serine Hydrolase Inhibitors: Discovery of a Uniquely Selective PNPLA4 Inhibitor

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Supporting Information

ABSTRACT: The design and examination of 4,1,2-benzoxathiazin-3-one 1,1-dioxides as candidate serine hydrolase inhibitors are disclosed, and represent the synthesis and study of a previously unexplored heterocycle. This new class of activated cyclic carbamates provided selective irreversible inhibition of a small subset of serine hydrolases without release of a leaving group, does not covalently modify active site catalytic cysteine and lysine residues of other enzyme classes, and was found to be amenable to predictable structural modifications that modulate intrinsic reactivity or active site recognition. Even more remarkable and within the small pilot series of candidate inhibitors examined in an initial study, an exquisitely selective inhibitor for a poorly characterized serine hydrolase (PNPLA4, patatin-like phospholipase domain-containing protein 4) involved in adipocyte triglyceride homeostasis was discovered.



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INTRODUCTION

Serine hydrolases play key roles in human physiology and disease and are an important class of therapeutic targets. Serine hydrolases represent more than 1% of predicted proteins in humans, making it one of the largest and most diverse class of mammalian enzyme families.¹ Not only do they make fundamental contributions to physiological and pathophysiological processes,² but a large number of the serine hydrolases remain uncharacterized or unannotated, lacking a known role, endogenous substrate, or specific inhibitor. Selective chemical inhibitors for members of the serine hydrolase family have uniquely contributed to an understanding of the biological function of individual enzyme members. They have also led to new therapeutics, including new treatments for obesity, diabetes, microbial infections, and Alzheimer's disease.¹ The active site catalytic triads that contain a serine nucleophile have inspired the design of many classes of small molecule inhibitors.³ The inhibitor classes include those that contain an electrophilic carbonyl such as trifluoromethyl ketones,^{4–6} α -ketoamides and esters,^{5,6} lactones,^{7,8} lactams,⁹ α -keto heterocycles,^{10–12} carbamates,^{13,14} ureas,^{15–17} and other activated carbonyl-containing compounds,¹⁸ which act through covalent modification of the serine nucleophile.

The use of activity-based protein profiling (ABPP)^{19–21} paired with such selective inhibitor classes has allowed the rapid analysis of target serine hydrolases and monitoring of enzyme activity in complex biological systems. The use of ABPP probes

developed for specific enzyme classes, including fluorophosphonate-rhodamine (FP-Rh)^{21,22} for selective serine hydrolase labeling, permits the rapid proteome-wide identification of inhibitor targets, assessment and optimization of inhibitor selectivity, and functional annotation of uncharacterized enzymes.^{23,24} This may be accomplished without recombinant enzyme expression, protein purification, knowledge of the endogenous substrate, or the development of specific substrate assays as required by traditional methods.^{24–27}

In efforts to interrogate serine hydrolases not yet successfully targeted by existing inhibitor classes, we have continued to explore new irreversible covalent inhibitor designs that might display a unique reaction selectivity among not only classes of enzymes, but also among a subset of the serine hydrolases.²⁸ A large body of work on irreversible inhibitors of serine, cysteine, and threonine proteases is available from which inspiration may be drawn.³ Tethered reactive moieties that acylate, phosphorylate, or sulfonylate active site nucleophiles were of particular interest, with the saccharin family of serine protease inhibitors serving as the inspiration for the new inhibitor class detailed herein.²⁹ The saccharin family of 1,2-benzisothiazol-3-one 1,1-dioxides has been shown to inhibit serine proteases such as human leukocyte elastase^{30–32} and human mast cell tryptase³³ through acylation of the nucleophilic serine active site residue

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(Figure 1). The mechanism of inhibition involves serine nucleophilic attack on the activated amide, collapse of the

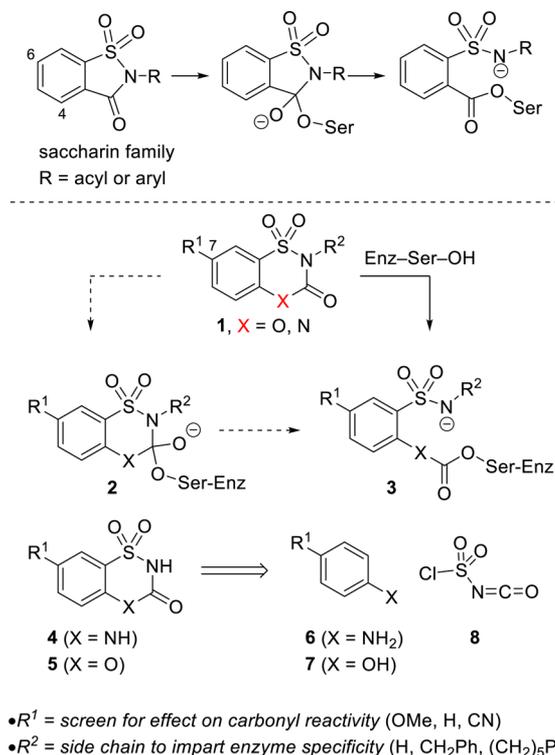


Figure 1. Saccharin family of protease inhibitors. Proposed mechanism of inhibition and synthetic design for new inhibitor class.

tetrahedral intermediate, and formation of an acyl enzyme intermediate in the form of an ester with release of the sulfonamide as the leaving group.³⁰ The reactivity of such saccharin derivatives, their intrinsic stability, and inhibitory potency can be modulated by core substitution. Activation of the leaving sulfonamide through *N*-acylation or *N*-arylation (*R*) or electronic modulation of the intrinsic carbonyl reactivity by C4/C6 aryl substitution has been detailed. However, the acyl bond formed with the active site residue is a serine ester that is susceptible to rapid deacylation and enzyme reactivation, limiting the utility of this inhibitor class for biological studies where sustained enzyme inhibition is required.

The redesigned scaffold (1) detailed herein represents a modification of the saccharin ring system with insertion of a heteroatom (O, NH) adjacent to the carbonyl (Figure 1). Nucleophilic attack of an active site serine on the inhibitor carbonyl followed by collapse of the tetrahedral intermediate 2 provides a covalently bound inhibitor 3 in the form of a more stable and potentially irreversible serine carbamate or carbonate, depending on the choice of heteroatom X (Figure 1). Remarkable in this day and age while the urea version of the redesigned scaffold (4, X = NH) represents a well-established heterocycle class,^{34–38} the carbamate version (5, X = O) constitutes a class with only a single report of its inadvertent synthesis.³⁹ The work detailed herein revealed that the former *N*-alkylated ureas may be insufficiently reactive to irreversibly inhibit serine hydrolases or other enzyme classes. However, the latter benzoxathiazin-3-one 1,1-dioxides (5, X = O) proved to be potent and remarkably selective irreversible serine hydrolase inhibitors. Moreover, these compounds constitute a class of serine hydrolase inhibitors that do not

release a leaving group, are capable of predictable electronic modulation of the intrinsic reactivity and selectivity by C7 aryl substitution (R^1), and can be tailored to bind individual enzyme targets through *N*-substitution (R^2). Their use in the discovery of the first selective inhibitor of patatin-like phospholipase domain containing protein 4 (PNPLA4) is disclosed.

RESULTS AND DISCUSSION

At the onset of our studies, we elected to probe the aryl substituent electronic effects ($R^1 = \text{OMe}$, H, CN), utilizing the parent core heterocycles ($R^2 = \text{H}$) and those that bear a small set of *N*-alkyl substituents that might impart representative selective enzyme active site targeting ($R^2 = \text{CH}_2\text{Ph}$, $(\text{CH}_2)_5\text{Ph}$ vs H). In particular, the inclusion of the 5-phenylpentyl side chain might impart binding and inhibition of fatty acid amide hydrolase (FAAH) for which we were especially well equipped to examine as part of a pilot examination.^{10,25} Proteome-wide evaluation by gel-based ABPP was used to provide an initial assessment of the impact of expected reactivity trends ($X = \text{O} > \text{NH}$, $R^1 = \text{CN} > \text{H} > \text{OMe}$), to define the utility and promiscuity of the parent core heterocycles ($R^2 = \text{H}$) in fragment-based screening, and to probe their utility in the identification of more selective inhibitors for existing (e.g., FAAH) or uncharacterized serine hydrolase targets with the *N*-substituted heterocycles ($R^2 = \text{CH}_2\text{Ph}$, $(\text{CH}_2)_5\text{Ph}$).

