Journal of Medicinal Chemistry

RICE Fondren Library

Article

Development and Characterization of a Fluorescent Probe for GLS1 and the Application for High-Throughput Screening of Allosteric Inhibitors

Xi Xu, Zijian Kuang, Jie Han, Ying Meng, Lei Li, Hongyu Luan, Pengfei Xu, Jubo Wang, Cheng Luo, Hong Ding, Zhiyu Li, and Jinlei Bian

J. Med. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jmedchem.9b01035 • Publication Date (Web): 11 Oct 2019

Downloaded from pubs.acs.org on October 12, 2019

Just Accepted

"Just Accepted" manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides "Just Accepted" as a service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. "Just Accepted" manuscripts appear in full in PDF format accompanied by an HTML abstract. "Just Accepted" manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are citable by the Digital Object Identifier (DOI®). "Just Accepted" is an optional service offered to authors. Therefore, the "Just Accepted" Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the "Just Accepted" Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these "Just Accepted" manuscripts.

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Published by American Chemical Society. Copyright © American Chemical Society. However, no copyright claim is made to original U.S. Government works, or works produced by employees of any Commonwealth realm Crown government in the course of their duties.

Development and Characterization of a Fluorescent Probe for GLS1 and the Application for High-Throughput Screening of Allosteric Inhibitors

Xi Xu,[†,‡] Zijian Kuang,[†] Jie Han,[‡] Ying Meng,[†] Lei Li,[†] Hongyu Luan,[†] Pengfei Xu,[†] Jubo Wang,[†] Cheng Luo,[‡] Hong Ding,[‡] Zhiyu Li,[*,†] Jinlei Bian[*,†]

[†]*Department of Medicinal Chemistry, China Pharmaceutical University, 24 Tongjiaxiang, Nanjing 210009, P.R.China*

[‡]*CAS Key Laboratory of Receptor Research, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zuchongzhi Road, Shanghai 201203, China*

**Corresponding author. Tel.: +86 13951678592 (Z. Li); +86 15151865295 (J. Bian)*

E-mail address: zhiyuli@cpu.edu.cn (Z. Li); bianjl@cpu.edu.cn (J. Bian)

ABSTRACT:

Glutaminase (GLS1) is a cancer energy metabolism protein which plays a predominant role in cell growth and proliferation. Due to its major involvement in malignant tumor, small-molecule GLS1 inhibitors are urgently needed to assess its therapeutic potential and for probing their underlying biology function. Recent studies showed that targeting the allosteric binding site represented a promising strategy for identifying potent and selective GLS1 inhibitors. Herein, we present the synthesis of two fluorescent probes targeting the allosteric binding site of GLS1 and their usage as mechanistic tools in multiple applicable assay platform. The fluorescence

polarization (FP)-based binding assay enables easy, fast and reliable screen of allosteric inhibitors from our in-house compound library obtained through click chemistry method. The obtained compound **C147** (named as **CPU-L1**) has been proved to be more potent and with greater solubility than the control compound **CB839**, which could serve as promising leads for further optimization as novel GLS1 inhibitors.

Keywords: glutaminase, fluorescence polarization, high-throughput screening, click chemistry

INTRODUCTION

Warburg effect, which was first proposed nearly a century ago, is the observation that even under conditions of sufficient oxygen, cancer cells are likely to convert glucose to lactic acid by glycolysis.[1] Cancer cells reprogram extensively novel cellular energy metabolism, including glycolysis and glutaminolysis instead of pyruvate oxidation.[2] Glutaminolysis serves as a significant role in many energy-generating and biosynthesis process for the growth and proliferation of cancer cells.[3] Glutamine is hydrolyzed by glutaminase (GLS) into glutamate, which is further oxidatively deaminated into α-ketoglutarate (α-KG) by glutamate dehydrogenase. Then, α-KG enters the tricarboxylic acid (TCA) cycle and involves in the production of nucleotides, ATP, certain amino acids, lipids and glutathione in mitochondria.[3] Glutaminase, as the first key enzyme in glutamine metabolism, has become a potential therapeutic target for the development of anti-cancer drug.

