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Design, Synthesis, and Pharmacological Evaluation of Bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl Sulfide 3 (BPTES) Analogs as Glutaminase Inhibitors

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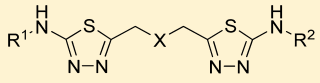
ABSTRACT: Bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES) is a potent and selective allosteric inhibitor of kidney-type glutaminase (GLS) that has served as a molecular probe to determine the therapeutic potential of GLS inhibition. In an attempt to identify more potent GLS inhibitors with improved drug-like molecular properties, a series of BPTES analogs were synthesized and evaluated. Our structure−activity relationship (SAR) studies revealed that some truncated analogs retained the potency of BPTES, presenting an opportunity to improve its aqueous solubility. One of the analogs, *N*-(5-{2-[2-(5-amino-[1,3,4]thiadiazol-2-yl)-ethylsulfanyl]-ethyl}-[1,3,4]thiadiazol-2-yl)-2-phenyl-acetamide **6**, exhibited similar potency and better solubility relative to BPTES and attenuated the growth of P493 human lymphoma B cells in vitro as well as in a mouse xenograft model.



3 ($R^1 = R^2 = PhCH_2CO$, $X = CH_2SCH_2$): IC_{50} = 3.3 μM
5 ($R^1 = R^2 = H$, $X = CH_2SCH_2$): IC_{50} = 100 μM
6 ($R^1 = H$, $R^2 = PhCH_2CO$, $X = CH_2SCH_2$): IC_{50} = 2.7 μM
11a ($R^1 = H$, $R^2 = PhCH_2CO$, $X = CH_2$): IC_{50} = 15 μM
11b ($R^1 = H$, $R^2 = PhCH_2CO$, $X = CH_2CH_2$): IC_{50} = 1.9 μM

INTRODUCTION

Glutaminase catalyzes the hydrolysis of glutamine into glutamate and ammonia. Mammalian tissues express two isoforms of glutaminase that are derived from distinct but structurally related genes.[1] Kidney-type glutaminase (GLS) is widely distributed throughout extra-hepatic tissues whereas liver-type glutaminase (GLS2) is found primarily in adult liver. GLS plays a critical role in glutaminolysis as an important energy source for many proliferating cells, especially rapidly growing malignant cells.[2,3] Recent studies have shown that Myc, an oncogenic transcription factor, increases glutamine metabolism by stimulating the expression of glutaminase directly or via suppression of miR23a and miR23b.[4] It is conceivable that, under hypoxic conditions, glutaminolysis provides the dominant means for cancer cell metabolism.[4] Indeed, in both P493 human B lymphoma cells and PC3 prostate cancer cells, the use of GLS siRNA diminished glutaminase levels and resulted in a remarkable decrease in cell proliferation. The antiproliferative effects could be recapitulated by removal of glutamine from the cell culture medium.[4] These findings indicate that small molecule GLS inhibitors might exert antiproliferative effects against Myc-dependent and other cancers.

In the nervous system, GLS is responsible for the production of intracellular glutamate, a key excitatory neurotransmitter, as a crucial part of the glutamine-glutamate cycle. However, accumulating evidence suggests glutamate formed by upregulated GLS in activated macrophages and microglia is playing a key pathogenic role in inflammatory neurological disorders such as HIV-1-associated dementia[5] and multiple sclerosis.[6] Therefore, small molecule GLS inhibitors may offer therapeutic potential in these devastating neurodegenerative diseases by reducing excess extracellular glutamate.

Despite the therapeutic potential of GLS inhibition, little effort has been made to explore the therapeutic utility of GLS inhibition for two key reasons. First, GLS plays a critical role in cellular metabolism, thus GLS inhibition could have major undesired physiological consequences. Indeed, recent studies showed that GLS knockouts die shortly after birth.[7] However, mice heterozygous for the GLS (GLS hets) are fully viable despite a mild yet significant reduction in glutamate levels in some tissues.[7] These findings suggest that GLS inhibition may provide a viable therapeutic option particularly for the treatment of cancer, where the clinical benefit outweighs the risk associated with a narrow therapeutic index. Second, little progress has been made in identifying potent and selective inhibitors of GLS. Although a conventional GLS inhibitor **1**, known as 6-diazo-5-oxo-L-norleucine (DON),[8] has been extensively utilized as a tool for studying physiological roles of GLS, its inherent chemical reactivity coupled with lack of selectivity and poor potency has hampered its use in establishing the therapeutic benefit of selective GLS inhibition.

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The renewed interest in GLS as a therapeutic target in recent years has prompted efforts to identify novel glutaminase inhibitors. For instance, dibenzophenanthridines represented by compound **2** are being explored as a new class of GLS inhibitors for the treatment of cancer.[9,10] Compound **3**, known as bis-2-[5-(phenylacetamido)-1,3,4-thiadiazol-2-yl]ethyl sulfide or BPTES, represents another class of GLS inhibitors structurally distinct from **1** (Figure 1).[11] Compound **3** offers some fundamental advantages over compounds **1** and **2**. Unlike **1**, compound **3** does not contain any reactive chemical group that might form a covalent bond and is unlikely to cause toxicity by irreversibly forming covalent adducts with endogenous proteins. Moreover, compound **3** bears no structural similarity to either glutamine or glutamate. This should minimize toxicological risk due to its interaction with other enzymes, transporters, or receptors that recognize glutamine or glutamate as substrates. For example, unlike **1**, compound **3** selectively inhibits GLS over GLS2 and γ-glutamyl transpeptidase.[12] A substrate concentration-dependent kinetic study revealed that **3** inhibits GLS in a uncompetitive manner by facilitating the formation of an inactive tetramer.[12,13] In addition compound **3** is capable of inhibiting the phosphate-activated GLS while compound **2** shows little ability to inhibit GLS in the presence of phosphate.[10] Recently, three independent groups solved crystal structures of GLS.[14−16] Some of these structures were determined as complexes with compound **3**,[15,16] providing compelling evidence for the previously proposed inactive tetramer formation by compound **3**. The new structural knowledge offers distinct advantages of compound **3** over other classes of GLS inhibitors as a template to conduct more rational structural optimizations.

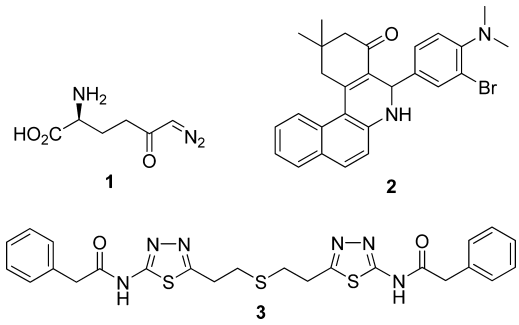


Figure 1. Structures of representative GLS inhibitors.

Because of its unique biochemical characteristics, compound **3** has been increasingly utilized to study the physiological role of GLS as well as the potential therapeutic effects of GLS inhibition.[17−19] Compound **3**, however, is highly hydrophobic with an aqueous solubility of less than 1 μg/mL. The poor solubility hinders its utility as a pharmacological probe and prompted us to explore whether structural modifications can be made to compound **3** without sacrificing its GLS inhibitory potency.

Herein is described systematic structure−activity relationship (SAR) analysis of analogs derived from **3** as a part of our ongoing efforts to identify drug-like GLS inhibitors. A series of truncated analogs were examined in an attempt to reduce the hydrophobicity and identify the minimal structural requirements for GLS binding. One of the truncated analogs **6** was also tested for its ability to attenuate lymphoma cell (P493) proliferation in vitro and tumor xenograft growth in vivo.

CHEMISTRY

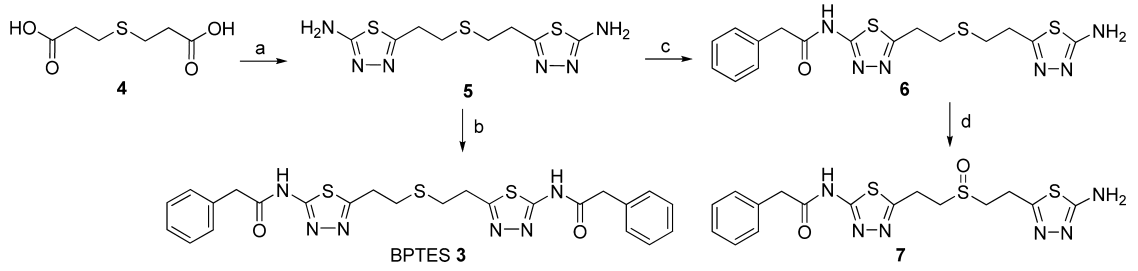
Two phases of SAR studies were carried out for analogs of compound **3**. First, various truncated analogs were explored to identify the pharmacophore required for GLS binding. Second, one of the truncated analogs was used as a template for further structural optimization in an attempt to improve the potency against GLS.

Scheme 1 illustrates the synthesis of **3** as well as its truncated analogs **5**, **6**, and **7**. Reaction of thiodipropionic acid **4** with thiosemicarbazide in the presence of phosphorus oxychloride gave bis-aminothiadiazole **5**.[11,16] Compound **3** was obtained by coupling **5** with 2.0 equiv of phenylacetyl chloride. On the other hand, monoacylated compound **6** was obtained by reacting **5** with only 1.0 equiv of phenylacetyl chloride. Sulfoxide **7** was prepared from sulfide **6** using hydrogen peroxide.

Truncated analogs lacking a sulfide group in the linker region (compounds **11a** and **11b**) were prepared from acid chlorides **8a** and **8b**, respectively (Scheme 2). Reaction of **8a-b** with thiosemicarbazide in the presence of phosphorus oxychloride followed by aqueous workup provided aminothiadiazole-containing carboxylic acids **9a-b**. The aminothiadiazole group of **9a-b** was acylated with phenylacetyl chloride to give **10a-b**. Subsequent reaction with thiosemicarbazide in the presence of phosphorus oxychloride afforded the desired products **11a** and **11b**.

Compound **9b** also served as an intermediate for analogs of **11b** where the phenylacetyl group is replaced with other acyl groups (Scheme 2). Compound **9b** was first converted to the corresponding methyl ester **12**. Subsequent reaction with acyl chlorides followed by hydrolysis gave **14a−f**. Reaction of **14a−f** with thiosemicarbazide in the presence of phosphorus oxychloride afforded the desired products **15a−f**.

Scheme 1. Synthesis of 3 and Truncated Analogs 5, 6 and 7[a]



[a]Reagents and conditions: (a) thiosemicarbazide, $POCl_3$, 90 °C, 79%; (b) 2.0 equiv phenylacetyl chloride, DMA, TEA 88%; (c) 1.0 equiv phenylacetyl chloride, DMA, TEA, 11%; (d) H_2O_2, MeOH, 60%.

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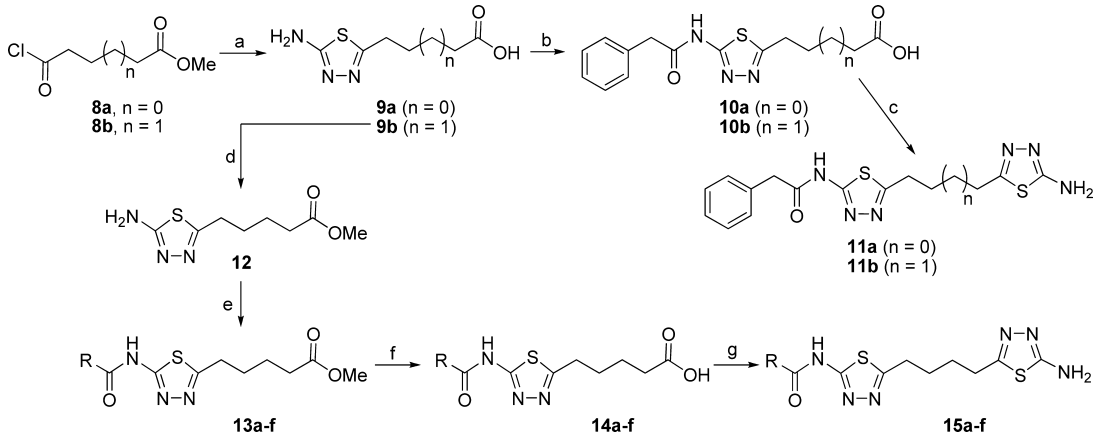
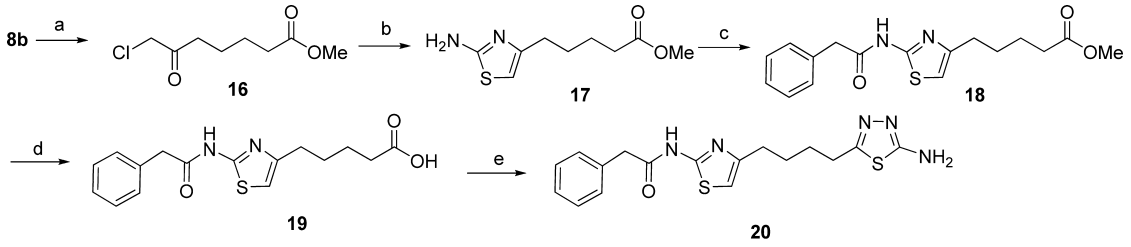
Scheme 2. Synthesis of 11a–b and 15a–f[a]