Their synthesis was anticipated to rely on a one-pot addition and cyclization of anilines (6) and phenols (7) with chlorosulfonyl isocyanate (8)⁴⁰ to produce the unsubstituted benzothiadiazin-3-one 1,1-dioxides (4, X = NH) or benzoxathiazin-3-one 1,1-dioxides (5, X = O) poised for *N*-alkylation (Figure 1). Benzothiadiazin-3-one 1,1-dioxides (4, X = NH) were targeted first because a two-step synthesis was known for similar compounds, allowing access to the desired candidate inhibitor set.³⁴ Interestingly, a number of compounds containing the substituted benzothiadiazin-3-one 1,1-dioxide core have been shown to exhibit effective biological activity,^{35–38,41} although none are reported to inhibit a hydrolytic enzyme target (Figure 2).

The use of reported conditions for the synthesis of the benzothiadiazin-3-one 1,1-dioxides (known for $R^1 = \text{OMe}$, Cl, Br),^{34,40,42,43} enlisting chlorosulfonyl isocyanate (CSI) and AlCl_3 with the electron-rich anilines, provided 9 and 10

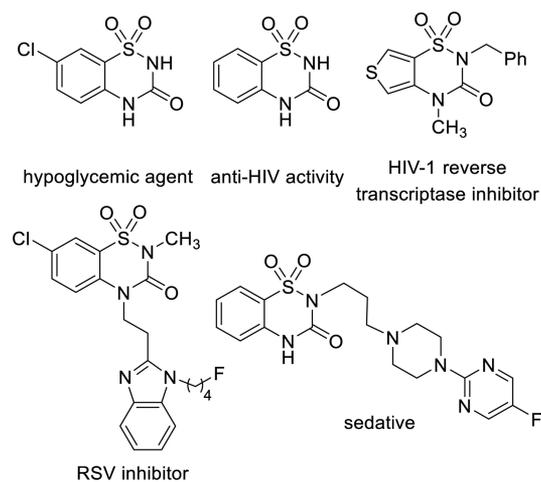
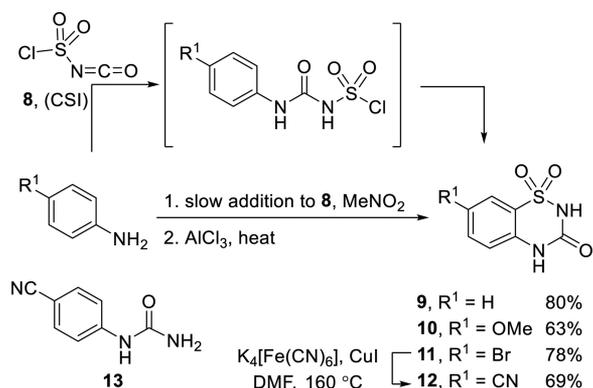


Figure 2. Benzothiadiazin-3-one 1,1-dioxides that display biological activity.

(Scheme 1), but failed to provide **12** with 4-aminobenzonitrile. Instead, after successful aniline addition to the isocyanate,

Scheme 1. Synthesis of Benzothiadiazin-3-one 1,1-Dioxide Cores



attempts to promote electrophilic aromatic substitution led only to isolation of the 4-cyanophenylurea (**13**, R¹ = CN) despite a screen of Lewis acids, reaction temperatures, and reaction solvents. However, the successful use of the method with *p*-bromoaniline provided **11** and permitted a subsequent cyanation reaction⁴⁴ for accessing **12** (Scheme 1).

The four urea-containing core scaffolds were subjected to alkylation to incorporate the two R² substituents (Figure 3).

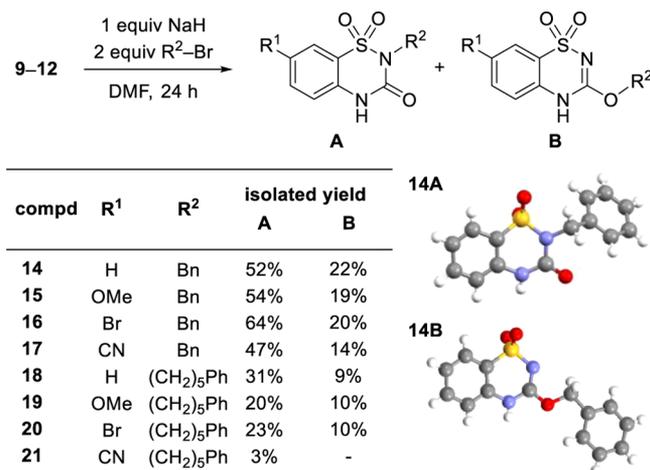


Figure 3. Alkylation of benzothiadiazin-3-one 1,1-dioxide cores and X-ray crystal structures of **14A** and **14B**.

Initial studies with **9** (R¹ = H), employing NaH and benzyl bromide, yielded a separable mixture of two products (**14A** and **14B**). They were tentatively assigned to be the products of the desired *N*-benzylation (**14A**, 52%) and *O*-benzylation (**14B**, 22%) on the basis of the ¹H/¹³C NMR chemical shifts, and their structures were unambiguously established by single-crystal X-ray structure determinations (Figure 3).⁴⁵ Extension of this method with **10-12** proceeded smoothly to give **15A-17A** in good yields (64–47%). Without optimization, the synthesis was also used to access the second R² series, using NaH and 1-bromo-5-phenylpentane to provide **18A-21A** (Figure 3).

Access to the targeted candidate inhibitors permitted their examination for inhibition of purified FAAH¹⁸ (Figure 4). The

compound	R ¹	N-alkyl group	K _i ^a
9	OMe	H	>100 μM ^b
10	H	H	>100 μM ^b
11	Br	H	>100 μM ^b
12	CN	H	>100 μM ^b
14A	OMe	Bn	>100 μM ^b
15A	H	Bn	>100 μM ^b
16A	Br	Bn	80 ± 1 μM
17A	CN	Bn	57 ± 0.7 μM
18A	OMe	(CH ₂) ₅ Ph	71 ± 1 μM
19A	H	(CH ₂) ₅ Ph	48 ± 0.5 μM
20A	Br	(CH ₂) ₅ Ph	29 ± 0.7 μM ^c
21A	CN	(CH ₂) ₅ Ph	45 ± 6 μM

^aPreincubation = 0 h. ^bHighest concentration tested. ^cAlso run at 3 h and 6 h preincubation to give K_i = 47.1 ± 0.5 μM and 82.1 ± 0.1 μM.

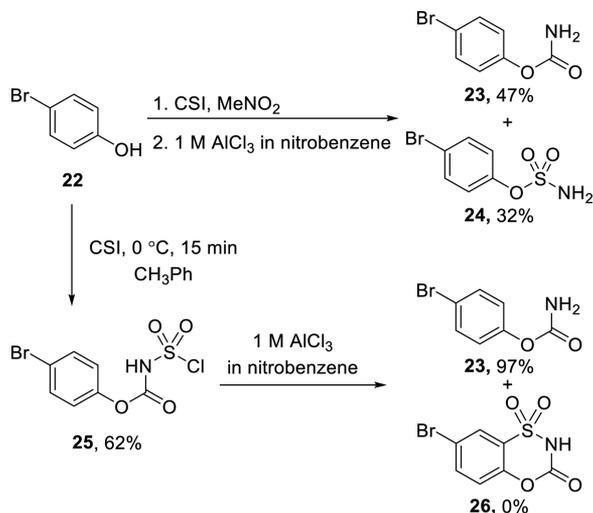
Figure 4. FAAH inhibition by benzothiadiazin-3-one 1,1-dioxides.

core scaffolds (R² = H, **9A-12A**) showed no activity (K_i > 100 μM), and only a limited set of the benzyl substituted benzothiadiazin-3-one 1,1-dioxides (**14A-17A**) showed modest activity (**16A** and **17A**). In the phenylpentyl series (**18A-21A**), all compounds exhibited modest activity. The most potent of the inhibitors (**20A**) was examined in detail. Compound **20A** was found to exhibit simple competitive inhibition kinetics, and it did not show a time-dependent increase in the inhibition potency, both of which are indicative of simple reversible competitive enzyme inhibition. Additionally, the full set of candidate inhibitors (**9A-21A**) was incubated with mouse brain membrane and soluble proteome and labeled with FP-rhodamine or iodoacetamide (IA)-rhodamine to examine serine and cysteine hydrolase inhibition (Figures S1 and S2). The reactivity with active site lysine-containing enzymes was also examined through use of a Kinativ kit. Ultimately, none of the gel-based ABPP studies showed evidence of significant target enzyme inhibition, and only weak reversible inhibition was detected at very high concentrations for a few compounds. The compounds also displayed a lack of hydrolytic reactivity of the potentially activated urea, being stable in methanol, DMSO, and organic solvents as well as in weakly acidic and basic aqueous media (>24 h). Thus, initial studies with the benzothiadiazin-3-one 1,1-dioxides, unlike those with the carbamate variant that followed, suggest they lack the structure and reactivity to generally act as irreversible serine hydrolase inhibitors.