In mammalian cells, there are two paralogous *GLS* genes, *GLS1* (or *GLS*) and *GLS2*. *GLS1* encodes two alternatively spliced isozymes: kidney glutaminase (KGA) and glutaminase C (GAC).

GLS2 also encodes two isozymes: liver glutaminase (LGA) and glutaminase B.[4] While the relationship of GLS2 and cancer is not certain, it has been intensively studied that GLS1 is linked to the progression of many malignant tumors.[4,5] More importantly, the expression of GLS1 appears to be highly upregulated and activated in some cancers that need much energy supply and nitrogen-rich substances.[6] In addition, some growing cancer cells exhibited "glutamine addiction", in which the proliferation of cancer cells are highly dependent on glutamine, with rapid dying after glutamine depletion.[7-9] Therefore, the development of inhibitors targeting GLS1 represents a novel and promising orientation for glutamine-addicted cancer therapeutics.

Currently, GLS1 inhibitors could be divided into two categories, including active site inhibitors and allosteric site inhibitors. The active site inhibitors 6-diazo-5-oxo-L-norleucine (**DON**), **azaserine**, and **acivicin** (**Figure 1A**), which showed similar chemical structures with glutamine, exhibited robust anti-cancer effects *in vitro* and *in vivo* by inhibiting glutaminase.[10,11] However, these glutamine mimetic have serious toxicity and side effects due to the low selectivity.[12-14] Therefore, the clinical development of active site GLS1 inhibitors are limited.[4]

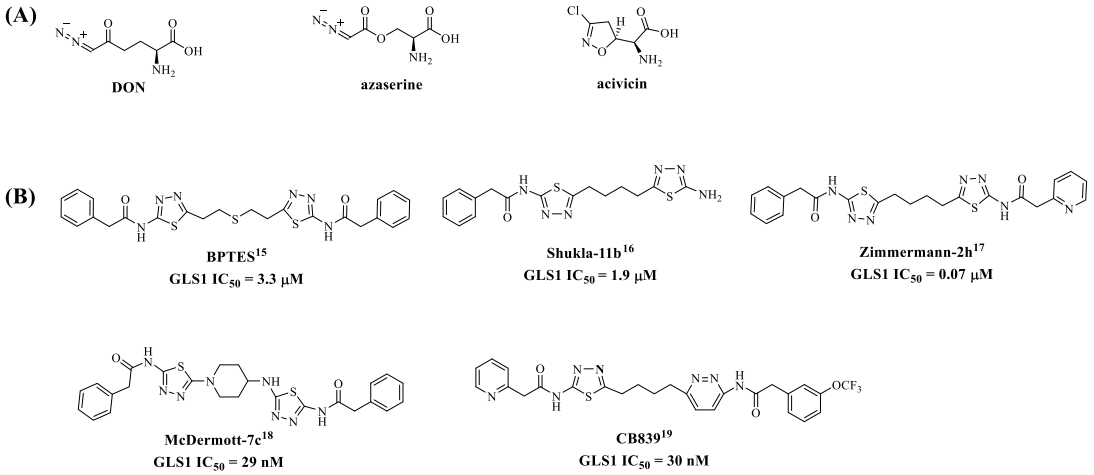


Figure 1. Representative chemical structures of GLS1 inhibitors. (**A**) active site GLS1 inhibitors; (**B**) allosteric site GLS1 inhibitors.

Bis-2-(5-phenylacetamido-1,2,4-thiadiazol)ethyl sulfide (**BPTES**), the first reported allosteric

inhibitors, showed potent and specific GLS1 inhibitory activity.[15] Based on the scaffold of **BPTES**, a great many of derivatives of it were designed and synthesized for the purposed of improving the drug-like properties and GLS1 inhibitory activity. Several representative compounds are listed in **Figure 1B**.[16-19] There is only one GLS1 allosteric inhibitor (**CB839**) in clinical trials currently.[4] Therefore, it is urgent to discover some potent GLS1 allosteric inhibitors with novel structures to provide more drug options for clinical researches.