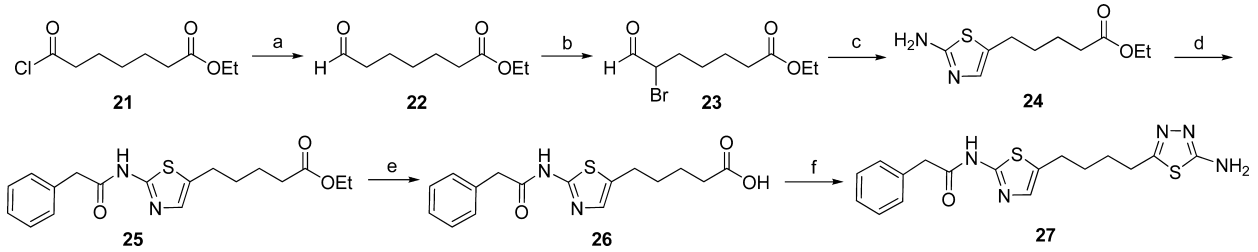
[a]Reagents and conditions: (a) thiosemicarbazide, $POCl_3$, 85 °C, 72% for **9a** and 89% for **9b**; (b) phenylacetyl chloride, TEA, THF, 15% for **10a** and 92% for **10b**; (c) thiosemicarbazide, $POCl_3$, 85 °C, 4% for **11a** and 45% for **11b**; (d) H_2SO_4, MeOH, 65 °C, 71%; (e) acid chloride, TEA, DCM, 48–95%; (f) LiOH, MeOH, H_2O, 40 °C, 63–91%; (g) thiosemicarbazide, $POCl_3$, 85 °C, 18–57%.

Scheme 3. Synthesis of 20[a]



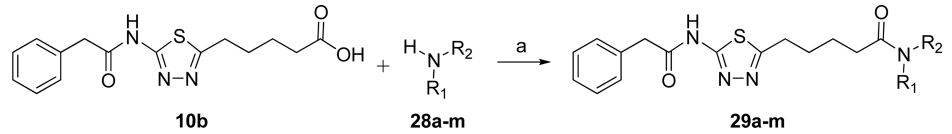
[a]Reagents and conditions: (a) CH_2N_2, Et_2O, DCM, 0 °C, 98%; (b) thiourea, EtOH, 60 °C, 81%; (c) phenylacetyl chloride, TEA, DCM, 81%; (d) 1 N NaOH, dioxane, 60 °C, 92%; (e) thiosemicarbazide, $POCl_3$, 85 °C, 29%.

Scheme 4. Synthesis of 27[a]



[a]Reagents and conditions: (a) H_2, 10% Pd/C, 2,6-lutidine, THF, 95%; (b) Br_2, $CHCl_3$, 62%; (c) thiourea, EtOH, 70 °C, 31%; (d) phenylacetyl chloride, TEA, DCM, 70%; (e) 1 M NaOH, dioxane, 60 °C, 91%; (f) thiosemicarbazide, $POCl_3$, 85 °C, 55%.

Scheme 5. Synthesis of Amides 29a–m[a]



[a]Reagents and conditions: (a) HATU, DMF, 0 °C to rt, 15–92%.

Scheme 3 illustrates the synthesis of compound **20**, where the internal thiadiazole ring of **11b** is replaced by a thiazole ring. Methyl adipoyl chloride **8b** was treated with diazomethane to afford an intermediate α-chloroketone **16**,[20] which was reacted with thiourea to form **17**.[21] The aminothiazole **17** was converted into the desired compound **20** by acetylation, hydrolysis and cyclization. Compound **27**, another thiazole-containing analog, was synthesized from acid chloride **21** as shown in Scheme 4. Rosenmund reduction of **21** gave aldehyde **22**, which was brominated to afford α-bromoaldehyde **23**.[22] The bromide **23** was cyclized with thiourea to produce the desired aminothiazole **24**, which was converted to analog **27** in a manner similar to the preparation of **20** from **17**.

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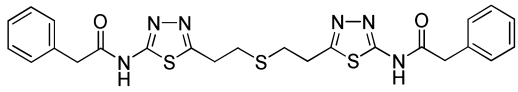
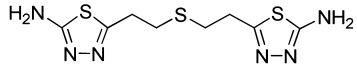
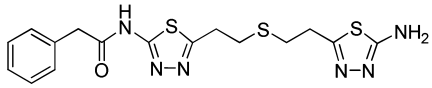
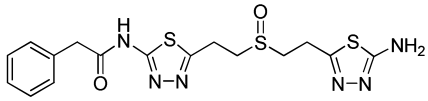
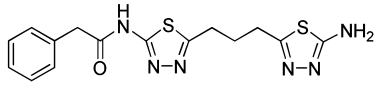
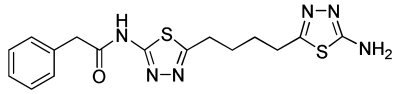
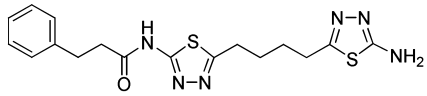
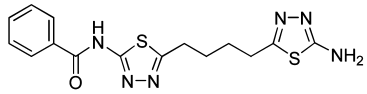
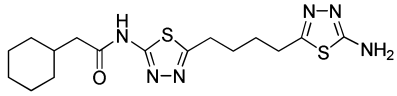
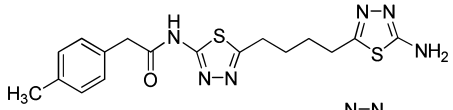
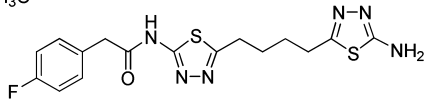
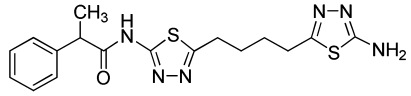
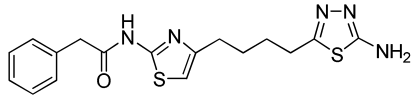
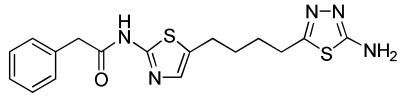
Table 1. Inhibition of GLS by Truncated Derivatives of Compound 3

Compd	Structure	IC_{50} (μM)[a]
3		3.3 ± 0.7
5		100
6		2.7 ± 1.7
7		61 ± 6
11a		15 ± 6
11b		1.9 ± 0.5
15a		>100
15b		7.2 ± 0.7
15c		22 ± 16
15d		4.5 ± 0.7
15e		2.6 ± 0.3
15f		12 ± 3
20		>100
27		>100

[a]Values are the means ± SD of three or more independent experiments.

As shown in Scheme 5, compounds **29a−m**, in which the terminal thiadiazole ring of **11b** is replaced by amide group, were prepared by coupling **10b** with a variety of amines **28a−m** using HATU as a coupling reagent.

RESULTS AND DISCUSSION

All new synthetic compounds were tested for their ability to inhibit GLS using L-[^{3}H]-glutamine as substrate and human kidney-type glutaminase ($hKGA_{124-669}$). Table 1 summarizes the GLS inhibitory potency of the truncated analogs of **3**. The removal of both phenylacetyl groups resulted in a significant loss of potency (compound **5**). A similar trend (50-fold increase in IC_{50} value from **3** to **5**) has been previously reported.[16] Removal of one phenylacetyl group from **3** (compound **6**), however, did not result in the significant change in the GLS inhibitory potency. Potent inhibition of GLS by an asymmetric compound such as **6** is rather surprising because the crystal structures of GLS in complex with symmetric compound **3** revealed that each identical half of compound **3** interacts with each GLS monomer in a highly symmetrical fashion.[15,16] While the possibility of a distinct binding site and/or mode for compound **6** cannot be

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completely ruled out, prior crystallographic studies have demonstrated that both compounds **3** (Figure 2A) and **5**

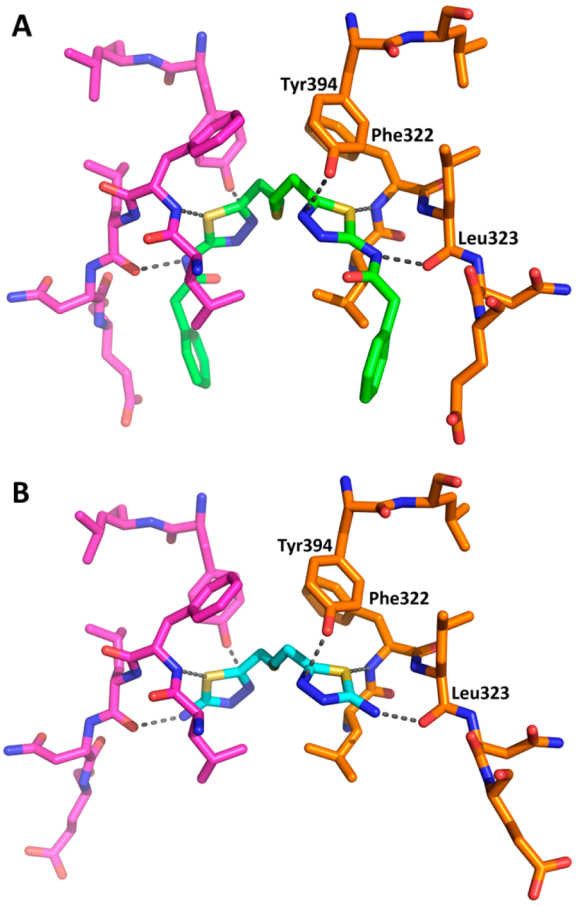

Figure 2. Allosteric sites of GLS in complex with (A) compound **3** (3VOZ) and (B) compound **5** (3VP2). Compounds **3** and **5** are shown in green and cyan, respectively. Two loops at the allosteric site (one from each GLS unit) are shown in orange and magenta, respectively. Key hydrogen-bonding interactions are shown as gray dashed lines.

(Figure 2B) bind to the same allosteric site of GLS.[16] Given the structure of **6** being an intermediate between compounds **3** and **5**, it is conceivable that compound **6** binds to the same allosteric site and preserves some of the key interactions with GLS that were identified in the crystallographic studies of compounds **3** and **5**. Furthermore, we conducted inhibitory kinetic studies of compound **6** at various concentrations of glutamine to determine its precise mode of GLS inhibition. As shown in Figure 3, a double reciprocal plot of glutaminase activity versus the glutamine concentrations produced parallel lines for different concentrations of compound **6**, indicative of uncompetitive inhibition with a K_i value of 2.0 μM. This is consistent with the mode of inhibition reported for compound **3**[13] and provides further supporting evidence that compounds **3** and **6** share the same allosteric binding site of GLS. It remains to be explored how asymmetric compounds such as **6** can bind to the highly symmetric GLS dimer interface region and lock the enzyme into an inactive conformation. A relevant precedent can be found in HIV-1 protease which forms a symmetrical dimer and yet accommodates both symmetric and asymmetric inhibitors. Nevertheless, this SAR trend presented a promising

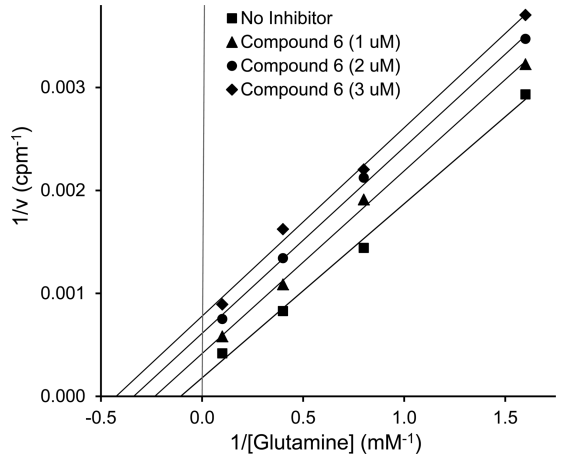

Figure 3. Double-reciprocal plot of the hydrolysis of glutamine by GLS in the presence of compound **6**. The straight lines represent the least-squares fit of the data obtained by plotting the reciprocal of glutaminase activity versus the reciprocal of the glutamine concentrations.

opportunity to identify GLS inhibitors of lower molecular weight and hydrophobicity.