Remarkably, there is only a single report of the preparation of a 4,1,2-benzoxathiazin-3-one 1,1-dioxide (**5**, X = O) with CSI, and it was isolated as a byproduct in the reaction with *p*-cresol.³⁹ This, along with the single report of one *N*-substituted derivative (R² ≠ H),⁴⁶ made this unexplored carbamate a compelling class of compounds to prepare, characterize, and evaluate. *p*-Bromophenol was chosen as an initial substrate for examination of its reactivity toward CSI as a route to the expectedly more reactive carbamate versus urea series. In the reported synthesis, the cyclized product of *p*-cresol was isolated

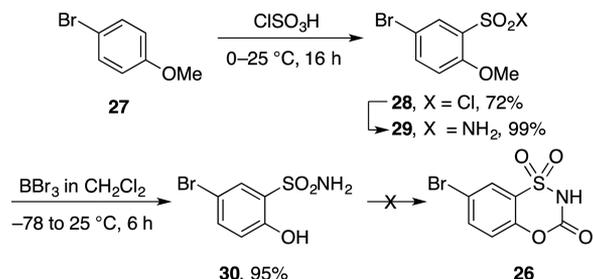
as a byproduct during sulfonylisocyanate formation.³⁹ Attempts to extend this synthesis to **22** were unsuccessful (Scheme 2), even upon isolation and resubjection of intermediate **25** to the Lewis acid-catalyzed cyclization conditions.

Scheme 2. Direct Reaction of Phenol **22** with CSI



An alternative approach to **26** via the 2-hydroxybenzenesulfonamide **30** was pursued in which aryl chlorosulfonylation *ortho* to a protected phenol preceded cyclic carbamate formation (Scheme 3). Although successful in providing **29**

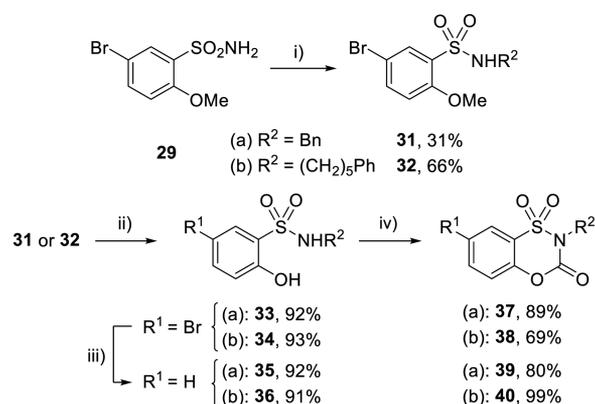
Scheme 3. Attempted Synthesis of Benzothiazin-3-one 1,1-Dioxide Core



and following methyl ether deprotection, attempts to form the final cyclized product from **30** with triphosgene, phosgene, or 4-nitrobenzoyl chloride were unsuccessful. The highly polar, acidic, and reactive nature of the product made its isolation challenging and led to examination of a variation on this route, introducing the amine substituent prior to cyclization. This provides nonacidic intermediates with increased organic solubility, avoiding the challenges of isolation of **26**.

N-Alkylation of **29** (NaH, BnBr, DMF), with a methyl ether serving as a phenol protecting group to prevent *O*-alkylation, proceeded to give **31** in 31% yield (Scheme 4), with recovered starting material and dialkylated product accounting for the remaining material. Methyl ether deprotection with boron tribromide provided **33** in 92% yield (Scheme 4). Formation of benzothiazin-3-one 1,1-dioxide was now successful when conducted with phosgene and NaH to yield **37** in 89% yield (Scheme 4). This provided a route to *N*-alkylated benzothiazin-3-one 1,1-dioxides and was applied to the additional targeted *N*-substitution product ($R^2 = (\text{CH}_2)_5\text{Ph}$), which proceeded even more smoothly to form **38** (42% over three

Scheme 4. Stepwise Synthesis of Substituted Benzothiazin-3-one 1,1-Dioxides^a



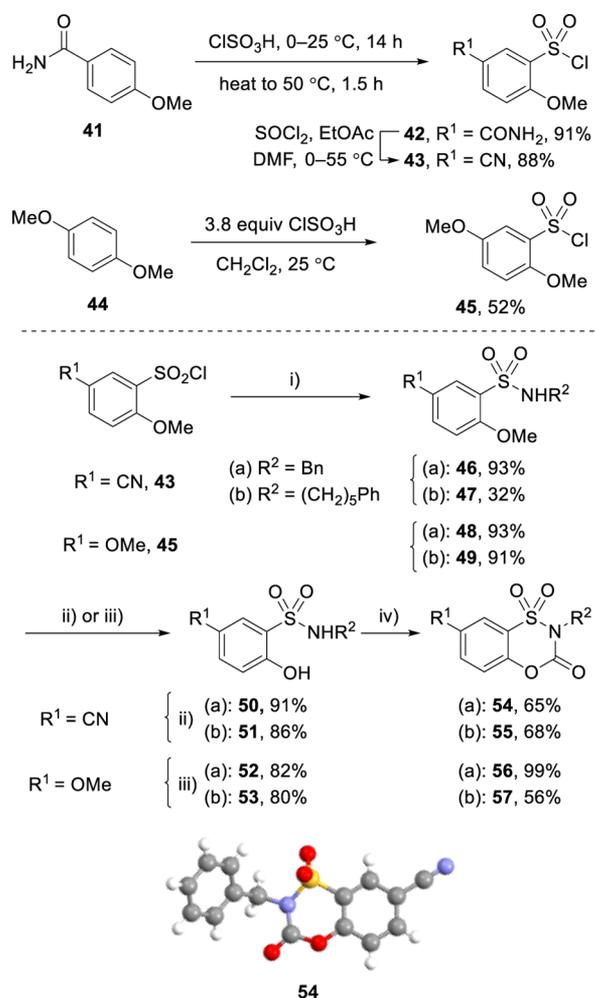
^aConditions: (i) 1 equiv of NaH, 2 equiv of $R^2\text{-Br}$, DMF; (ii) BBr_3 , CH_2Cl_2 , -78 to 25 °C; (iii) Pd/C, H_2 , MeOH; (iv) 5 equiv of NaH, 2 equiv of phosgene, CH_2Cl_2 , 25 °C.

steps, Scheme 4). Hydrogenolysis of **33** and **34** ($R^1 = \text{Br}$) proceeded smoothly to yield the C7-unsubstituted ($R^1 = \text{H}$) precursors **35** and **36** in 92% and 91% yield, respectively, and subsequent carbamate formation yielded **39** and **40** (Scheme 4).

Completion of the series with preparation of the C7-cyano and C7-methoxy derivatives is summarized in Scheme 5. Chlorosulfonylation of 4-methoxybenzamide (**41**) followed by subsequent dehydration of **42** and chlorosulfonylation of 1,4-dimethoxybenzene (**44**) were successfully utilized to access the sulfonyl chlorides **43** and **45** (Scheme 5). To further streamline the approach, a direct addition of the desired substituted amine to the sulfonyl chloride was used. Reaction of **43** and **45** with benzylamine and 5-phenylpentylamine produced the substituted sulfonamides **46–49** in high yield. Subjection of the methyl ethers **48** and **49** to the boron tribromide deprotection conditions selectively provided **52** and **53**, whereas phenol deprotection in the *p*-cyano series required stronger reaction conditions (LiCl, 140 °C, DMF) to form hydroxysulfonamides **50** and **51**. Preparation of the four additional benzothiazin-3-one 1,1-dioxides (**54–57**) proceeded upon treatment of **50–53** with phosgene and NaH. The structure of the candidate inhibitors was confirmed with a single-crystal X-ray structure determination⁴⁵ of **54** (Scheme 5).