It is significant to develop efficacious assay technology platforms for discovering GLS1 inhibitors due to the potential mechanism of compound action and compound-mediated assay interference. The most common method employed to evaluate the GLS1 inhibitors was the enzymatic-based assay, which was hydrolysis of glutamine to glutamate under the catalysis of GLS1. The available enzymatic-based assay has quite a few deficiencies. Firstly, the activity of GLS1 can be quantitatively reflected by measuring the absorbance of NADH/NADPH at 340 nm in glutamine hydrolysis assay. The disadvantage of the method is the relatively short and unspecific wavelengths at which many small molecule compounds absorb. Secondly, the amount of GLS1 protein required for glutamine hydrolysis assay is relatively large. Thirdly, the glutamine hydrolysis assay involves two steps, which are cumbersome and unsuitable for high-throughput screening (HTS). Moreover, many other reagents are required for glutamine hydrolysis assay, such as: glutamate dehydrogenase (GDH), NAD/NADH and ADP. These additional reagents could reduce the stability of the experiment and increase the cost. Thus, an easy, fast and reliable binding assay for HTS of allosteric GLS1 inhibitors were urgent to be developed, which could be used to combine with the glutamine hydrolysis assays to ensure the precise assessment of GLS1 inhibitors and provide useful information for further optimization. Recently, fluorescence-based techniques

have undergone a huge development for studying biochemical process and employed for drug discovery.[27,29] In the present study, we report the first small-molecule fluorescent probe based on affinity, which can be used for the fluorescence polarization (FP) method to quantitatively ascertain *in vitro* binding affinity of allosteric inhibitors targeting GLS1. We have optimized the experimental conditions and demonstrated the feasibility of conducting the method for HTS of GLS1 inhibitors. Take advantage of this fluorescent probe, we discovered several novel GLS1 inhibitors through screening our in-house compound library which was obtained through click chemistry method.

RESULTS AND DISCUSSION

Designation of fluorescent-labeled CB839 probes.

It has robustly been demonstrated that **CB839** is a potent, selective and orally bioavailable GLS1 inhibitor.[19] **CB839** exhibits extremely strong allosteric binding mechanism and slow-on/slow-off kinetic behavior with GLS1.[19] In this work, the cocrystal structure of **CB839** bound to the allosteric site of hKGA (PDB ID: 5JYO) was deeply analyzed to guide the design of small molecule probes.[21] The structure revealed that the nitrogen atoms in thiadiazole and pyridazinyl group of **CB839** could form hydrogen bonds with the protein backbone amide groups of the Phe322 and Leu323. The pyridazinyl and acetyl groups made hydrogen bonds with Tyr394, Lys320 and Asn324. The thiadiazole group was also involved in a water-mediated interaction with Asp327. However, the trifluoromethoxyphenyl group in the end of **CB839** was not engaged in any interactions with GLS1 and orientated towards the solvent site (**Figure 2A** and **2B**). Thus, the fragment **I** was selected as the recognition group (**Figure 2C**). For the fluorophores, we selected

the fluorescein isothiocyanate (**FITC**) and 7-nitro-1,2,3-benzoxadiazole (**NBD**), which have been widely used in many probes.[22,23] In addition, different linkers with varying composition and length were evaluated as survey of the potential chemical space. We conceived that a fluorescent tag, *i.e.* **FITC** and **NBD**, conjugated via a flexible linker to **CB839** will not only afford a fluorescent probe but also recapitulate the binding performance of the parent ligand **CB839** (**Figure 2C**). In this paper, we just reported the best two molecules probe **1** and probe **2** among our designed probes. Data of other molecules are not shown here.