Subsequently, we conducted further structural optimization of compound **6**. In an attempt to improve aqueous solubility, sulfoxide analog **7** was prepared, though it turned out to be a weak inhibitor of GLS with an IC_{50} value of 61 μM. In the next set of SAR studies, we tested analogs of **6**, in which the middle linker was shortened by removing sulfide and one methylene unit (compound **11a**) or sulfide only (compound **11b**). While significant loss of GLS inhibitory potency was observed with **11a**, compound **11b** exhibited potency similar to **6**, indicating that the middle linker can be shortened to some degree without compromising the affinity to GLS. This is consistent with the crystal structure of KGA complexed with 5,5′-butane-1,4-diylbis(1,3,4-thiadiazol-2-amine), another 4-carbon linker containing GLS inhibitor (3VP4).[16] The linker was able to adopt stretched conformation so that the two thiadiazole rings can be positioned in a manner similar to those of compound **3**.

Compounds **15a–f** represent a variety of analogs in which the phenylacetyl group of **11b** was replaced by various acyl groups. With the exception of compound **15a**, modifications at this site were generally well tolerated with IC_{50} values ranging from 2.6 to 12 μM. Only modest loss of potency in compound **15c** bearing a cyclohexylacetyl group indicates that the terminal aromatic ring is not an essential component for GLS inhibition. In fact, crystal structures indicate that the phenyl groups of compound **3** do not form pi-stacking interactions with any aromatic residues of GLS (Figure 2A).[16] The flat SAR trend within the various amide analogs may be attributed to the lack of specific interaction between the terminal acyl group and the allosteric site.

In compounds **20** and **27**, the internal thiadiazole group of **11b** was replaced by a 1,3-thiazole ring. Neither compounds inhibited GLS up to 100 μM. The complete loss of potency seen in compound **27** particularly highlights the crucial role played by N_4 of the thiadiazole ring most likely through its interaction with Phe322[15] and/or Tyr394[16] residue(s). Indeed, mutant forms of GLS (F322S, Y394I, or Y394L) exhibited similar enzymatic activity compared to the wild-type form but showed significantly reduced sensitivity to **3**,[15,16] illuminating

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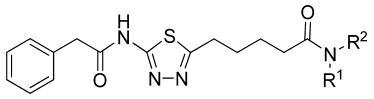
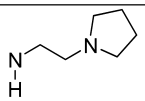
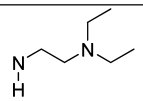
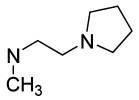
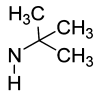
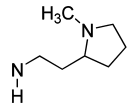
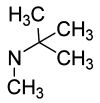
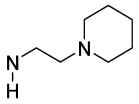
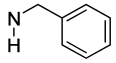
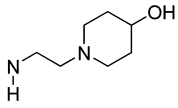
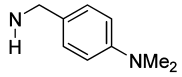
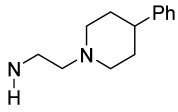
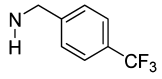
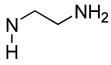
Table 2. Structure–Activity Relationships of Amides 29a–m



Compd	NR^1R^2	IC_{50} (μM)[a]	Compd	NR^1R^2	IC_{50} (μM)[a]
29a		4.6 ± 1.2	**29h**		26 ± 6
29b		>100	**29i**		11 ± 1
29c		8.1 ± 2.4	**29j**		5.2 ± 0.9
29d		10 ± 1	**29k**		>100
29e		3.8 ± 0.6	**29l**		48 ± 13
29f		1.4 ± 0.3	**29m**		>100
29g		>100			

[a]Values are the means ± SD of three or more independent experiments.

an important role played by these residues in the allosteric binding site of GLS.

Subsequent to the identification of **11b** as a potent GLS inhibitor, we explored the structural modification of its terminal thiadiazole ring. In analogs **29a–m**, the terminal thiadiazole of **11b** was replaced by a variety of amide groups. As shown in Table 2, some of these compounds exhibited potent GLS inhibitory activity. Most notable inhibitors were amides **29a** and **29c–f** which contain a pyrrolidine or piperidine moiety. These compounds inhibited GLS with IC_{50} values of 10 μM or below. It appears the cyclic amino group is preferred for GLS inhibitory activity as analogs **29g** and **29h** lacking a ring system showed significantly lower potency. Among other amides synthesized, only tert-butyl amide derivative **29i** exhibited reasonably potent GLS inhibition. Tertiary amide **29b** was completely devoid of GLS inhibitory activity while another tertiary amide **29j** was equally potent as its close analog **29i**. Overall, these findings demonstrate that the terminal thiadiazole can be replaced without significant loss of potency. While the mode that these amide groups interact with the allosteric binding site of GLS remains unclear, it is conceivable that the extra nitrogen atom of **29a** and **29c–f** participates in hydrogen bonding with some of the key residues (Phe322, Tyr394, Leu323) identified in complexes of GLS with compound **3** (Figure 2A). Since this series of compounds can be synthesized from the common intermediate **10b** in one step, SAR studies can be extended to a variety of amines to provide further insights into the molecular features required for the high affinity to GLS.

To examine the effect of the structural modifications on the physicochemical properties, we assessed the aqueous solubility of GLS inhibitors **6**, **11b**, and **29a** relative to that of compound **3**. Using the LC/MS based shake-flask method,[23] aqueous solubility of **3**, **6**, **11b** and **29a** was determined to be 0.144, 13, 3.4, and 683 μg/mL, respectively. The improved solubility over compound **3** makes the new GLS inhibitors not only more valuable pharmacological tools but also more tractable leads for further structural optimization.

Subsequent pharmacological characterization was conducted with one of the truncated analogs, compound **6**. At concentrations up to 100 μM, compound **6** showed no inhibition of mouse liver-type glutaminase (mouse GLS2), whose sequence is highly homologous to that of a human GLS2.[24] Although speculative, it is conceivable that the highly specific inhibition of GLS by compound **6** can be attributed to its ability to retain the critical interaction with Phe322, one of the residues unique to GLS allosteric loop. In vitro metabolic stability studies using mouse liver microsomes showed the loss of compound **6** over time (42 and 16% remaining after 30 and 60 min incubation, respectively) in the presence of NADPH. LC/MS analysis identified compound **7** as one of the major metabolites which formed through the oxidation of the sulfide group in the linker region of compound **6**. Consistent with the metabolic site of **6**, nonsulfide GLS inhibitors **11b** and **29a** were found to be completely stable against microsomes in the presence of NADPH. Since compound **6** possesses a primary amino group at its terminal thiadiazole ring, glucuronidation might also play a role in the liver metabolism of this compound.

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Fortification of the microsome incubation with UGPGA, however, resulted in negligible loss of the parent compound.

Antitumor effects of compound **6** were assessed using the human P493 B cells, in which Myc is overexpressed in the absence of tetracycline.[25] As shown in Figure 4A, compound **6**

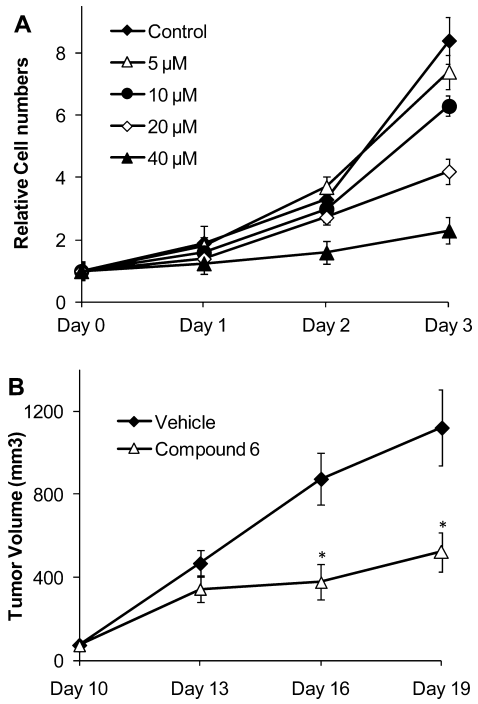

Figure 4. Antiproliferative effects of GLS inhibitor **6**. (A) Effect of GLS inhibitor **6** on aerobic P493 human B cell growth. Error bars represent ± SD (n = 3). (B) Antitumor effect of GLS inhibitor **6** (200 μg/mice, ~12.5 mg/kg, every other day, i.p.) on P493 human B cell xenograft transplanted into SCID mice. Error bars represent ± SEM (n = 4, *p < 0.05 compared to the vehicle-treated control).

attenuated the growth of P493 cells in vitro in a dose-dependent manner with an IC_{50} value near 20 μM. Previous studies have shown that similar antiproliferative effects can be achieved by GLS siRNA,[4] glutamine deprivation,[4] or treatment with compound **3**.[18] In the same assay, compound **29a** inhibited the growth with IC_{50} value near 40 μM. An attempt to assess antiproliferative activity of compound **11b** was unsuccessful due to its poor aqueous solubility.

Compound **6** was also tested in an animal model in which compound **3** was previously reported to exhibit antiproliferative efficacy.[18] Given the instability in liver microsomes, intraperitoneal injection was chosen as a route of administration to avoid the first pass metabolism. As shown in Figure 4B, P493 tumor xenograft-bearing SCID mice treated with compound **6** showed significantly diminished tumor growth compared to the vehicle-treated mice. The degree of efficacy achieved by compound **6** is comparable with that of compound **3** reported previously.[18] As mentioned before, compound **6** can be metabolized in liver into the inactive metabolite **7**. Similar metabolism is expected for compound **3** since it also contains a metabolically liable sulfide group. It would be interesting to test equally potent nonsulfide GLS inhibitors such as **11b** and **29a** to determine the effects of improved metabolic stability on the degree of efficacy.

CONCLUSIONS

While kidney-type glutaminase (GLS) has recently gained increasing attention as a therapeutic target for the treatment of cancer and neuroinflammatory diseases, little progress has been made, to date, in efforts to identify new GLS inhibitors mechanistically distinct from the conventional inhibitor **1**. Allosteric GLS inhibitor **3** has presented an unprecedented opportunity to address the needs to identify a new series of inhibitors selective to GLS over GLS2. Recently reported crystal structures of GLS, particularly those in complex with compound **3**, has provided new insights into the allosteric binding mode of compound **3** and its implication for the design of new inhibitors. Incremental truncation of compound **3** led us to identify analogs equally potent as compound **3** with improved solubility. While it is promising that the new GLS inhibitor **6** retains antiproliferative activity of compound **3** both in vitro and in vivo, further improvement, particularly in potency, is necessary to translate GLS inhibitors into practical therapeutic interventions. Although there was no apparent toxicity following the repeated intraperitoneal injection of compound **6** in P493 tumor xenograft-bearing SCID mice, more extensive toxicity studies are required to better define the therapeutic window of GLS inhibitors. Such investigation, however, should await GLS inhibitors with better potency and ADME profile. SAR and metabolic stability data provided herein coupled with the new structural insights gained from the recently published GLS crystal structures should allow more refined structural optimization to maximize the interaction with the allosteric binding site, leading to the discovery of more desirable GLS inhibitors.

EXPERIMENTAL SECTION

General. All solvents were reagent grade or HPLC grade. Unless otherwise noted, all materials were obtained from commercial suppliers (Aldrich, Sigma, TCI, or Matrix) and used without further purification. All reactions were performed under nitrogen. Preparative HPLC purification was performed on an Agilent 1200 Series HPLC system equipped with a multiwavelength detector using a Phenomenex Luna 5 μm C18 (2) column (250 × 4.6 mm) with a flow rate of 15 mL/min. The solvent system consisted of distilled water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B); 40% B was used from 0 to 5 min followed by a linear gradient of 40% to 85% B over 35 min. Melting points were obtained on a Mel-Temp apparatus and are uncorrected. ^{1}H NMR spectra were recorded at 400 MHz. ^{13}C NMR spectra were recorded at 100 MHz. Elemental analyses were obtained from Atlantic Microlabs, Norcross, GA. The purity of test compounds was determined by elemental analysis (within ±0.4% of the calculated value).