Access to the eight benzothiazin-3-one 1,1-dioxides allowed the examination of this carbamate series by gel-based ABPP. The use of mouse brain proteome (membrane fractions of proteome) and FP-rhodamine labeling showed clear inhibition of the serine hydrolase ABHD10 by nearly all compounds in the series, as well as low micromolar inhibitor activity against LYPLA1/2 by **55** (Figure 5). Interestingly, FAAH was not effectively inhibited by members of this class even with the candidate inhibitors that bear the 5-phenylpentyl side chain. Although not extensively profiled, IA-rhodamine and Alexa Fluor labeling did not detect evidence of cysteine protease/hydrolase or lysine-containing enzyme inhibition (Figures S3 and S4).

The results from this initial gel-based screen displayed interesting trends. First, the new inhibitor class displayed surprisingly selective inhibition among the serine hydrolases, displaying full inhibition of only three enzymes and partial inhibition of three more (Figure 5). Moreover, even within this

Scheme 5. Streamlined Synthesis of Candidate Inhibitors and X-ray Crystal Structure of Candidate Inhibitor 54^a

^aConditions: (i) 2 equiv of R^2-NH_2 , 0.15 equiv of Et_3N , CH_2Cl_2 ; (ii) 4 equiv of LiCl , DMF , $140\text{ }^\circ\text{C}$; (iii) BBr_3 , CH_2Cl_2 , -78 to $25\text{ }^\circ\text{C}$; (iv) 5 equiv of NaH , 2 equiv of phosgene, CH_2Cl_2 .

small set of inhibitors, distinctive selectivity trends were observed toward these enzymes. Low micromolar inhibition against ABHD10 was observed for nearly all of the inhibitors ($\text{R}^2 = (\text{CH}_2)_5\text{Ph} > \text{CH}_2\text{Ph}$) regardless of core substituent ($\text{R}^1 = \text{CN}$, Br , H , OMe). Only the more reactive inhibitor **55** ($\text{R}^1 = \text{CN}$) bearing only the phenylpentyl R^2 substituent in the set of eight displayed LYPLA1/2 inhibition. Similarly, only inhibitors that contain the benzyl substituent ($\text{R}^2 = \text{CH}_2\text{Ph}$) inhibited APEH with core substituent trends reflecting the expected intrinsic reactivity of the inhibitors ($\text{R}^1 = \text{CN} > \text{H} > \text{OMe}$). Finally, the inhibition was established to be irreversible against ABHD10 and LYPLA1/2 in studies analogous to those detailed latter herein (see Figure 12). As a result of these initial observations, a detailed examination of both the chemical and the biochemical reactivity of these compounds was conducted to more comprehensively establish their behavior and the potential electronic effects of the varied substituents.

The stability and reactivity of the benzoxathiazin-3-one 1,1-dioxides were briefly and initially surveyed with **37** ($\text{R}^1 = \text{Br}$, $\text{R}^2 = \text{Bn}$) and **54** ($\text{R}^1 = \text{CN}$, $\text{R}^2 = \text{Bn}$), and both were found to be stable in DPBS, H_2O , THF , and ethanol at $25\text{ }^\circ\text{C}$ for over 24 h (Figure 6). Hydrolysis to provide **33** or **50** was observed in

DMEM buffer (1–6 h at 25 or $37\text{ }^\circ\text{C}$, Figure 6), whereas reaction to produce unknown products was observed in Tris-HCl buffer (in less than 1 h at $25\text{ }^\circ\text{C}$). Finally, samples stored in DMSO showed slow conversion to the hydrolysis products **33** and **50** at both $25\text{ }^\circ\text{C}$ (50% conversion in approximately 7 or 14 days, respectively) and at $-78\text{ }^\circ\text{C}$ (50% conversion in approximately 14 or 21 days, respectively).

Their reactivity toward methanol as solvent provided the clearest indication of not only the relative reactivity ($\mathbf{54} > \mathbf{37}$), but also the reaction course. The reaction product derived from **37** and **54** in methanol was shown to be **58** or **59**, and indicates that the phenol acts as the leaving group (vs $-\text{SO}_2\text{NHR}^2$). In this respect, the behavior of the benzoxathiazin-3-one 1,1-dioxides differs from that of the saccharin-based inhibitors where only the sulfonamide can serve as the leaving group. Subjection of the full set of phenylpentyl-substituted compounds ($\text{R}^1 = \text{OMe}$, H , and CN) to the reaction with methanol allowed a direct comparison of the magnitude of the electronic effect of the 7-substitution on the carbamate carbonyl reactivity (Figure 7). The compounds displayed predictable relative reaction rates ($\text{R}^1 = \text{CN} > \text{H} > \text{OMe}$), where compound **55** with the electronic-withdrawing nitrile substituent exhibited the greatest reactivity observed even at room temperature. Compound **57** with the electron-donating methoxy substituent exhibited the lowest reactivity, requiring elevated temperatures to observe methanol addition, and the differences were of a magnitude indicative of a pronounced C7 substituent electronic effect (Figure 7).

The reactivity toward nucleophiles was also examined through subjection of the same series of compounds to reactions with benzylamine, benzyl alcohol, and benzyl mercaptan (1 equiv, Figure 7). The reactions were performed in deuterated chloroform (0.1 M) with dibromomethane as an internal standard and analyzed by ^1H NMR initially to determine the reaction time required for 75% conversion to products **60** (Figure 7). Neither benzyl alcohol nor benzyl mercaptan reacted with the compounds under these conditions. However, the reactivity profile with benzylamine exhibited a clear trend (Figure 7). Very rapid reaction was observed with the 7-cyano derivative **55** ($t_{1/2} = 1.3\text{ min}$, $k = 5.3\text{ M}^{-1}\text{ s}^{-1}$), and a slower reaction was seen for the C7 unsubstituted compound **40** ($t_{1/2} = 13\text{ min}$, $k = 1.8\text{ M}^{-1}\text{ s}^{-1}$). The 7-methoxy derivative **57** exhibited a much slower rate of reaction ($t_{1/2} = 66\text{ min}$, $k = 0.24\text{ M}^{-1}\text{ s}^{-1}$) with full conversion observed only after 12 h. A Hammett plot ($\log k$ vs σ_p) of the results provided a slope (ρ value) of 1.2, indicating that the electronic effect of the substituent on this reactivity exceeds even that observed on benzoic acid acidity.

Because of their cyclic structure, the designed inhibitors are unique among previously explored carbamates in that there are not only two potential leaving groups (the sulfonamide or phenol), but no group is released from the enzyme upon initial covalent acylation of an active site serine due to the tethered nature of the carbonyl. Moreover, the compounds are also potentially capable of a subsequent cross-linking modification at the active site, where a second nucleophilic residue could attack the carbonyl of the covalently bound inhibitor, leaving a carbonyl bound to two residues in the active site with release of the remainder of the molecule. To probe these two possibilities, compound **57** was incubated with purified ABHD10 (5-fold excess of inhibitor) for 30 min at $25\text{ }^\circ\text{C}$ and examined by ESI-MS (Figure 8). Accordingly, the measured mass of ABHD10, established 1–4 h after the initial 30 min incubation, increased