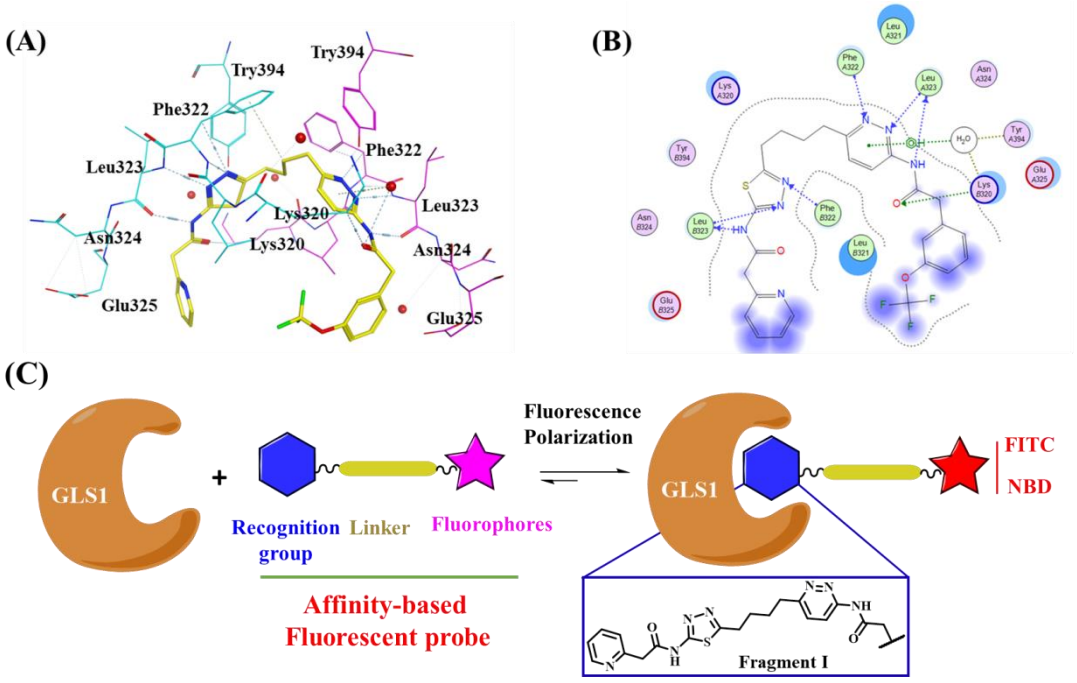


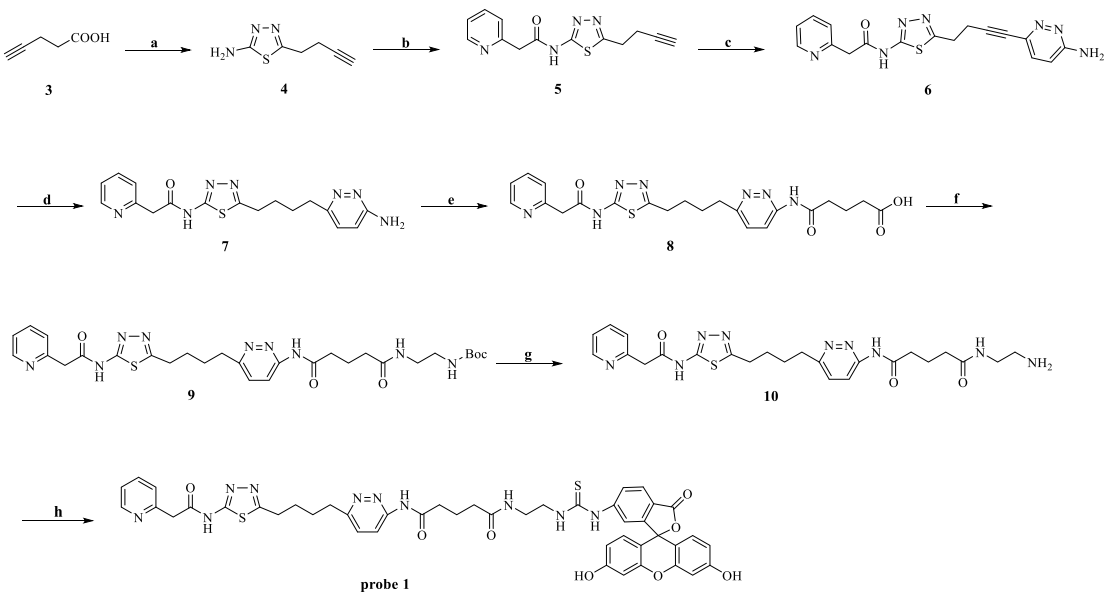
Figure 2. (**A**) Cocrystal structure of **CB839** (yellow) bound to the allosteric site of GLS1 (PDB: 5JYO). (**B**) The 2D diagram of binding mode of **CB839** in complex with GLS1 (PDB: 5JYO). (**C**) The principle of affinity-based fluorescent probe and the structures of fluorophores used in this paper.