*N,N′-(5,5′-(2,2′-Thiobis(ethane-2,1-diyl))bis(1,3,4-thiadiazole-5,2-diyl))bis(2-phenylacetamide) (**3**).* The preparation of **3** from bisthiadiazole **5** has already been reported.[11,16] We used a similar procedure to synthesize **3**. To a solution of compound **5** (3.61 g, 12.5 mmol) in DMA (100 mL) was added NEt_3 (3.8 mL, 27.5 mmol) and phenylacetyl chloride (3.2 mL, 25 mmol). The reaction was stirred overnight at room temperature. DMA was removed in vacuo and the resulting solid was triturated with 10% $NaHCO_3$ and boiling MeOH to obtain **3** as a white solid (5.8 g, 88% yield): mp 231−234 °C; ^{1}H NMR (DMSO-d_6) δ 2.92 (t, J = 7.2 Hz, 4H), 3.24 (t, J = 7.1 Hz, 4H), 3.79 (s, 4H), 7.23−7.34 (m, 10H), 12.69 (s, 2H); ^{13}C NMR (DMSO-d_6) δ 29.4, 30.1, 41.6, 126.9, 128.5, 129.3, 134.7, 158.6, 162.3, 169.4. Anal. Calcd for $C_{24}H_{24}N_6O_2S_3$·0.5H_2O: C, 54.01; H, 4.72; N, 15.75; S, 18.02. Found: C, 54.00; H, 4.54; N, 16.0; S, 18.41.

*Bis-[2-(5-amino-[1,3,4]thiadiazol-2-yl)-ethylsulfide] (**5**).* The preparation of compound **5** from thiodipropionic acid **4** has already been reported.[16] We used a similar procedure to synthesize **5**. Thiodipropionic acid **4** (10 g, 56 mmol) and thiosemicarbazide (10

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g, 56 mmol) were dissolved in $POCl_3$ (30 mL) and heated to 90 °C for 3 h. The reaction was cooled and the viscous oil was poured onto ∼100 g ice. The filtrate was basified using solid NaOH until pH 10. The solid that precipitated out of solution was filtered off and washed several times with H_2O. The solid was then resuspended in boiling MeOH and filtered hot. This final filtration led to desired bis-aminothiadiazole **5** as a white solid (12.7 g, 79% yield): mp 222 °C (dec); ^{1}H NMR (DMSO-d_6) δ 2.84 (t, J = 7.2 Hz, 4H), 3.06 (t, J = 7.2 Hz, 4H), 7.04 (s, 4H); ^{13}C NMR (DMSO-d_6) δ 30.0, 30.3, 156.4, 168.5. Anal. Calcd for $C_8H_{12}N_6S_3\cdot0.1H_2O\cdot0.02$MeOH: C, 33.12; H, 4.26; N, 28.89; S, 33.07. Found: C, 33.49; H, 4.11; N, 28.51; S, 32.84.

*N-(5-{2-[2-(5-Amino-[1,3,4]thiadiazol-2-yl)-ethylsulfanyl]-ethyl}-[1,3,4]thiadiazol-2-yl)-2-phenyl-acetamide (**6**).* Compound **5** (5.0 g, 17.3 mmol) was dissolved in hot DMA (∼20−30 mL) until in solution. The solution was cooled down and triethylamine (7.25 mL, 52 mmol) was added followed by dropwise addition of phenylacetyl chloride (2.6 g, 17.3 mmol). After stirring for 3 h, the reaction was concentrated in vacuo to remove DMA and the residue was triturated with water (∼100 mL). The resulting solid was triturated with boiling THF (∼300 mL). The insoluble solid was filtered off (starting material) and the filtrate was concentrated, absorbed onto silica gel and chromatographed (50% EtOAc/DCM to DCM to 20% MeOH/DCM) to afford compound **6** as a white solid (R_f = 0.4, 10% MeOH/EtOAc, 750 mg, 11% yield): mp 198 °C (dec); ^{1}H NMR (DMSO-d_6) δ 2.85 (t, J = 7.2 Hz, 2H), 2.92 (t, J = 7.1 Hz, 2H), 3.06 (t, J = 7.2 Hz, 2H), 3.25 (t, J = 7.2 Hz, 2H), 3.80 (s, 2H), 7.05 (s, 2H), 7.18−7.40 (m, 5H), 12.69 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 29.4, 30.0, 30.1, 30.2, 41.6, 126.9, 128.5, 129.3, 134.7, 156.4, 158.6, 162.3, 168.5, 169.4. Anal. Calcd for $C_{16}H_{18}N_6O_1S_3\cdot0.25$MeOH: C, 47.08; H, 4.62; N, 20.27; S, 23.20. Found: C, 47.41; H, 4.38; N, 19.97; S, 22.83.

*N-(5-(2-(2-(5-Amino-1,3,4-thiadiazol-2-yl)ethylsulfinyl)ethyl)-1,3,4-thiadiazol-2-yl)-2-phenylacetamide (**7**).* To a solution of sulfide **6** (40 mg, 0.1 mmol) in MeOH (4 mL) was added 35% H_2O_2 (5 drops). After 18 h, MeOH was removed. The resulting residue was purified by preparative HPLC to obtain sulfoxide **7** as a white solid (25 mg, 60% yield): mp 181 °C (dec); ^{1}H NMR (DMSO-d_6) δ 3.06−3.15 (m, 2H), 3.17−3.29 (m, 4H), 3.36−3.40 (m, 2H), 3.80 (s, 2H), 7.10 (s, 2H), 7.26−7.35 (m, 5H), 12.74 (br s, 1H). Anal. Calcd for $C_{16}H_{18}N_6O_2S_3\cdot0.8H_2O\cdot0.35$NaCl: C, 42.01; H, 4.32; N, 18.37; S, 21.03. Found: C, 42.39; H, 4.56; N, 18.21; S, 20.65.

*4-(5-Amino-1,3,4-thiadiazol-2-yl)butanoic acid (**9a**).* Methyl 5-chloro-5-oxopentanoate **8a** (10 mL, 72 mmol) and thiosemicarbazide (7.0 g, 72 mmol) were dissolved in $POCl_3$ (25 mL) and heated to 85 °C for 5 h. The reaction was cooled and the viscous oil was poured onto ice. The filtrate was basified using NaOH pellets to pH ∼5. The precipitate was filtered off to obtain **9a** as an off-white solid (9.7 g, 72% yield): ^{1}H NMR (DMSO-d_6) δ 1.82 (quint, J = 7.6 Hz, 2H), 2.28 (t, J = 7.3 Hz, 2H), 2.80 (t, J = 7.6 Hz, 2H), 7.03 (br s, 2H).

*4-(5-(2-Phenylacetamido)-1,3,4-thiadiazol-2-yl)butanoic Acid (**10a**).* To compound **9a** (4.0 g, 22 mmol) in DCM (100 mL), was added TEA (6.6 mL, 47 mmol). Next, phenylacetyl chloride (3.1 mL, 24 mmol) was added dropwise. After stirring for 4 d, the product was extracted with DCM (3 × 100 mL) and brine (100 mL). The organic layer was dried over $MgSO_4$ and concentrated in vacuo to obtain an orange oil. The crude material was purified by column chromatography (5% MeOH/DCM) to obtain an off-white solid. The solid was triturated with EtOAc to afford **10a** as a white solid (1.0 g, 15% yield): ^{1}H NMR (DMSO-d_6) δ 1.89 (quint, J = 7.6 Hz, 2H), 2.29 (t, J = 7.3 Hz, 2H), 2.98 (t, J = 7.6 Hz, 2H), 3.79 (s, 2H), 7.24−7.35 (m, 5H), 12.13 (br s, 1H), 12.68 (br s, 1H).

*N-(5-(3-(5-Amino-1,3,4-thiadiazol-2-yl)propyl)-1,3,4-thiadiazol-2-yl)-2-phenylacetamide (**11a**).* Compound **10a** (600 mg, 2.0 mmol) and thiosemicarbazide (180 mg, 2.0 mmol) were dissolved in $POCl_3$ (6 mL) and heated to 85 °C for 4 h. The reaction was cooled and the viscous oil was poured onto ice. The solution was basified using NaOH pellets to pH ∼5. The precipitate was filtered off and triturated with H_2O, followed by EtOAc to obtain **11a** as a tan solid (25 mg, 4% yield): mp 185 °C (dec); ^{1}H NMR (DMSO-d_6) δ 2.05 (quint, J = 7.3 Hz, 2H), 2.87 (t, J = 7.5 Hz, 2H), 3.03 (t, J = 7.5 Hz, 2H), 3.81 (s, 2H), 7.04 (s, 2H), 7.26−7.36 (m, 5H); ^{13}C NMR (DMSO-d_6) δ 28.1, 28.6, 28.6, 41.6, 126.9, 128.4, 129.3, 134.7, 157.4, 158.3, 163.4, 168.3, 169.4. Anal. Calcd. for $C_{15}H_{16}N_6O_1S_2\cdot0.02H_2O\cdot0.1$EtOAc$\cdot0.06$NaCl: C, 49.57; H, 4.55; N, 22.52; S, 17.19. Found: C, 49.81; H, 4.46; N, 22.92; S, 16.79.

*5-(5-Amino-1,3,4-thiadiazol-2-yl)pentanoic Acid (**9b**).* Methyl adipoyl chloride **8b** (25 g, 0.14 mol) and thiosemicarbazide (12.8 g, 0.14 mol) were dissolved in $POCl_3$ (50 mL) and heated to 85 °C for 5 h. The reaction was cooled and the viscous oil was poured onto ∼100 g of ice. The filtrate was basified using 50% NaOH to pH ∼5. The precipitate was filtered off to obtain **9b** as a white solid (25 g, 89% yield): ^{1}H NMR (DMSO-d_6) δ 1.49−1.65 (m, 4H), 2.23 (t, J = 7.2 Hz, 2H), 2.78 (t, J = 7.2 Hz, 2H), 7.00 (br s, 2H), 12.02 (br s, 1H).

*5-(5-(2-Phenylacetamido)-1,3,4-thiadiazol-2-yl)pentanoic Acid (**10b**).* To compound **9b** (30 g, 0.15 mol) in THF (300 mL), was added TEA (46 mL, 0.33 mol). Next, phenylacetyl chloride (22 mL, 0.16 mmol) was added via addition funnel. After stirring for 2 d, the reaction was concentrated in vacuo to remove THF. The residue was triturated with hot water (∼1000 mL) for 30 min and the solid was filtered to obtain **10b** as a off-white solid (44 g, 92% yield): ^{1}H NMR (DMSO-d_6) δ 1.53 (quint, J = 7.6 Hz, 2H), 1.68 (quint, J = 7.6 Hz, 2H), 2.23 (t, J = 7.6 Hz, 2H), 2.96 (t, J = 7.6 Hz, 2H), 3.79 (s, 2H), 7.24−7.35 (m, 5H), 12.04 (br s, 1H), 12.67 (br s, 1H).

*N-(5-(4-(5-Amino-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)-2-phenylacetamide (**11b**).* Compound **10b** (1.2 g, 3.8 mmol) and thiosemicarbazide (0.34 g, 3.8 mmol) were dissolved in $POCl_3$ (5 mL) and heated to 85 °C for 4 h. The reaction was cooled and the viscous oil was poured onto ice. The solution was basified using NaOH pellets to pH ∼5. The precipitate was filtered off to obtain off-white solid. The solid was triturated with boiling water (∼100 mL), followed by hot EtOAc to afford **11b** as a off-white solid (0.63 g, 45% yield): mp 210 °C (dec); ^{1}H NMR (DMSO-d_6) δ 1.64−1.75 (m, 4H), 2.82 (t, J = 7.2 Hz, 2H), 2.99 (t, J = 7.2 Hz, 2H), 3.79 (s, 2H), 7.07 (br s, 2H), 7.23−7.35 (m, 5H), 12.67 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 28.1, 28.2, 28.4, 28.9, 41.5, 126.9, 128.4, 129.2, 134.6, 158.0, 158.2, 163.8, 168.2, 169.3. Anal. Calcd for $C_{16}H_{18}N_6O_1S_2\cdot0.4H_2O$: C, 50.35; H, 4.96; N, 22.02; S, 16.80. Found: C, 50.67; H, 4.73; N, 21.71; S, 16.40.

*Methyl 5-(5-Amino-1,3,4-thiadiazol-2-yl)pentanoate (**12**).* To a solution of carboxylic acid **9b** (4.0 g, 20 mmol) in MeOH (100 mL), was added concentrated H_2SO_4 (4 mL). The reaction mixture was stirred at 65 °C for 2 d. MeOH was removed and the product was extracted with saturated $NaHCO_3$ (100 mL) and DCM (4 × 50 mL). The organic layer was dried over $MgSO_4$ and concentrated in vacuo to obtain a white residue. The residue was triturated with Et_2O to afford **12** as a white solid (3.0 g, 71% yield): ^{1}H NMR (DMSO-d_6) δ 1.56−1.62 (m, 4H), 2.34 (t, J = 7.2 Hz, 2H), 2.79 (t, J = 7.2 Hz, 2H), 3.58 (s, 3H), 7.00 (s, 2H).