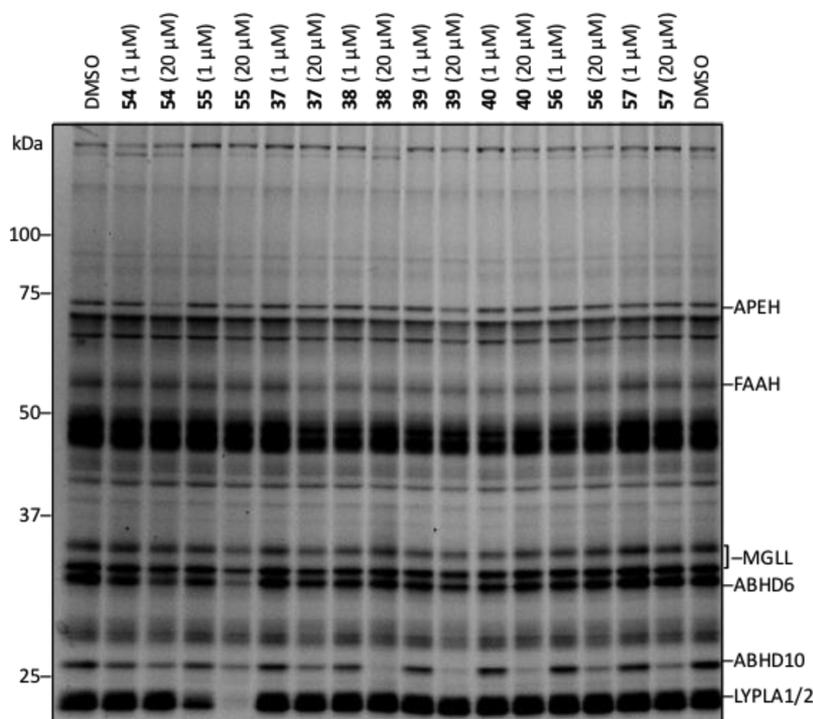
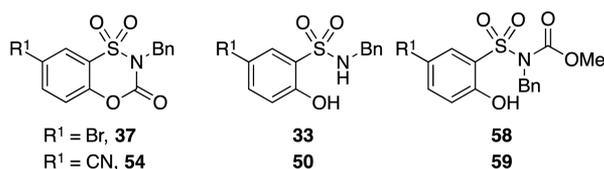


Figure 5. Screen of benzothiazin-3-one 1,1-dioxides by competitive gel-based ABPP. Mouse brain membrane proteome treated with compounds (at 1 and 20 μM , 30 min incubation) followed by FP-rhodamine (1 μM , 30 min). Fluorescent images shown in gray.



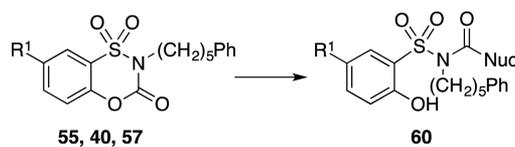
Conditions	Stability (at 25 °C) ^a	Stability (at 25 °C) ^a
	37, R ¹ = Br, R ² = Bn	54, R ¹ = CN, R ² = Bn
H ₂ O	>24 h	>24 h
EtOH	>24 h	>24 h
MeOH	4 h ^b	1 h ^b
DMSO	14 days	7 days
DMSO (-78 °C)	21 days	14 days
DPBS	>24 h	>24 h
Tris-HCl	1 h	1 h
DMEM	6 h	2 h
DMEM (37 °C)	2 h	<2 h

^aTime to approximately 50% conversion to either the open-form **33** or **50**, or to an unknown product. ^bProduct observed was **58** or **59**

Figure 6. Stability and reactivity of **37** and **54**.

by 376 Da, indicative of only initial acylation of the enzyme with no evidence of a subsequent cross-linking reaction (no increase of 26 Da). Thus, **57** serves as an irreversible inhibitor of ABHD10 that acts by monoacylation of an active site serine without release of a leaving group under the conditions of our examinations. Although prolonged incubation conceivably could lead to cross-linking, these studies suggest it is unlikely to be observed with ABHD10. However, we would not want to rule out such behavior against other serine hydrolases with members of this inhibitor class.

Additional proteome-wide screens of the compounds allowed the further comparison of their reactivity and serine hydrolase inhibition in vitro, by examining ABHD10 and LYPLA1/2



Nuc-H	Reactivity (at 25 °C) ^a		
	55 (R ¹ = CN)	40 (R ¹ = H)	57 (R ¹ = OMe)
MeOH	32 h	>72 h (5 h) ^b	>72 h (>18 h) ^b
BnNH ₂	<0.1 h	0.5 h	3.1 h
BnOH	>24 h	>24 h	>24 h
BnSH	>24 h	>24 h	>24 h

^aTime until 75% conversion to **60**. ^bTime in brackets indicates result at 37 °C.

Figure 7. Reactivity of inhibitors.

activity in cell lysate proteome samples from mouse neuroblastomas (N2A cells, Figure 9a) and human embryonic kidney cells (HEK 293T cells, Figure 9b). Treatment with the three related compounds **55**, **40**, and **57** (R² = (CH₂)₅Ph), at 1, 50, and 100 μM , showed a clear trend of strongest inhibition observed with R¹ = CN, and progressively less inhibition with R¹ = H and R¹ = OMe (Figure 9).

To more comprehensively define the relative selectivity of the inhibitors, the set comprised of **55**, **40**, and **57**, bearing the same R² group ((CH₂)₅Ph) and the varied R¹ group (CN, H, OMe, respectively), was analyzed by ABPP-SILAC (Stable Isotopic Labeling with Amino Acids in Cell culture) (Figure 10).^{14,47} Whole cell proteome samples from isotopically labeled HEK cell lysates were incubated with the compounds (at 25 μM) or DMSO control, then the activity-based probe FP-biotin. The FP-biotin-labeled proteins were enriched on streptavidin beads and then subjected to on-bead trypsin digestion. The resulting tryptic peptide mixtures were analyzed

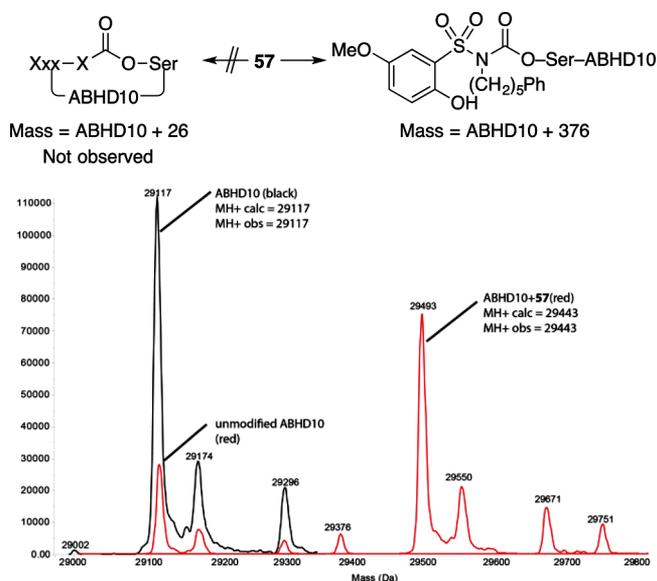


Figure 8. ESI-TOF-MS analysis of purified ABHD10 incubated with **57** (red), starting purified ABHD10 (black), and summary of results (top).

by multidimensional liquid chromatography–tandem mass spectrometry (LC/LC–MS/MS). The raw data were searched with the ProLuCID algorithm, and SILAC ratios were quantified with CIMAGE,⁴⁸ generating the data graphically shown in Figure 10. The greatest spectrum of inhibition was seen with the 7-cyano-substituted inhibitor **55**, with 10 enzymes inhibited at greater than 50% (Figure 10a), while only three enzymes were inhibited at this level by **40** (Figure 10b) and selective inhibition of only a single enzyme (PNPLA4) was observed for **57** (Figure 10c). These remarkable results beautifully display the C7 substituent electronic effect on the reactivity of the carbamate carbonyl of the benzoxathiazin-3-one 1,1-dioxides and its impact on the promiscuity of the inhibitor activity (Figure 10d). Additionally, the selectivity of the inhibition of LYPLA1/2 by **55**, observed in the gel-based ABPP assay, was confirmed by the MudPIT analysis, as no inhibition of these two enzymes is observed for **40** and **57** (Figure 10). Like most but not all of the identified enzyme targets, ABHD10 inhibition followed the trends (CN > H > OMe) first observed by gel-based ABPP (Figure 10d). Most impressively, the analysis revealed the exquisite selectivity of **57** for a single human enzyme (not present in mouse): PNPLA4. Interestingly, both **57** and **40** inhibit PNPLA4 preferentially and completely, whereas the more reactive inhibitor **55** does so incompletely and with a greater preference for eight other enzymes. Clearly the more reactive inhibitor **55**

identified a larger subset of serine hydrolases that can be targeted by the new inhibitor class, which most prominently also includes PNPLA8, AIG1, ABHD6, LIPE, and ABHD11, but the less reactive members **40** and **57** uniquely identified an enzyme (PNPLA4) that would have been overlooked by screening with **55** alone.