Synthesis of two fluorescent probes.

Scheme 1 illustrates the synthesis of probe **1**. 4-Pentynoic acid (**3**) was reacted with thiosemicarbazide in the presence of phosphorus oxychloride to give aminothiadiazole **4**.[24] Intermediate **5** was obtained by coupling **4** with 2-pyridine acetic acid. Under the catalysis of

(beta-4)-platinum and CuI, **5** was reacted with 6-iodopyridazin-3-amine by a C-C coupling reaction[25] to give intermediate **6**, which was further hydrogenated to intermediate **7** by Raney-Ni and hydrogen. **7** was converted to intermediate **8** by treating with 1,5-pentanedioic acid. Subsequent reaction with *N*-boc-ethylenediamine followed by hydrolysis gave intermediate **10**. Finally, compound **10** was condensed with **FITC** in the presence of TEA afforded the desired probe **1**.

Scheme 1. Synthesis of probe 1[a]

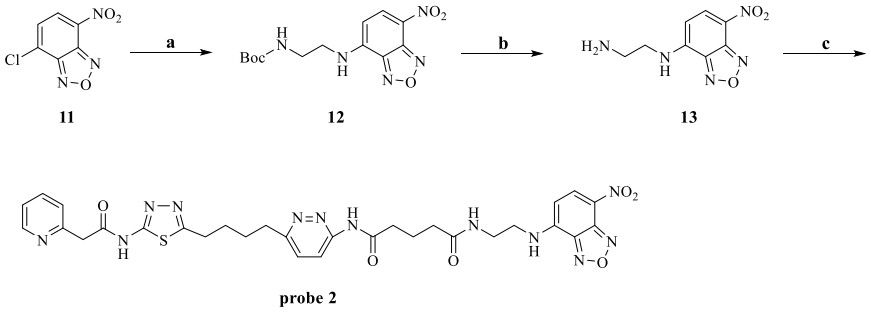


[a]Reagents and conditions: (a) thiosemicarbazide, $POCl_3$, 80 °C, 4 h, 80%; (b) 2-pyridine acetic acid hydrochloride, HATU, DIPEA, DMF, rt, 2 h, 92%; (c) 6-iodopyridazin-3-amine, (beta-4)-platinum, CuI, TEA, DMA, 60 °C, 3.5 h, 68%; (d) Raney-Ni, H_2, MeOH, rt, 12 h, 97%; (e) 1,5-pentanedioic acid, DMTMM, DMF, 35 °C, 12 h, 45%; (f) *N*-boc-ethylenediamine, HATU, DIPEA, DMF, rt, 2 h, 69%; (g) trifluoroacetic acid, DCM, rt, 1.5 h, 72%; (h) **FITC**, TEA, THF, 35 °C, 2 h, 31%.

The synthesis of target probe **2** was exhibited in **Scheme 2**. Starting from commercially available 4-chloro-7-nitro-1,2,3-benzoxadiazole (**11**), it was treated with *N*-boc-ethylenediamine and trifluoroacetic acid to give intermediate **13**. Reaction of **13** with intermediate **8** in the presence

of HATU and DIPEA afforded the desired probe **2**.

Scheme 2. Synthesis of probe 2[a]



[a]Reagents and conditions: (a) *N*-boc-ethylenediamine, TEA, DMF, DCM, rt, 1 h, 76%; (b) trifluoroacetic acid, DCM, rt, 10 h, 81%; (c) **8**, HATU, DIPEA, DMF, 35 °C, 1 h, 52%.