*Methyl 5-(5-(3-Phenylpropanamido)-1,3,4-thiadiazol-2-yl)-pentanoate (**13a**).* To compound **12** (1.0 g, 4.6 mmol) in DCM (20 mL) was added NEt_3 (1.4 mL, 10 mmol). This was followed by addition of 3-phenylpropanoyl chloride (0.76 mL, 5.1 mmol) dropwise. The reaction mixture was stirred at room temperature for 24 h. The product was extracted with DCM (3 × 50 mL) and brine (30 mL). The organic layer was dried over $MgSO_4$, concentrated in vacuo to obtain off-white solid. The solid was triturated with Et_2O to obtain **13a** as a white solid (1.5 g, 95% yield): ^{1}H NMR (DMSO-d_6) δ 1.54−1.61 (m, 2H), 1.66−1.72 (m, 2H), 2.35 (t, J = 7.3 Hz, 2H), 2.77 (t, J = 7.8 Hz, 2H), 2.92−2.99 (m, 4H), 3.58 (s, 3H), 7.17−7.30 (m, 5H), 12.42 (s, 1H).

*Methyl 5-(5-Benzamido-1,3,4-thiadiazol-2-yl)pentanoate (**13b**).* Compound **13b** was prepared from compound **12** as described for the preparation of **13a** except benzoyl chloride was used in place of 3-phenylpropanoyl chloride. The crude material was purified by trituration with EtOAc: white solid (48% yield); ^{1}H NMR (DMSO-d_6) δ 1.57−1.64 (m, 2H), 1.70−1.78 (m, 2H), 2.37 (t, J = 7.3 Hz, 2H), 3.02 (t, J = 7.3 Hz, 2H), 3.59 (s, 3H), 7.55 (t, J = 8.0 Hz, 2H), 7.66 (t, J = 7.2 Hz, 1H), 8.09 (d, J = 7.6 Hz, 2H), 12.87 (br s, 1H).

*Methyl 5-(5-(2-Cyclohexylacetamido)-1,3,4-thiadiazol-2-yl)-pentanoate (**13c**).* Compound **13c** was prepared from compound **12** as described for the preparation of **13a** except 2-cyclohexylacetyl chloride was used in place of 3-phenylpropanoyl chloride: white solid

(59% yield); ^{1}H NMR ($CDCl_3$) δ 1.01−1.29 (m, 5H), 1.61−1.94 (m, 10H), 2.35 (t, J = 7.2 Hz, 2H), 2.54 (d, J = 7.3 Hz, 2H), 3.02 (t, J = 7.5 Hz, 2H), 3.65 (s, 3H), 12.45 (br s, 1H).

*Methyl 5-(5-(2-p-Tolylacetamido)-1,3,4-thiadiazol-2-yl)-pentanoate (**13d**).* Compound **13d** was prepared from compound **12** as described for the preparation of **13a** except 2-*p*-tolylacetyl chloride was used in place of 3-phenylpropanoyl chloride: white solid (86% yield); ^{1}H NMR (DMSO-d_6) δ 1.54−1.60 (m, 2H), 1.65−1.71 (m, 2H), 2.27 (s, 3H), 2.34 (t, J = 7.2 Hz, 2H), 2.96 (t, J = 7.3 Hz, 2H), 3.57 (s, 3H), 3.74 (s, 2H), 7.13 (d, J = 8.1 Hz, 2H), 7.20 (d, J = 7.8 Hz, 2H), 12.64 (s, 1H).

*Methyl 5-(5-(2-(4-Fluorophenyl)acetamido)-1,3,4-thiadiazol-2-yl)pentanoate (**13e**).* Compound **13e** was prepared from compound **12** as described for the preparation of **13a** except 2-(4-fluorophenyl)-acetyl chloride was used in place of 3-phenylpropanoyl chloride: white solid (84% yield); ^{1}H NMR ($CDCl_3$) δ 1.70−1.76 (m, 2H), 1.79−1.84 (m, 2H), 2.34 (t, J = 7.2 Hz, 2H), 3.04 (t, J = 7.3 Hz, 2H), 3.63 (s, 3H), 3.99 (s, 2H), 7.00 (t, J = 8.7 Hz, 2H), 7.39−7.42 (dd, J = 5.4, 8.7 Hz, 2H), 13.02 (br s, 1H).

*Methyl 5-(5-(2-Phenylpropanamido)-1,3,4-thiadiazol-2-yl)-pentanoate (**13f**).* Compound **13f** was prepared from compound **12** as described for the preparation of **13a** except 2-phenylpropanoyl chloride was used in place of 3-phenylpropanoyl chloride: white solid (74% yield); ^{1}H NMR ($CDCl_3$) δ 1.61 (d, J = 6.8 Hz, 3H), 1.72−1.77 (m, 2H), 1.80−1.86 (m, 2H), 2.35 (t, J = 7.2 Hz, 2H), 3.05 (t, J = 7.3 Hz, 2H), 3.64 (s, 3H), 4.11 (q, J = 7.1 Hz, 2H), 7.26−7.32 (m, 3H), 7.42 (dd, J = 1.3, 8.1 Hz, 2H), 11.47 (br s, 1H).

*5-(5-(3-Phenylpropanamido)-1,3,4-thiadiazol-2-yl)pentanoic Acid (**14a**).* To **13a** (1.5 g, 4.4 mmol) in MeOH (70 mL) and H_2O (30 mL) was added LiOH (11 mmol). The reaction mixture was stirred at 40 °C for 24 h. MeOH was removed and solution was diluted with ∼20 mL H_2O. The reaction was acidified to pH ∼2. The precipitate was collected by vacuum filtration to obtain **14a** as a white solid (1.0 g, 69% yield): ^{1}H NMR (DMSO-d_6) δ 1.51−1.58 (t, J = 7.5 Hz, 2H), 1.66−1.74 (t, J = 7.6 Hz, 2H), 2.25 (t, J = 7.4 Hz, 2H), 2.77 (t, J = 7.6 Hz, 2H), 2.90−2.98 (m, 4H), 7.16−7.30 (m, 5H), 12.05 (br s, 1H), 12.41 (br s, 1H).

*5-(5-Benzamido-1,3,4-thiadiazol-2-yl)pentanoic Acid (**14b**).* Compound **14b** was prepared as described for the preparation of **14a** except methyl 5-(5-benzamido-1,3,4-thiadiazol-2-yl)pentanoate **13b** was used in place of **13a**: off-white solid (83% yield); ^{1}H NMR (DMSO-d_6) δ 1.58 (m, 2H), 1.74 (m, 2H), 2.27 (t, J = 7.3 Hz, 2H), 3.02 (t, J = 7.5 Hz, 2H), 7.56 (t, J = 7.8 Hz, 2H), 7.66 (t, J = 7.0 Hz, 1H), 8.09 (d, J = 7.1 Hz, 2H), 12.18 (br s, 1H), 12.93 (br s, 1H).

*5-(5-(2-Cyclohexylacetamido)-1,3,4-thiadiazol-2-yl)pentanoic Acid (**14c**).* Compound **14c** was prepared as described for the preparation of **14a** except methyl 5-(5-(2-cyclohexylacetamido)-1,3,4-thiadiazol-2-yl)pentanoate **13c** was used in place of **13a**: white solid (79% yield); ^{1}H NMR (DMSO-d_6) δ 0.90−0.99 (m, 2H), 1.13−1.25 (m, 3H), 1.51−1.78 (m, 10H), 2.25 (t, J = 7.3 Hz, 2H), 2.33 (d, J = 7.1 Hz, 2H), 2.96 (t, J = 7.3 Hz, 2H), 12.04 (br s, 1H), 12.36 (br s, 1H).

*5-(5-(2-p-Tolylacetamido)-1,3,4-thiadiazol-2-yl)pentanoic Acid (**14d**).* Compound **14d** was prepared as described for the preparation of **14a** except methyl 5-(5-(2-*p*-tolylacetamido)-1,3,4-thiadiazol-2-yl)pentanoate **13d** was used in place of **13a**: white solid (71% yield); ^{1}H NMR (DMSO-d_6) δ 1.49−1.57 (m, 2H), 1.64−1.72 (m, 2H), 2.24 (t, J = 7.3 Hz, 2H), 2.27 (s, 3H), 2.96 (t, J = 7.5 Hz, 2H), 3.73 (s, 2H), 7.13 (d, J = 8.3 Hz, 2H), 7.20 (d, J = 7.8 Hz, 2H), 12.04 (br s, 1H), 12.62 (br s, 1H).

*5-(5-(2-(4-Fluorophenyl)acetamido)-1,3,4-thiadiazol-2-yl)-pentanoic Acid (**14e**).* Compound **14e** was prepared as described for the preparation of **14a** except methyl 5-(5-(2-(4-fluorophenyl)-acetamido)-1,3,4-thiadiazol-2-yl)pentanoate **13e** was used in place of **13a**: white solid (63% yield); ^{1}H NMR (DMSO-d_6) δ 1.54 (m, 2H), 1.69 (m, 2H), 2.24 (t, J = 7.3 Hz, 2H), 2.97 (t, J = 7.5 Hz, 2H), 3.80 (s, 2H), 7.16 (t, J = 8.9 Hz, 2H), 7.36 (dd, J = 3.1, 8.6 Hz, 2H), 12.05 (br s, 1H), 12.66 (br s, 1H).

*5-(5-(2-Phenylpropanamido)-1,3,4-thiadiazol-2-yl)pentanoic Acid (**14f**).* Compound **14f** was prepared as described for the preparation of **14a** except methyl 5-(5-(2-phenylpropanamido)-1,3,4-thiadiazol-2-yl)pentanoate **13f** was used in place of **13a**: white solid (91% yield); ^{1}H NMR DMSO-d_6) δ 1.44 (d, J = 7.1 Hz, 3H), 1.51−1.55 (m, 2H), 1.66−1.70 (m, 2H), 2.24 (t, J = 7.2 Hz, 2H), 2.95 (t, J = 7.2 Hz, 2H), 4.00 (q, J = 7.1 Hz, 1H), 7.25−7.35 (m, 5H), 12.05 (br s, 1H), 12.61 (br s, 1H).

*N-(5-(4-(5-Amino-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)-3-phenylpropanamide (**15a**).* Compound **15a** was prepared as described for the preparation of **11b** except 5-(5-(3-phenylpropanamido)-1,3,4-thiadiazol-2-yl)pentanoic acid **14a** was used in place of **10b**. The crude material was purified by trituration with mildly basic water (pH ∼10, 10 mL): off-white solid (33% yield); mp 212 °C (dec); ^{1}H NMR (DMSO-d_6) δ 1.67−1.66 (m, 4H), 2.75−2.84 (m, 4H), 2.91 (t, J = 7.6 Hz, 2H), 2.99 (t, J = 7.2 Hz, 2H), 7.03 (s, 2H), 7.16−7.30 (m, 5H), 12.45 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 28.2, 28.3, 28.4, 29.0, 30.3, 36.5, 126.1, 128.3, 128.4, 140.6, 158.0, 158.1, 163.4, 168.2, 170.6. Anal. Calcd for $C_{17}H_{20}N_6O_2S{\cdot}0.7H_2O{\cdot}0.03$NaCl: C, 50.68; H, 5.35; N, 20.68; S, 15.92; Found: C, 51.08; H, 4.95; N, 20.44; S, 15.59.

*N-(5-(4-(5-Amino-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)benzamide (**15b**).* Compound **15b** was prepared as described for the preparation of **11b** except 5-(5-benzamido-1,3,4-thiadiazol-2-yl)pentanoic acid **14b** was used in place of **10b**. The crude material was purified by trituration with mildly basic water (pH ∼10, 2 × 10 mL): tan solid (38% yield); mp 215 °C (dec); ^{1}H NMR (DMSO-d_6) δ 1.68−1.82 (m, 4H), 2.85 (t, J = 7.2 Hz, 2H), 3.04 (t, J = 7.2 Hz, 2H), 7.01 (s, 2H), 7.55 (t, J = 7.8 Hz, 2H), 7.65 (t, J = 7.5 Hz, 1H), 8.10 (d, J = 7.3 Hz, 2H), 12.83 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 28.3, 28.3, 28.5, 29.0, 128.4, 128.6, 131.8, 132.9, 158.0, 159.4, 164.0, 165.3, 168.2. Anal. Calcd for $C_{15}H_{16}N_6O_2S{\cdot}0.19$EtOAc·0.2NaCl: C, 48.67; H, 4.54; N, 21.61; S,16.49; Found: C, 49.06; H, 4.52; N, 22.00; S, 16.45.