PNPLA4 (patatin-like phospholipase domain-containing protein 4), also known as GS2,⁴⁹ is present in humans, but is absent in mouse.⁵⁰ It constitutes a poorly characterized serine hydrolase that is known to hydrolyze triglycerides and retinol esters and is thought to be involved in adipocyte homeostasis. Thus, the discovery of a selective inhibitor of PNPLA4 constitutes a critical advance for characterizing the physiological role of PNPLA4. The use of overexpressed PNPLA4 in HEK cells allowed selective PNPLA4 inhibition by **57** to be observed in gel-based ABPP assays with whole cell lysates of the soluble and membrane-bound proteome fractions, with a calculated IC₅₀ of 1.8 μM determined from the separated membrane proteome fraction (Figure 11).

Subsequent studies were conducted that demonstrated the irreversibility of serine hydrolase inhibition through a gel-based ABPP study. After 30 min of incubation of compound **57** with the same HEK cell membrane proteome as Figure 11, filtration through a desalting column removed excess compound and allowed isolation of the proteome containing the enzyme–inhibitor complex. Aliquots of the complex in the full proteome were subjected to FP-rhodamine labeling at various time points to show the persistence of inhibition of PNPLA4, indicating both that binding is covalent, because the complex survives column filtration, and irreversible over the time scale because little enzyme reactivation was observed (<10% at 8 h postfiltration, Figure 12).

In preliminary studies, subjection of these same HEK cells to **57** in FBS-free media provided in-cell PNPLA4 inhibition, albeit at a 10-fold higher concentration (calculated IC₅₀ = 11.7 μM), whereas treatment (50 μM **57**) in the presence of fetal bovine serum (FBS) failed to display activity. At present, we do not know whether this loss of in-cell activity in the presence of FBS is due to serum protein binding or serum instability, although we suspect it is not due to serum instability (see below). These results are remarkable for a pilot study of a new serine hydrolase inhibitor chemotype, and further optimization of **57** can be expected to provide a more potent, cellularly active inhibitor of PNPLA4 capable of use in defining its physiological role. Notably, **57** is among the most stable benzoxathiazin-3-one 1,1-dioxides examined, bearing an aryl electron-donating substituent that moderates its reactivity. Compound **57** is stable in DMSO at room temperature for at least 2 weeks, solid samples could be stored on the bench open to the air for greater than 6 months, and it was found to be stable to both

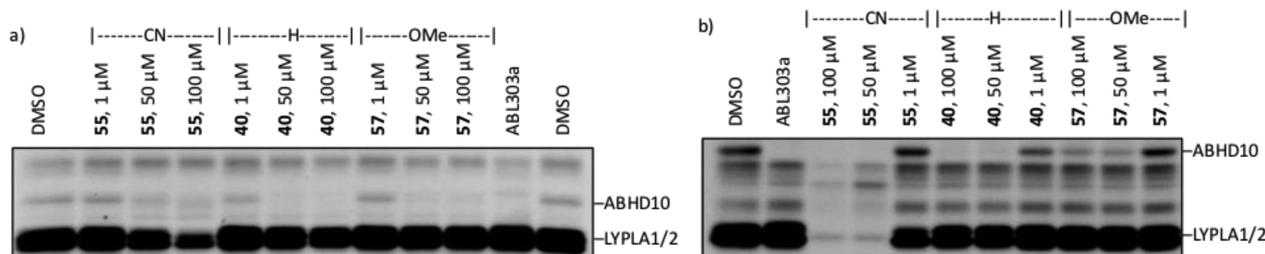


Figure 9. In vitro analysis of compounds **55**, **40**, and **57** (at 1, 50, 100 μM, R² = (CH₂)₅Ph) by gel-based ABPP with (a) processed N2A cells and (b) processed HEK cells showing the electronic effect of aromatic core C7 substituents (R¹ = CN, H, OMe) on inhibition of ABHD10 and LYPLA1/2.

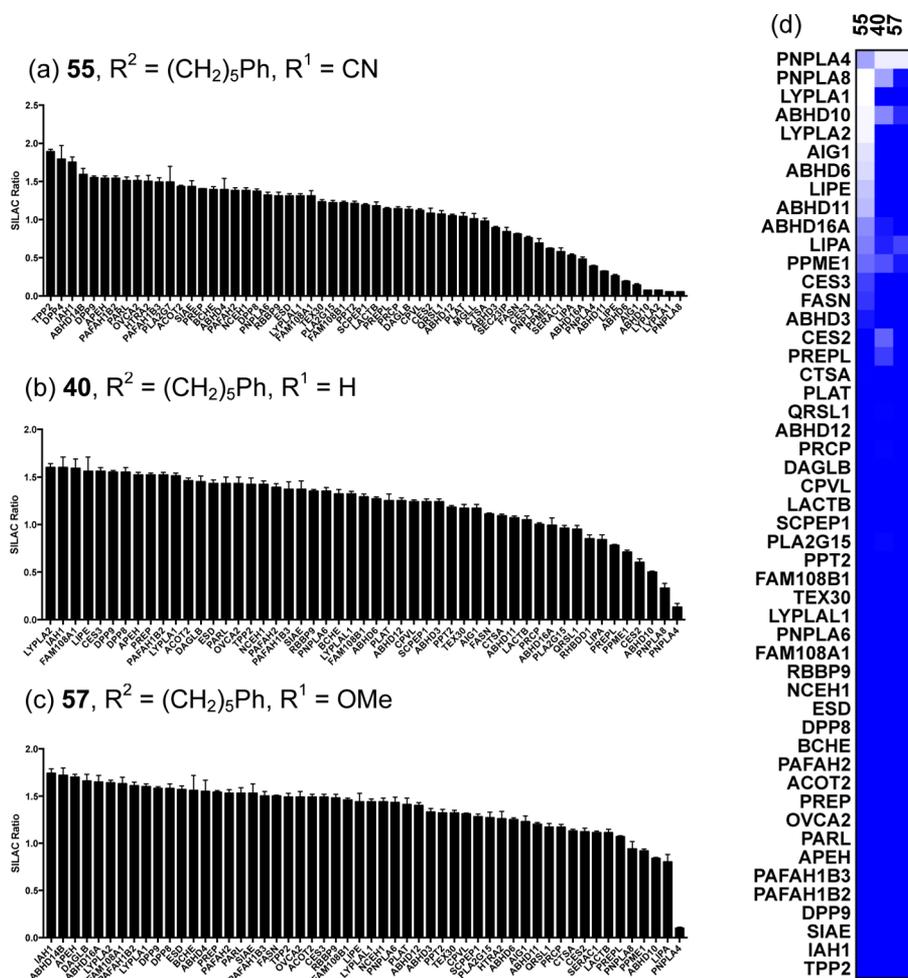


Figure 10. ABPP-SILAC analysis of compounds (**55** (a), **40** (b), **57** (c)) using HEK cell lysates showing the effect of substituents on inhibitor potency and selectivity and (d) heat map showing trends for the three inhibitors and the selectivity for PNPLA4 inhibition.

DPBS (25 °C) and DMEM (25 and 37 °C) buffers over the time frames used in our assays (1–4 h, longer times not examined). Finally, and although IA-rhodamine and Alexa Fluor labeling did not detect evidence of competitive cysteine protease/hydrolase or lysine-containing enzyme inhibition by **57**, it and members of this class have not been examined for activity against or reactivity toward other cellular targets.