*N-(5-(4-(5-Amino-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)-2-cyclohexylacetamide (**15c**).* Compound **15c** was prepared as described for the preparation of **11b** except 5-(5-(2-cyclohexylacetamido)-1,3,4-thiadiazol-2-yl)pentanoic acid **14c** was used in place of **10b**. The crude material was purified by trituration with mildly basic water (pH ∼10, 2 × 10 mL), followed by trituration with EtOAc: off-white solid (18% yield); mp 218 °C (dec); ^{1}H NMR (DMSO-d_6) δ 0.90−0.99 (m, 2H), 1.10−1.22 (m, 4H), 1.58−1.74 (m, 9H), 2.33 (d, J = 7.1 Hz, 2H), 2.83 (t, J = 7.2 Hz, 2H), 2.99 (t, J = 7.2 Hz, 2H), 7.00 (s, 2H), 12.34 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 25.6, 25.7, 28.3, 28.3, 28.5, 29.0, 32.4, 34.7, 42.6, 158.0, 158.3, 163.5, 168.2, 170.7. Anal. Calcd for $C_{16}H_{24}N_6O_1S_2{\cdot}0.53$EtOAc: C, 50.94; H, 6.66; N, 19.67; S, 15.01; Found: C, 51.35; H, 6.25; N, 20.03; S, 14.90.

*N-(5-(4-(5-Amino-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)-2-p-tolylacetamide (**15d**).* Compound **15d** was prepared as described for the preparation of **11b** except 5-(5-(2-p-tolylacetamido)-1,3,4-thiadiazol-2-yl)pentanoic acid **14d** was used in place of **10b**. The crude material was purified by trituration with boiling water (2 × 50 mL), followed by trituration with MeOH: off-white solid (57% yield); mp 225 °C (dec); ^{1}H NMR (DMSO-d_6) δ 1.64−1.75 (m, 2H), 2.26 (s, 3H), 2.81 (t, J = 7.1 Hz, 2H), 2.98 (t, J = 7.2 Hz, 2H), 3.73 (s, 2H), 7.00 (s, 2H), 7.12 (d, J = 8.1 Hz, 2H), 7.20 (d, J = 7.8 Hz, 2H), 12.63 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 20.7, 28.2, 28.3, 28.4, 29.0, 41.2, 129.0, 129.1, 131.6, 136.0, 158.0, 158.2, 163.8, 168.2, 169.5. Anal. Calcd for $C_{17}H_{20}N_6O_1S_2{\cdot}0.35$MeOH·$0.1H_2O$: C, 51.90; H, 5.42; N, 20.93; S, 15.97; Found: C, 52.23; H, 5.09; N, 20.57; S, 16.20.

*N-(5-(4-(5-Amino-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)-2-(4-fluorophenyl)acetamide (**15e**).* Compound **15e** was prepared as described for the preparation of **11b** except 5-(5-(2-(4-fluorophenyl)acetamido)-1,3,4-thiadiazol-2-yl)pentanoic acid **14e** was used in place of **10b**. The crude material was purified by trituration with mildly basic water (pH ∼9, 2 × 30 mL), followed by trituration with EtOAc: tan solid (56%); mp 203 °C (dec); ^{1}H NMR (DMSO-d_6) δ 1.66−1.73 (m, 4H), 2.81 (t, J = 7.2 Hz, 2H), 2.99 (t, J = 7.1 Hz, 2H), 3.80 (s, 2H), 7.03 (br s, 2H), 7.15 (t, J = 8.8 Hz, 2H), 7.33−7.37 (dd, J = 5.7, 8.5 Hz, 2H), 12.69 (br s, 1H). Anal. Calcd for $C_{16}H_{17}N_6O_1FS_2{\cdot}0.2$EtOAc·$0.09H_2O{\cdot}0.4$NaCl: C, 46.38; H, 4.35; N, 19.32; S, 14.74; Found: C, 46.78; H, 4.31; N, 18.92; S, 14.36.

*N-(5-(4-(5-Amino-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)-2-phenylpropanamide (**15f**).* Compound **15f** was prepared as described for the preparation of **11b** except 5-(5-(2-phenylpropana-

mido)-1,3,4-thiadiazol-2-yl)pentanoic acid **14f** was used in place of **10b**. The crude material was purified by trituration with boiling water (~50 mL), followed by trituration with MeOH: tan solid (40%); mp 213 °C (dec); ^{1}H NMR (DMSO-d_6) δ 1.43 (d, J = 8 Hz, 3H), 1.64−1.75 (m, 4H), 2.81 (t, J = 7.2 Hz, 2H), 2.97 (t, J = 7.1 Hz, 2H), 3.98 (q, J = 7.0 Hz, 1H), 7.01 (s, 2H), 7.22−7.26 (m, 1H), 7.30−7.37 (m, 4H), 12.56 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 18.3, 28.2, 28.3, 28.5, 29.0, 45.0, 127.0, 127.4, 128.5, 140.9, 158.0, 158.9, 163.5, 168.2, 172.7. Anal. Calcd for $C_{17}H_{20}N_6O_1S_2$·0.5H_2O·0.3MeOH: C, 51.04; H, 5.50; N, 20.64; S, 15.75; Found: C, 50.86; H, 5.23; N, 20.49; S, 15.34.

*Methyl 7-Chloro-6-oxoheptanoate (**16**).* The methyl adipoyl chloride **8b** (2.5 mL, 15.3 mmol) was dissolved in DCM (15 mL) followed by slow addition to an ethereal solution of diazomethane (generated from 25 g of Diazald) at 0 °C. The solution was allowed to stir at 0 °C for 1.5 h followed by quenching with 10 mL of 48% HBr (bubbling). The solution was diluted with 40 mL DCM then put in a separatory funnel and the HBr was siphoned off. The organic layer was washed with 50 mL of satd $NaHCO_3$ and 50 mL brine. The organic layer was concentrated and chromatographed in 20% EtOAc/hex with the desired product R_f = 0.4 (20% EtOAc/hex, sulfuric acid charring) to afford **16** (2.9 g, 98%): ^{1}H NMR ($CDCl_3$) δ 1.66 (m, 4H), 2.35 (m, 2H), 2.67 (m, 2H), 3.67 (s, 3H), 4.08 (s, 2H).

*Methyl 5-(2-Aminothiazol-4-yl)pentanoate (**17**).* The α-chloroketone **16** (2.8 g, 14.5 mmol) was dissolved in EtOH (20 mL) followed by addition of thiourea (1.1 g, 14.5 mmol) and heated at 60 °C for 18 h. The solvent was removed and the crude mixture was partitioned between DCM and satd $NaHCO_3$ (20 mL). The DCM layer was dried and concentrated to afford a white solid that was triturated with Et_2O to afford 2.5 g of the desired aminothiazole **17** (81%): mp 123−128 °C; ^{1}H NMR ($CDCl_3$) δ, 1.67 (m, 4H), 2.33 (m, 2H), 2.54 (m, 2H), 3.66 (s, 3H), 4.95 (br s, 2H), 6.10 (s, 1H).

*Methyl 5-(2-(2-Phenylacetamido)thiazol-4-yl)pentanoate (**18**).* The aminothiazole **17** (2.0 g, 9.35 mmol) was dissolved in DCM (30 mL) followed by addition of triethylamine (1.4 mL, 10.3 mmol) and dropwise addition of phenylacetyl chloride (1.45 g, 9.35 mmol). The reaction was stirred overnight then quenched with 20 mL of 1N HCl. The crude mixture was partitioned between DCM and satd $NaHCO_3$ (20 mL). The DCM layer was dried and concentrated, then chromotographed on silica gel (1/1 hexanes/EtOAc) to afford 2.5 g (81%) of the desired methyl ester **18**: ^{1}H NMR ($CDCl_3$) δ 1.64 (m, 4H), 2.31 (m, 2H), 2.61 (m, 2H), 3.65 (s, 3H), 3.81 (s, 2H), 6.52 (s, 1H), 7.39−7.27 (m, 5H), 8.95 (br s, 1H).

*5-(2-(2-Phenylacetamido)thiazol-4-yl)pentanoic Acid (**19**).* The methyl ester **18** (2.0 g) was dissolved in 1N NaOH (20 mL) and dioxane (10 mL) followed by stirring for 2 h at 60 °C. The dioxane was removed and the basic solution was acidified with conc. HCl after which a white precipitate crashed out of solution. This precipitate collected by vacuum filtration, was washed with water and then triturated with ether to afford **19** (1.75 g, 92% yield): ^{1}H NMR (DMSO-d_6) δ 1.48 (m, 2H), 1.61 (m, 2H), 2.22 (t, J = 7.2 Hz, 2H), 2.58 (t, J = 7.3 Hz, 2H), 3.72 (s, 2H), 6.75 (s, 1H), 7.32 (m, 5H), 12.20 (br s, 1H), 12.25 (br s, 1H).

*N-(4-(4-(5-Amino-1,3,4-thiadiazol-2-yl)butyl)thiazol-2-yl)-2-phenylacetamide (**20**).* Compound **20** was prepared as described for the preparation of **11b** except 5-(2-(2-phenylacetamido)thiazol-4-yl)-pentanoic acid **19** was used in place of **10b**: off-white solid (29% yield); mp 192−197 °C; ^{1}H NMR (DMSO-d_6) δ 1.63−1.65 (m, 4H), 2.61 (t, J = 6.8 Hz, 2H), 2.80 (t, J = 6.8 Hz, 2H), 3.72 (s, 2H), 6.75 (s, 1H), 7.00 (s, 2H), 7.25−7.33 (m, 5H), 12.32 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 27.8, 28.5, 29.2, 30.4, 41.7, 107.4, 126.8, 128.4, 129.2, 135.0, 151.0, 157.3, 158.2, 168.1, 169.0. Anal. Calcd for $C_{17}H_{19}N_5O_1S_2$·0.5H_2O·0.02EtOAc: C, 53.39; H, 5.29; N, 18.22; S, 16.69. Found: C, 53.75; H, 5.11; N, 17.88; S, 16.31.

*Ethyl 7-Oxoheptanoate (**22**).* Compound **22** was prepared from acid chloride **21** as previously described.[22] The aldehyde **22** was used without further purification: red oil (95% yield); ^{1}H NMR ($CDCl_3$) δ 1.22 (t, J = 7.2 Hz, 3H), 1.32−1.40 (m, 2H), 1.58−1.66 (m, 4H), 2.28 (t, J = 7.5 Hz, 2H), 2.45 (tt, J = 2.2, 7.3 Hz, 2H), 4.08 (q, J = 7.1 Hz, 2H), 9.74 (t, J = 1.8 Hz, 1H).

*Ethyl 6-Bromo-7-oxoheptanoate (**23**).* Compound **23** was prepared from aldehyde **22** as was previously described.[22] The bromide **23** was used without further purification: orange oil (62% yield); ^{1}H NMR ($CDCl_3$) δ 1.24 (t, J = 7.2 Hz, 3H) 1.62−1.75 (m, 4H), 2.28 (t, J = 7.5 Hz, 1H), 2.34 (t, J = 7.1 Hz, 2H), 2.39−2.43 (m, 2H), 4.12 (q, J = 7.1 Hz, 2H), 9.20 (s, 1H).

*Ethyl 5-(2-Aminothiazol-5-yl)pentanoate (**24**).* Compound **24** was prepared from α-bromoaldehyde **23** as was previously described.[22] The crude material was purified by column chromatography (99.9% EtOAc, 0.1% NH_4OH): brown oil (31% yield); ^{1}H NMR ($CDCl_3$) δ 1.22 (t, J = 7.2 Hz, 3H), 1.54−1.69 (m, 4H), 2.32 (t, J = 7.2 Hz, 2H), 2.62 (t, J = 6.8 Hz, 2H), 4.08 (q, J = 7.2 Hz, 2H), 4.85 (br s, 2H), 6.70 (s, 1H).

*Ethyl 5-(2-(2-Phenylacetamido)thiazol-5-yl)pentanoate (**25**).* Compound **25** was prepared as described for the preparation of **18** except ethyl 5-(2-aminothiazol-5-yl)pentanoate **24** was used in place of **17**: white powder (70% yield); ^{1}H NMR ($CDCl_3$) δ 1.22 (t, J = 7.1 Hz, 3H) 1.63−1.67 (m, 4H), 2.27−2.30 (m, 2H), 2.72−2.75 (m, 2H), 3.80 (s, 2H), 4.09 (q, J = 7.1 Hz, 2H), 7.03 (s, 1H), 7.28−7.38 (m, 5H), 9.66 (br s, 1H).

*5-(2-(2-Phenylacetamido)thiazol-5-yl)pentanoic Acid (**26**).* Compound **26** was prepared as described for the preparation of **19** except ethyl 5-(2-(2-phenylacetamido)thiazol-5-yl)pentanoate **25** was used in place of **18**: white powder (91% yield); ^{1}H NMR (DMSO-d_6) δ 1.50−1.56 (m, 4H), 2.22 (t, J = 6.9 Hz, 2H), 2.71 (t, J = 6.9 Hz, 2H), 3.72 (s, 2H), 7.16 (s, 1H), 7.23−7.35 (m, 5H), 12.02 (br s, 1H), 12.18 (br s, 1H).

*N-(5-(4-(5-Amino-1,3,4-thiadiazol-2-yl)butyl)thiazol-2-yl)-2-phenylacetamide (**27**).* Compound **27** was prepared as described for the preparation of **11b** except 5-(2-(2-phenylacetamido)thiazol-5-yl)-pentanoic acid **26** was used in place of **10b:** off-white solid (55% yield); ^{1}H NMR (DMSO-d_6) δ 1.63−1.65 (m, 4H), 2.61 (t, J = 6.7 Hz, 2H), 2.80 (t, J = 6.8 Hz, 2H), 3.72 (s, 2H), 6.75 (s, 1H), 7.00 (br s, 2H), 7.24−7.33 (m, 5H), 12.02 (br s, 1H), 12.18 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 27.8, 28.5, 29.1, 30.4, 41.7, 107.4, 126.8, 128.4, 129.2, 135.0, 151.0, 157.3, 158.2, 168.1, 169.0. Anal. Calcd for $C_{17}H_{19}N_5O_1S_2$·0.01H_2O·0.27MeOH: C, 54.25; H, 5.30; N, 18.32; S, 16.77. Found: C, 54.49; H, 5.05; N, 17.97; S, 16.38.

*5-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)-N-(2-(pyrrolidin-1-yl)ethyl)pentanamide (**29a**).* To carboxylic acid **10b** (2.5 g, 7.9 mmol) dissolved in DMF was added DIEA (5.5 mL, 32 mmol). The reaction mixture was cooled to 0 °C. HATU (3.0 g, 7.9 mmol) was added and stirred for 10 min, followed by addition of 2-(pyrrolidin-1-yl)ethanamine **28a** (1.1 mL, 8.6 mmol). After 24 h, the DMF was removed. The product was extracted with DCM (4 × 50 mL) and washed with saturated $NaHCO_3$ (50 mL). The organic layer was dried over $MgSO_4$ and concentrated to obtain off-white solid. The solid was triturated with EtOAc to obtain **29a** as an off-white solid (2.1 g, 65%): mp 152 °C (dec); ^{1}H NMR (DMSO-d_6) δ 1.52 (quint, J = 3 Hz, 2H), 1.61−1.68 (m, 6H), 2.08 (t, J = 7.2 Hz, 2H), 2.44−2.48 (m, 6H), 2.95 (t, J = 7.5 Hz, 2H), 3.14 (q, J = 6.6 Hz, 2H), 3.79 (s, 2H), 7.24−7.35 (m, 5H), 7.79 (t, J = 5.4 Hz, 1H), 12.28 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 23.0, 24.6, 28.6, 28.7, 34.5, 37.6, 41.6, 53.6, 54.9, 126.9, 128.4, 129.3, 134.7, 158.2, 164.0, 169.3, 171.7. Anal. Calcd for $C_{21}H_{29}N_5O_2$ S_1·0.15H_2O·0.2NaCl: C, 58.67; H, 6.87; N, 16.29; S, 7.46. Found: C, 58.28; H, 6.74; N, 16.26; S, 7.30.

*N-Methyl-5-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)-N-(2-(pyrrolidin-1-yl)ethyl)pentanamide (**29b**).* Amide **29b** was prepared from **10b** as described for the preparation of **29a** except *N*-methyl-2-(pyrrolidin-1-yl)ethanamine **28b** was used in place of 2-(pyrrolidin-1-yl)ethanamine **28a**. The crude material was purified using column chromatography (5% MeOH, 94% DCM, 1% NH_4OH) to obtain **28a** as an orange oil (50% yield): ^{1}H NMR (MeOD) δ 1.65−1.71 (m, 2H), 1.78−1.85 (m, 6H), 2.40−2.45 (m, 2H), 2.59 (m, 1H), 2.67−2.77 (m, 5H), 2.89−2.91 (m, 1H), 3.02−3.06 (m, 4H), 3.46−3.50 (m, 1H), 3.53−3.56 (m, 1H), 3.81 (s, 2H), 7.25−7.33 (m, 5H). Anal. Calcd for $C_{22}H_{31}N_5O_2S_1$·0.3H_2O·0.2NaCl: C, 58.55; H, 7.28; N, 15.24; S, 6.98. Found: C, 58.17; H, 7.11; N, 15.31; S, 7.12.

*N-(2-(1-Methylpyrrolidin-2-yl)ethyl)-5-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)pentanamide (**29c**).* Amide **29c** was prepared

from **10b** as described for the preparation of **29a** except 2-(1-methylpyrrolidin-2-yl)ethanamine **28c** was used in place of 2-(pyrrolidin-1-yl)ethanamine **28a**: tan solid (30% yield); mp 121−131 °C; ^{1}H NMR (DMSO-d_6) δ 1.25−1.37 (m, 2H), 1.52−1.70 (m, 7H), 1.85−1.90 (m, 1H), 2.01−2.08 (m, 4H), 2.18 (s, 3H), 2.89−2.97 (m, 3H), 2.99−3.06 (m, 2H), 3.79 (s, 2H), 7.23−7.35 (m, 5H), 7.79 (t, J = 5.6 Hz, 1H), 12.47 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 21.5, 24.6, 28.6, 28.7, 30.0, 32.8, 34.9, 35.9, 40.0, 41.6, 56.5, 63.7, 126.9, 128.5, 129.3, 134.7, 158.2, 164.0, 169.3, 171.5. Anal. Calcd for $C_{22}H_{31}N_5O_2S_1$·0.45H_2O: C, 60.37; H, 7.35; N, 16.00; S, 7.33. Found: C, 59.98; H, 7.11; N, 15.65; S, 7.17.

5-(5-(2-Phenylacetamido)-1,3,4-thiadiazol-2-yl)-N-(2-(piperidin-1-yl)ethyl)pentanamide ***29d***. Amide **29d** was prepared from **10b** as described for the preparation of **29a** except 2-(piperidin-1-yl)-ethanamine **28d** was used in place of 2-(pyrrolidin-1-yl)ethanamine **28a:** white solid (39% yield); mp 132 °C (dec); ^{1}H NMR (DMSO-d_6) δ 1.32−1.35 (m, 2H), 1.41−1.46 (m, 4H), 1.49−1.56 (m, 2H), 1.58−1.64 (m, 2H), 1.61−1.68 (m, 2H), 2.07 (t, J = 7.1 Hz, 2H), 2.25−2.30 (m, 6H), 2.95 (t, J = 7.3 Hz, 2H), 3.12 (q, J = 6.8 Hz, 2H), 3.79 (s, 2H), 7.26−7.35 (m, 5 H), 7.70 (t, J = 5.6 Hz, 1H), 12.61 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 24.0, 24.6, 25.5, 28.6, 28.7, 34.9, 36.1, 41.6, 54.0, 57.9, 126.9, 128.4, 129.3, 134.7, 158.2, 164.0, 169.3, 171.7. Anal. Calcd for $C_{22}H_{31}N_5O_2S$·0.12H_2O: C, 61.2; H, 7.29; N, 16.22; S, 7.43. Found: C, 60.80; H, 7.09; N, 16.21; S, 7.52.

N-(2-(4-Hydroxypiperidin-1-yl)ethyl)-5-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)pentanamide ***(29e)***. Amide **29e** was prepared from **10b** as described for the preparation of **29a** except 1-(2-aminoethyl)piperidin-4-ol **28e** was used in place of 2-(pyrrolidin-1-yl)ethanamine **28a**: white solid (15% yield); mp 94−99 °C; ^{1}H NMR (DMSO-d_6) δ 1.38 (m, 2H), 1.50−1.56 (m, 2H), 1.61−1.68 (m, 4H), 2.08 (t, J = 7.2 Hz, 3H), 2.33−2.34 (m, 2H), 2.67−2.72 (m, 2H), 2.95 (t, J = 7.1 Hz, 3H), 3.13 (m, 2H), 3.43 (m, 1H), 3.79 (s, 2H), 4.57 (br s, 1H), 7.24−7.35 (m, 5H), 7.75 (br s, 1H), 12.67 (br s, 1H). Anal. Calcd for $C_{22}H_{31}N_5O_3S$·0.25H_2O·0.3NaCl: C, 56.51; H, 6.79 ; N, 14.98; S, 6.86. Found: C, 56.27; H, 6.84; N, 14.93; S, 6.94.

5-(5-(2-Phenylacetamido)-1,3,4-thiadiazol-2-yl)-N-(2-(4-phenylpiperidin-1-yl)ethyl)pentanamide ***(29f)***. Amide **29f** was prepared from **10b** as described for the preparation of **29a** except 2-(4-phenylpiperidin-1-yl)ethanamine **28f** was used in place of 2-(pyrrolidin-1-yl)ethanamine **28a**: off-white solid (18% yield); mp 123−126 °C; ^{1}H NMR (DMSO-d_6) δ 1.33−1.43 (m, 1H), 1.49−1.56 (m, 3H), 1.62−1.67 (m, 3H), 1.74−1.77 (m, 1H), 1.90−1.98 (m, 2H), 2.08 (t, J = 7.2 Hz, 2H), 2.34 (t, J = 6.3 Hz, 2H), 2.64−2.70 (m, 1H), 2.83−2.86 (m, 2H), 2.95 (t, J = 7.3 Hz, 2H), 3.15 (q, J = 6.7 Hz, 2H), 3.78 (s, 2H), 7.16−7.35 (m, 10H), 7.73 (t, J = 5.7 Hz, 1H), 12.66 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 24.6, 25.2, 28.6, 31.2, 34.9, 36.1, 41.5, 42.1, 53.2, 57.4, 126.1, 126.9, 127.1, 128.2, 128.4, 129.2, 134.6, 144.6, 158.1, 164.0, 169.3, 171.6. Anal. Calcd for $C_{28}H_{35}N_5O_2S$·0.22H_2O·0.08NaCl: C, 65.39; H, 6.95; N, 13.62. Found: C, 64.94; H, 6.81; N, 14.10.

N-(2-Aminoethyl)-5-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)pentanamide ***(29g)***. To synthesize **29g**, tert-butyl 2-(5-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)pentanamido)ethylcarbamate was first prepared from **10b** as described for the preparation of **29a** except tert-butyl 2-aminoethylcarbamate was used in place of 2-(pyrrolidin-1-yl)ethanamine **28a**. To the solution of 2-(5-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)pentanamido)ethylcarbamate (100 mg, 0.22 mmol) in DCM (2 mL) was added TFA (0.5 mL). The reaction mixture was stirred at room temperature for 2 h. H_2O (20 mL) was added and organic layer was separated. The aqueous layer was washed with DCM (3 × 20 mL) and concentrated to obtain a white solid. The solid was triturated with Et_2O to obtain 95 mg of **29g** as a white solid (92% yield): mp 167−172 °C; ^{1}H NMR (DMSO-d_6) δ 1.54 (quint, J = 7.6 Hz, 2H), 1.67 (quint, J = 7.5 Hz, 2H), 2.13 (t, J = 7.3 Hz, 2H), 2.83 (m, 2H), 2.96 (t, J = 7.2 Hz, 2H), 3.25 (q, J = 6.0 Hz, 2H), 3.80 (s, 2H), 7.26−7.34 (m, 5H), 7.76 (br s, 3H), 8.01 (br s, 1H), 12.69 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 24.2, 28.6, 28.7, 34.8, 36.4, 38.7, 41.5, 126.9, 128.4, 129.2, 134.6, 158.1, 164.0, 169.3, 172.7. Anal. Calcd for $C_{17}H_{23}N_5O_1S_2$·TFA·0.2H_2O: C, 47.73; H, 4.93; N, 14.65; S, 6.71. Found: C, 47.38; H, 5.04; N, 14.50; S, 6.91.

(2-(Diethylamino)ethyl)-5-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)pentanamide ***(29h)***. Amide **29h** was prepared from **10b** as described for the preparation of **29a** except *N*,*N*-diethylethane-1,2-diamine **28h** was used in place of 2-(pyrrolidin-1-yl)ethanamine **28a**: white solid (43% yield); mp 126−132 °C; ^{1}H NMR (DMSO-d_6) δ 0.91(t, J = 7.1 Hz, 6H), 1.52 (quint, J = 7.6 Hz, 2H), 1.65 (quint, J = 7.6 Hz, 2H), 2.08 (t, J = 7.2 Hz, 2H), 2.38 (t, J = 7.2 Hz, 2H), 2.44 (q, J = 7.1 Hz, 4H), 2.95 (t, J = 7.4 Hz, 2H), 3.07 (q, J = 6.1 Hz, 2H), 3.79 (s, 2H), 7.24−7.35 (m, 5H), 7.69 (t, J = 5.7 Hz, 1H), 12.62 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 11.7, 24.6, 28.6, 28.6, 34.9, 36.8, 41.6, 46.6, 51.7, 126.9, 128.4, 129.2, 134.7, 158.2, 163.9, 169.3, 171.6. Anal. Calcd for $C_{21}H_{31}N_5O_1S_2$: C, 60.40; H, 7.48; N, 16.77; S, 7.68. Found: C, 60.15; H, 7.43; N, 16.66; S, 7.92.