CONCLUSIONS

A new class of serine hydrolase inhibitors was discovered, bearing a previously unexplored chemotype, based on a ring-expanded saccharin core obtained by introduction of a ring heteroatom that converted the saccharin amide to a carbamate. Although the analogous urea core displayed insufficient reactivity for use as candidate irreversible inhibitors of the serine hydrolases, the benzoxathiazin-3-one 1,1-dioxides, containing the carbamate core and a more reactive carbonyl, were found to be effective and selective irreversible serine hydrolase inhibitors. The benzoxathiazin-3-one 1,1-dioxides displayed tunable reactivity capable of C7-substituent electronic modulation of the inherent reactivity and proved amenable for active site targeting by variation of the N-substituent. Additionally and unique among the more traditional carbamate and urea-based serine hydrolase inhibitors, they do not release a leaving group upon active site serine acylation.³ This latter feature avoids unknown or unanticipated pharmacology or

toxicity derived from a released leaving group. In this regard, the inhibitors more closely parallel irreversible cysteine protease inhibition derived from halide displacement, conjugate addition, addition to epoxides or aziridines, or nucleophilic cleavage of weak bonds.³ Even more remarkable and within the limited series of candidate inhibitors examined, an exquisitely selective inhibitor was discovered for an uncharacterized hydrolase, PNPLA4. This enzyme bears a Ser-Asp catalytic dyad and is likely involved in adipocyte triglyceride homeostasis.^{51–53} Also known as protein GS2,⁴⁹ it is present in a variety of human tissues, but is absent in mouse.⁵⁰ PNPLA4 is known to catalyze the hydrolysis of triglycerides and participate in retinol ester hydrolytic metabolism.^{54,55} Further exploration of this unique PNPLA4-selective inhibition by **57** should allow the optimization and development of an even more potent and selective inhibitor suitable for detailed examination of this underexplored serine hydrolase. Just as significantly, the benzoxathiazin-3-one 1,1-dioxides represent a new and unexplored class of candidate enzyme inhibitors capable of selective irreversible inhibition of a subclass of serine hydrolases and amenable to structural modifications that substantially modulate intrinsic reactivity and/or active site recognition.

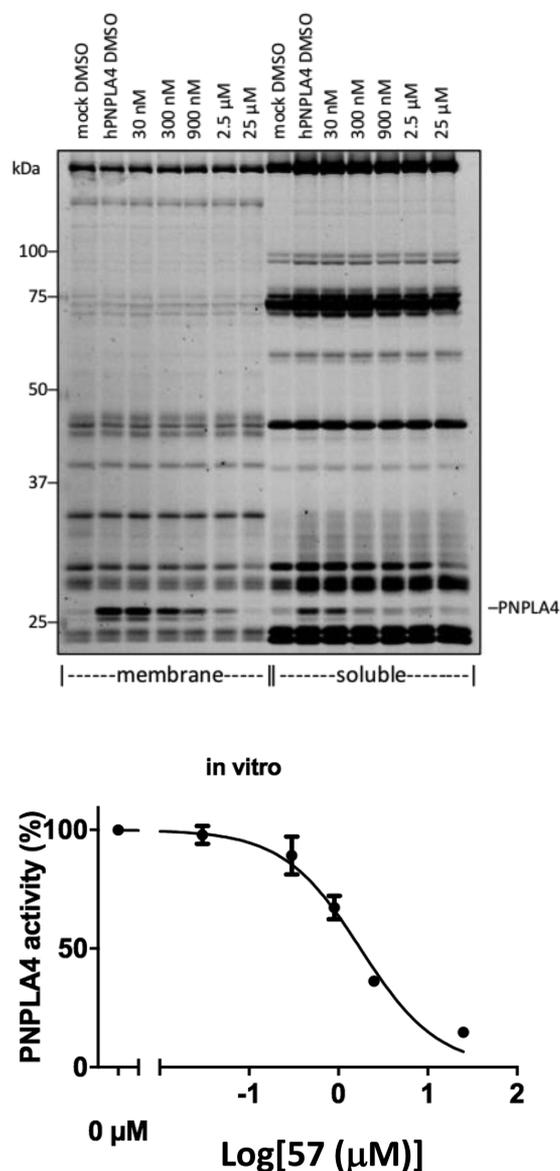


Figure 11. Selective PNPLA4 inhibition of cell lysates of transfected HEK cells by 57.

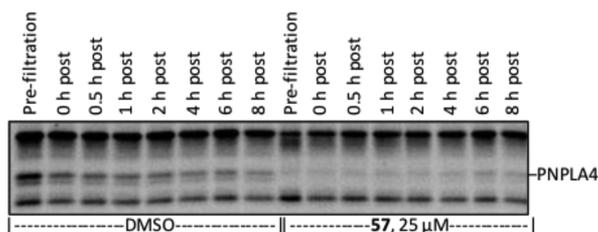


Figure 12. Irreversibility of the inhibition of PNPLA4 by 57. Incubation of HEK cell membrane proteome with 57 (25 μM, 30 min) and column filtration provided the covalent complex that was then subjected to FP-rhodamine labeling (1 μM, 30 min) at various time points postfiltration. Full gel shown in Figure S5.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b02985.

Full experimental details, characterization data of all compounds, supplemental figures, and ¹H and ¹³C NMR spectra of all compounds (PDF)

X-ray crystallographic data for compound 14A (CIF)

X-ray crystallographic data for compound 14B (CIF)

X-ray crystallographic data for compound 54 (CIF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

- (1) Bachovchin, D. A.; Cravatt, B. F. *Nat. Rev. Drug Discovery* **2012**, *11*, 52–68.
- (2) Long, J. Z.; Cravatt, B. F. *Chem. Rev.* **2011**, *111*, 6022–6063.
- (3) Powers, J. C.; Asgjan, J. L.; Ekici, O. D.; James, K. E. *Chem. Rev.* **2002**, *102*, 4639–4750.
- (4) Boger, D. L.; Sato, H.; Lerner, A. E.; Austin, B. J.; Patterson, J. E.; Patricelli, M. P.; Cravatt, B. F. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 265–270.
- (5) Koutek, B.; Prestwich, G. D.; Howlett, A. C.; Chin, S. A.; Salehani, D.; Akhavan, N.; Deutsch, D. G. *J. Biol. Chem.* **1994**, *269*, 22937–22940.
- (6) (a) Patterson, J. E.; Ollmann, I. R.; Cravatt, B. F.; Boger, D. L.; Wong, C. H.; Lerner, R. A. *J. Am. Chem. Soc.* **1996**, *118*, 5938–5945. (b) Cravatt, B. F.; Lerner, R. A.; Boger, D. L. *J. Am. Chem. Soc.* **1996**, *118*, 580–590.
- (7) Bottcher, T.; Sieber, S. A. *Angew. Chem., Int. Ed.* **2008**, *47*, 4600–4603.
- (8) Hoover, H. S.; Blankman, J. L.; Niessen, S.; Cravatt, B. F. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5838–5841.
- (9) Tew, D. G.; Boyd, H. F.; Ashman, S.; Theobald, C.; Leach, C. A. *Biochemistry* **1998**, *37*, 10087–10093.
- (10) Boger, D. L.; Sato, H.; Lerner, A. E.; Hedrick, M. P.; Fecik, R. A.; Miyauchi, H.; Wilkie, G. D.; Austin, B. J.; Patricelli, M. P.; Cravatt, B. F. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97*, 5044–5049.
- (11) Boger, D. L.; Miyauchi, H.; Hedrick, M. P. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1517–1520.
- (12) Otrubova, K.; Boger, D. L. *ACS Chem. Neurosci.* **2012**, *3*, 340–348.
- (13) Bachovchin, D. A.; Ji, T.; Li, W.; Simon, G. M.; Blankman, J. L.; Adibekian, A.; Hoover, H.; Niessen, S.; Cravatt, B. F. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 20941–20946.
- (14) Li, W.; Blankman, J. L.; Cravatt, B. F. *J. Am. Chem. Soc.* **2007**, *129*, 9594–9595.
- (15) Ahn, K.; Johnson, D. S.; Mileni, M.; Beidler, D.; Long, J. Z.; McKinney, M. K.; Weerapana, E.; Sadagopan, N.; Liimatta, M.; Smith, S. E.; Lazerwith, S.; Stiff, C.; Kamtekar, S.; Bhattacharya, K.; Zhang, Y.;

Swaney, S.; Van Becelaere, K.; Stevens, R. C.; Cravatt, B. F. *Chem. Biol.* **2009**, *16*, 411–420.

(16) Adibekian, A.; Martin, B. R.; Wang, C.; Hsu, K.-L.; Bachovchin, D. A.; Niessen, S.; Hoover, H.; Cravatt, B. F. *Nat. Chem. Biol.* **2011**, *7*, 469–478.

(17) Otrubova, K.; Ezzili, C.; Boger, D. L. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 4674–4685.

(18) Patricelli, M. P.; Patterson, J. E.; Boger, D. L.; Cravatt, B. F. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 613–618.

(19) Cravatt, B. F.; Wright, A. T.; Kozarich, J. W. *Annu. Rev. Biochem.* **2008**, *77*, 383–414.