N-tert-Butyl-5-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)-pentanamide ***(29i)***. Amide **29i** was prepared from **10b** as described for the preparation of **29a** except 2-methylpropan-2-amine **28i** was used in place of 2-(pyrrolidin-1-yl)ethanamine **28a**. The crude material was triturated with DCM and Et_2O: white solid (41% yield), mp 158−160 °C; ^{1}H NMR (DMSO-d_6) δ 1.21 (s, 9H), 1.46−1.53 (m, 2H), 1.59−1.67 (m, 2H), 2.03 (t, J = 7.2 Hz, 2H), 2.95 (t, J = 7.3 Hz, 2H), 3.79 (s, 2H), 7.24−7.35 (m, 5 H), 7.38 (s, 1H), 12.7 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 24.7, 28.6, 28.6, 35.5, 38.9, 41.6, 49.8, 126.9, 128.5, 129.3, 134.7, 158.2, 164.1, 169.4, 171.4. Anal. Calcd for $C_{19}H_{26}N_4O_2S$·0.05H_2O: C, 60.79; H, 7.01; N, 14.92; S, 8.54. Found: C, 60.44; H, 6.95; N, 14.98; S, 8.89.

N-tert-butyl-N-methyl-5-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)pentanamide ***(29j)***. Amide **29j** was prepared from **10b** as described for the preparation of **29a** except *N*-2-dimethylpropan-2-amine **28j** was used in place of 2-(pyrrolidin-1-yl)ethanamine **28a**. The crude material was purified using column chromatography (3% MeOH, 96% EtOAc, 1% NH_4OH) to obtain **29j** as an off-white solid (55% yield): mp 103−109 °C; ^{1}H NMR (DMSO-d_6) δ 1.30 (s, 9H), 1.50 (quint, J = 7.7 Hz, 2H), 1.68 (quint, J = 7.5 Hz, 2H), 2.28 (t, J = 7.2 Hz, 2H), 2.81 (s, 3H), 2.96 (t, J = 7.3 Hz, 2H), 3.79 (s, 2H), 7.25−7.33 (m, 5H), 12.67 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 24.3, 27.9, 28.8, 31.6, 35.2, 38.3, 41.6, 55.8, 126.9, 128.5, 129.3, 134.7, 158.2, 164.2, 169.3, 172.3. Anal. Calcd for $C_{20}H_{28}N_4O_2S$·0.55H_2O: C, 60.29; H, 7.36; N, 14.06; S, 8.05. Found: C, 59.91; H, 7.04; N, 13.94; S, 7.80.

N-Benzyl-5-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)-pentanamide ***(29k)***. Amide **29k** was prepared from **10b** as described for the preparation of **29a** except benzylamine **28k** was used in place of 2-(pyrrolidin-1-yl)ethanamine **28a**: white solid (72% yield), mp 186−188 °C; ^{1}H NMR (DMSO-d_6) δ 1.54−1.61 (m, 2H), 1.63−1.71 (m, 2H), 2.17 (t, J = 7.2 Hz, 2H), 2.96 (t, J = 7.3 Hz, 2H), 3.80 (s, 2H), 4.25 (d, J = 6.1 Hz, 2H), 7.18−7.35 (m, 10H), 8.33 (t, J = 5.8 Hz, 1H), 12.65 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 24.7, 28.6, 28.7, 34.9, 41.6, 42.0, 126.7, 126.9, 127.2, 128.3, 128.5, 129.3, 134.7, 139.7, 158.3, 164.0, 169.4, 171.8. Anal. Calcd for $C_{22}H_{24}N_4O_2S$·0.16H_2O: C, 64.23; H, 5.96; N, 13.62; S, 7.79. Found: C, 63.85; H, 5.85; N, 13.52; S, 8.00.

N-(4-(Dimethylamino)benzyl)-5-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)pentanamide ***(29l)***. Amide **29l** was prepared from **10b** as described for the preparation of **29a** except 4-(aminomethyl)-*N*,*N*-dimethylaniline **28l** was used in place of 2-(pyrrolidin-1-yl)ethanamine **28a**: white solid (32% yield), mp 206 °C (dec); ^{1}H NMR (DMSO-d_6) δ 1.55−1.67 (m, 4H), 2.13 (t, J = 7.0 Hz, 2H), 2.82 (s, 6H), 2.96 (t, J = 7.3 Hz, 2H), 3.80 (s, 2H), 4.11 (d, J = 5.6 Hz, 2H), 6.65 (d, J = 8.6 Hz, 2H), 7.04 (d, J = 8.6 Hz, 2H), 7.25−7.34 (m, 5H), 8.17 (br s, 1H), 12.67 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 24.7, 28.6, 28.7, 34.8, 40.30, 41.55, 41.57, 112.4, 126.9, 127.1, 128.1, 128.5, 129.3, 134.7, 149.5, 158.2, 164.0, 169.3, 171.5. Anal. Calcd. for $C_{24}H_{29}N_5O_2S$·0.01H_2O: C, 63.81; H, 6.47; N, 15.50; S, 7.10. Found: C, 63.41; H, 6.42; N, 15.50; S, 6.95.

5-(5-(2-Phenylacetamido)-1,3,4-thiadiazol-2-yl)-N-(4-(trifluoromethyl)benzyl)pentanamide ***(29m)***. Amide **29m** was prepared from **10b** as described for the preparation of **29a** except (4-(trifluoromethyl)phenyl)methanamine **28m** was used in place of 2-(pyrrolidin-1-yl)ethanamine **28a**: white solid (51% yield), mp 214−218 °C; ^{1}H NMR (DMSO-d_6) δ 1.59 (m, 2H), 1.66 (m, 2H), 2.19 (t, J = 7.2 Hz, 2H), 2.97 (t, J = 7.3 Hz, 2H), 3.79 (s, 2H), 4.33 (d, J = 6.1 Hz, 2H), 7.25−7.33 (m, 5H), 7.44 (d, J = 7.8 Hz, 2H), 7.67 (d, J = 8.1

Hz, 2H), 8.46 (t, J = 5.9 Hz, 2H), 12.67 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 24.6, 28.6, 28.7, 34.8, 41.6, 41.6, 124.4 (q, J = 272 Hz), 125.2 (q, J = 3.7 Hz), 126.9, 127.4 (q, J = 32 Hz), 127.8, 128.5, 129.3, 134.7, 144.7 (q, J = 1.5 Hz), 158.2, 164.0, 169.4, 172.0. Anal. Calcd for $C_{23}H_{23}N_4O_2F_3S{\cdot}0.2H_2O$: C, 57.54; H, 4.91; N, 11.67; S, 6.68. Found: C, 57.21; H, 4.82; N, 11.67; S, 7.02.

GLS Assay. A 96-well microplate-based glutaminase assay was used to determine the activity of various glutaminase inhibitors. The hKGAd1 construct was prepared from the hKGA cDNA[26] by deleting the sequence encoded in exon 1 and cloning into pET15b as described for the rat KGAd1 construct.[27] Purified human kidney glutaminase ($hKGA_{124-669}$; 250 nM) was used as the source of enzyme and radiolabeled glutamine (L-[^{3}H]-glutamine, American Radiolabeled Chemicals, Saint Louis, MO; 2 mM and at a specific activity of 0.91 μCi/μmol) was used as the substrate. The assay was conducted in the presence and absence of inhibitors, at room temperature (45 min incubation), in 45 mM phosphate buffer (pH 8.2). At the end of the reaction period, the assay was terminated upon the addition of 20 mM imidazole buffer (pH 7). 96-well spin columns packed with strong anion ion-exchange resin (Bio-Rad, Hercules, CA) were used to separate the substrate and the reaction product. Unreacted [^{3}H]-glutamine was removed by washing with imidazole buffer. [^{3}H]-Glutamate, the reaction product, was then eluted with 0.1 M HCl and analyzed for radioactivity using Perkin-Elmer's TopCount instrument in conjunction with their 96-well LumaPlates (Waltham, MA).

GLS2 Assay. Mouse liver glutaminase assay (mLGA) was carried out using a three enzyme assay system. In the first reaction, glutamine was hydrolyzed to glutamate and ammonia by mouse liver glutaminase.[14] In the second reaction, glutamate was oxidized by glutamate oxidase (Sigma, St. Louis, MO) to α-ketoglutarate, ammonia and hydrogen peroxide. Finally, in the third reaction, hydrogen peroxide was complexed with fluorogenic Amplex red (colorless; Life Technologies, Grand Island, NY) in the presence of horse radish peroxidase (HRP; Worthington Biochemical Corp., Lakewood, NJ) to produce highly fluorescent resorufin (colored product; ex 530, em 590). Enzyme assays were conducted at room temperature in 45 mM phosphate buffer (pH 8.2) containing 0.04 U/mL glutamate oxidase, 0.125 U/mL HRP, 50 μM Amplex UltraRed and glutaminase. The half maximal inhibitory constant (IC_{50}) of various compounds were ascertained in the presence of 1 mM glutamine. In order to rule out assay method dependent variability, we tested compound **6** in fluorescence-based GLS assay and obtained IC_{50} value of 5.5 ± 0.9 μM.

Determination of Aqueous Solubility using Conventional Shake-flask Method. The solubility was determined using modified shake-flask procedure.[23] The solid was added to PBS buffer (pH = 7.4) until saturation occurred. To ensure equilibrium is achieved the mixture was shaken for at least 72 h at 37 °C. At the end of the incubation period, the saturated solution was filtered using a syringe filter (0.45 μm PTFE). The filtrate was diluted appropriately and concentration of compound was determined by LC/MS using a calibration curve of DMSO stock solution.

Metabolic Stability Studies in Mouse Microsomes. Compounds **6**, **11b** and **29a** were evaluated for phase I and phase II metabolic stability using mouse liver microsomes. For phase I metabolism, the reaction was carried out with 100 mM potassium phosphate buffer, pH 7.4, in the presence of NADPH regenerating system (1.3 mM NADPH, 3.3 mM glucose 6-phosphate, 3.3 mM $MgCl_2$, 0.4 U/mL glucose-6-phosphate dehydrogenase, 50 μM sodium citrate). Reactions in duplicate were initiated by addition of the liver microsomes to the incubation mixture (compound final concentration was 5 μM; 0.5 mg/mL microsomes). For phase II glucuronidation reaction, the compound was added to TRIS-HCl buffer (50 mM, pH 7.5) with microsomes (0.5 mg/mL), along with $MgCl_2$ (8 mM), and alamethicin (25 μg/mL) and preincubated at 37 °C. The reaction was initiated (in duplicate) with UDPGA (2 mM; final concentration). Controls in the absence cofactors were carried out to determine the specific cofactor-free degradation. At predetermined times up to 1 h, aliquots of the mixture were removed and the reaction quenched by addition of two times the volume of ice cold acetonitrile spiked with the internal standard. Compound disappearance was monitored over time using a liquid chromatography and tandem mass spectrometry (LC/MS/MS) method.

Cell Proliferation Assay. P493 cells were cultured in RPMI in aerobic conditions in the presence of various concentrations of compound **6**. Live cells were counted manually in a hemacytometer using trypan blue dye exclusion method.[28]

Animal Studies. The animal studies were performed according to the protocols approved by the Animal Care and Use Committee at The Johns Hopkins University. To generate tumorigenesis study in xenograft model, 2.0 × 10^7 P493 human lymphoma B cells were injected subcutaneously into male SCID mice (National Cancer Institute) as previously described.[28] Intraperitoneal (i.p.) injection of compound **6** (200 μg, ~12.5 mg/kg) was performed every other day starting from day 10. Tumor volumes were measured on days 10, 13, 16, and 19. Control animals were treated with i.p. injection of vehicle (2% [vol/vol] DMSO in PBS). The tumor volumes were measured using digital calipers and were calculated using the following formula: [length (mm) × width (mm) × width (mm) × 0.52].

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

GLS, kidney-type glutaminase; GLS2, liver glutaminase; BPTES, bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide; UDPGA, UDP-glucuronic acid; HATU, 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

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