(20) Evans, M. J.; Cravatt, B. F. *Chem. Rev.* **2006**, *106*, 3279–3301.

(21) Liu, Y. S.; Patricelli, M. P.; Cravatt, B. F. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 14694–14699.

(22) Patricelli, M. P.; Giang, D. K.; Stamp, L. M.; Burbaum, J. J. *Proteomics* **2001**, *1*, 1067–1071.

(23) Leung, D.; Hardouin, C.; Boger, D. L.; Cravatt, B. F. *Nat. Biotechnol.* **2003**, *21*, 687–691.

(24) Leung, D.; Du, W.; Hardouin, C.; Cheng, H.; Hwang, I.; Cravatt, B. F.; Boger, D. L. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1423–1428.

(25) Boger, D. L.; Miyauchi, H.; Du, W.; Hardouin, C.; Fecik, R. A.; Cheng, H.; Hwang, I.; Hedrick, M. P.; Leung, D.; Acevedo, O.; Guimarães, C. R. W.; Jorgensen, W. L.; Cravatt, B. F. *J. Med. Chem.* **2005**, *48*, 1849–1856.

(26) Lichtman, A. H.; Leung, D.; Shelton, C. C.; Hardouin, C.; Boger, D. L.; Cravatt, B. F.; Saghatelian, A. *J. Pharmacol. Exp. Ther.* **2004**, *311*, 441–448.

(27) Kinsey, S. G.; Long, J. Z.; O'Neal, S. T.; Abdullah, R. A.; Poklis, J. L.; Boger, D. L.; Cravatt, B. F.; Lichtman, A. H. *J. Pharmacol. Exp. Ther.* **2009**, *330*, 902–910.

(28) Otrubova, K.; Srinivasan, V.; Boger, D. L. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 3807–3813.

(29) Martyn, D. C.; Moore, M.; Abell, A. D. *Curr. Pharm. Des.* **1999**, *5*, 405–415.

(30) Zimmerman, M.; Morman, H.; Mulvey, D.; Jones, H.; Frankshun, R.; Ashe, B. M. *J. Biol. Chem.* **1980**, *255*, 9848–9851.

(31) Ashe, B. M.; Clark, R. L.; Jones, H.; Zimmerman, M. *J. Biol. Chem.* **1981**, *256*, 11603–11606.

(32) Hlasta, D. J.; Subramanyam, C.; Bell, M. R. *J. Med. Chem.* **1995**, *38*, 739–744.

(33) Combrink, K. D.; Gülgeze, H. B.; Meanwell, N. A.; Pearce, B. C.; Zulan, P.; Bisacchi, G. S.; Roberts, D. G.; Stanley, P.; Seiler, S. M. *J. Med. Chem.* **1998**, *41*, 4854–4860.

(34) Boverie, S.; Antoine, M.-H.; Somers, F.; Becker, B.; Seville, S.; Ouedraogo, R.; Counerotte, S.; Pirotte, B.; Lebrun, P.; de Tullio, P. *J. Med. Chem.* **2005**, *48*, 3492–3503.

(35) Majumdar, K. C.; Ganai, S. *Beilstein J. Org. Chem.* **2013**, *9*, 503–509.

(36) Hayao, S.; Stryker, W.; Phillips, B.; Fujimori, H.; Vidrio, H. *J. Med. Chem.* **1968**, *11*, 1246–1248.

(37) Buckheit, R. W., Jr.; Fliakas-Boltz, V.; Decker, W. D.; Roberson, J. L.; Pyle, C. A.; White, E. L.; Bowdon, B. J.; McMahon, J. B.; Boyd, M. R.; Bader, J. P.; Nickell, D. G.; Barth, H.; Antonucci, T. K. *Antiviral Res.* **1994**, *25*, 43–56.

(38) Arranz, M. E.; Díaz, J. A.; Ingate, S. T.; Witvrouw, M.; Pannecouque, C.; Balzarini, J.; De Clercq, E.; Vega, S. *Bioorg. Med. Chem.* **1999**, *7*, 2811–2822.

(39) Lohaus, G. *Chem. Ber.* **1972**, *105*, 2791–2799.

(40) Girard, Y.; Atkinson, J. G.; Rokach, J. J. *Chem. Soc., Perkin Trans. I* **1979**, 1043–1047.

(41) Combrink, K. D.; Gulgeze, H. B.; Thuring, J. W.; Yu, K.-L.; Civiello, R. L.; Zhang, Y.; Pearce, B. C.; Yin, Z.; Langley, D. R.; Kadow, K. F.; Cianci, C. W.; Li, Z.; Clarke, J.; Genovesi, E. V.; Medina, L.; Lamb, L.; Yang, Z.; Zadajura, L.; Krystal, M.; Meanwell, N. A. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 4784–4790.

(42) de Tullio, P.; Becker, B.; Boverie, S.; Dabrowski, M.; Wahl, P.; Antoine, M.-H.; Somers, F.; Seville, S.; Ouedraogo, R.; Hansen, J. B.; Lebrun, P.; Pirotte, B. *J. Med. Chem.* **2003**, *46*, 3342–3353.

(43) de Tullio, P.; Boverie, S.; Becker, B.; Antoine, M.-H.; Nguyen, Q.-A.; Francotte, P.; Counerotte, S.; Seville, S.; Pirotte, B.; Lebrun, P. *J. Med. Chem.* **2005**, *48*, 4990–5000.

(44) Schareina, T.; Zapf, A.; Mägerlein, W.; Müller, N.; Beller, M. *Chem. - Eur. J.* **2007**, *13*, 6249–6254.

(45) The structures **14A** (CCDC 1501165), **14B** (CCDC 1501166), and **55** (CCDC 1501167) were established by single-crystal X-ray structure determinations.

(46) Cremlyn, R. J.; Cronje, T. *Phosphorus Sulfur Relat. Elem.* **1979**, *6*, 413–419.

(47) Long, J. Z.; Li, W.; Booker, L.; Burstson, J. J.; Kinsey, S. G.; Schlosburg, J. E.; Pavón, F. J.; Serrano, A. M.; Selley, D. E.; Parsons, L. H.; Lichtman, A. H.; Cravatt, B. F. *Nat. Chem. Biol.* **2009**, *5*, 37–44.

(48) Weerapana, E.; Wang, C.; Simon, G. M.; Richter, F.; Khare, S.; Dillon, M. B. D.; Bachovchin, D. A.; Mowen, K.; Baker, D.; Cravatt, B. F. *Nature* **2010**, *468*, 790–797.

(49) Gao, J.; Simon, M. *J. Invest. Dermatol.* **2005**, *124*, 1259–1266.

(50) Kienesberger, P. C.; Oberer, M.; Lass, A.; Zechner, R. *J. Lipid Res.* **2009**, *50*, S63–S68.

(51) Cognetta, A. B.; Niphakis, M. J.; Lee, H.-C.; Martini, M. L.; Hulce, J. J.; Cravatt, B. F. *Chem. Biol.* **2015**, *22*, 1–10.

(52) Kohda, M.; Tokuzawa, Y.; Kishita, Y.; Nyuzuki, H.; Moriyama, Y.; Mizuno, Y.; Hirata, T.; Yatsuka, Y.; Yamashita-Sugahara, Y.; Nakachi, Y.; Kato, H.; Okuda, A.; Tamaru, S.; Bornha, N. N.; Banskoya, K.; Aigaki, T.; Sato-Miyata, Y.; Ohnuma, K.; Suzuki, T.; Nagao, A.; Maehata, H.; Matsuda, F.; Higasa, K.; Nagasaki, M.; Yasuda, J.; Yamamoto, M.; Fushimi, T.; Shimura, M.; Kaiho-Ichimoto, K.; Harashima, H.; Yamazaki, T.; Mori, M.; Murayama, K.; Ohtake, A.; Okazaki, Y. *PLoS Genet.* **2016**, *12*, e1005679.

(53) Gao, J. G.; Simon, M. *J. Invest. Dermatol.* **2006**, *126*, 2087–2095.

(54) Gao, J. G.; Shih, A.; Gruber, R.; Schmuth, M.; Simon, M. *Mol. Genet. Metab.* **2009**, *96*, 253–260.

(55) Holmes, R. S. *3 Biotech* **2012**, *2*, 277–286.