Characterization and Screening of Potential Probes for GLS1.

Absorption and fluorescence spectra of probe **1** and probe **2** were measured to investigate the spectroscopic properties of the ligand and how these were affected by the environment. **Figure S1** showed that different solvents had a certain impact on the spectroscopic properties. Probe **1** had a maximum UV absorption at 481 nm, an excitation wavelength at 486 nm, an emission wavelength at 529 nm with a Stokes shift of 43 nm and a considerable high fluorescence quantum yield (Φ) of 88.27% in PBS (**Table 1** and **Figure S1**). The wavelength difference and fluorescence quantum yield were suitable for further biological evaluation *in vitro*.

Table 1. Absorption and Fluorescence Properties of probe 1 and 2 in PBS.

probe	λ_{max} [nm]	λ_{ex} [nm]	λ_{em} [nm]	SS[a] [nm]	Φ[b] [%]
1	481	486	529	43	88.27
2	470	475	546	71	9.86

[a]SS, Stokes shift; [b]Φ, quantum yield.

The absorption spectrum of probe **2** was almost similar in four solvents with a maximum UV absorption at 470 nm (**Figure S2**). However, the excitation and emission spectrum of probe **2** were

different in various solvents. The increase in polarity of the environment (from 60% dioxane in PBS to aqueous buffer) induced an increase in the fluorescence intensity. Probe **2** had an excitation wavelength at 475 nm, an emission wavelength at 546 nm, with a Stokes shift of 71 nm and a quantum yield of 9.86% in PBS. Because the fluorescence quantum yield of probe **1** is significantly higher than that of probe **2**, probe **1** was selected to be mainly evaluated in following experiments.

*Fluorescent Properties of Probe **1** combined with GLS1 and Nonspecific Protein (BSA).* For further determination of the affinity of the probe **1**, it was incubated with a series of concentrations of GLS1 protein. As showed in **Figure S3A**, the fluorescence intensity was gradually enhanced with increased GLS1 protein concentrations. When incubated with 1 μM GLS1 protein, the fluorescence intensity was 2-fold higher than that of the blank group. In order to study the selectivity of probe **1** for GLS1, bovine-serum albumin (BSA) was employed to evaluate. Though the probe **1** presented a slightly higher fluorescence response when treated with BSA than blank group, it was much lower than the probe **1** treating with GLS1 (**Figure S3B** and **Figure S3C**). The results indicated that probe **1** exhibited potent fluorescence selectivity with GLS1 and had very weak nonspecific binding with BSA.

Fluorescence Polarization (FP) assay.

Then, probe **1** was employed as a fluorescent tracer to develop a competition binding FP assay. It was well known that the change of mP (ΔmP) reflects the interaction between the fluorescent-labeled compound (in the case probe **1**) and the protein (GLS1).[27] To develop a powerful and steady fluorescence polarization assay, the binding affinity of the probe to the

protein should be high and the binding range from maximum mP at saturation to minimal mP with no protein should be relatively large.

Figure 3A indicated that probe **1** and probe **2** exhibited obvious concentration-dependent FP response signal in assay buffer-1 with 10 mM Tris pH = 8.5 and 150 mM NaCl. The ΔmP value of probe **1** between maximum mP and minimal mP was about 120, which was in a reasonable assay window.[39] Compared with probe **1**, probe **2** exhibited a lower assay window with ΔmP value being 85. To investigate whether the assay buffer with different pH had an effect on ΔmP value, FP response signal of probe **1** and probe **2** were examined in assay buffer-2 with 10 mM Tris pH = 7.4 and 150 mM NaCl and assay buffer-3 with 10 mM Tris pH = 6.0 and 150 mM NaCl. Experimental results (**Figure S4**) indicated probe **1** and probe **2** showed almost similar FP response signal in various pH assay buffer, respectively. Combined with the spectroscopic properties, probe **1** was employed for further HTS of novel GLS1 inhibitors by FP assay.

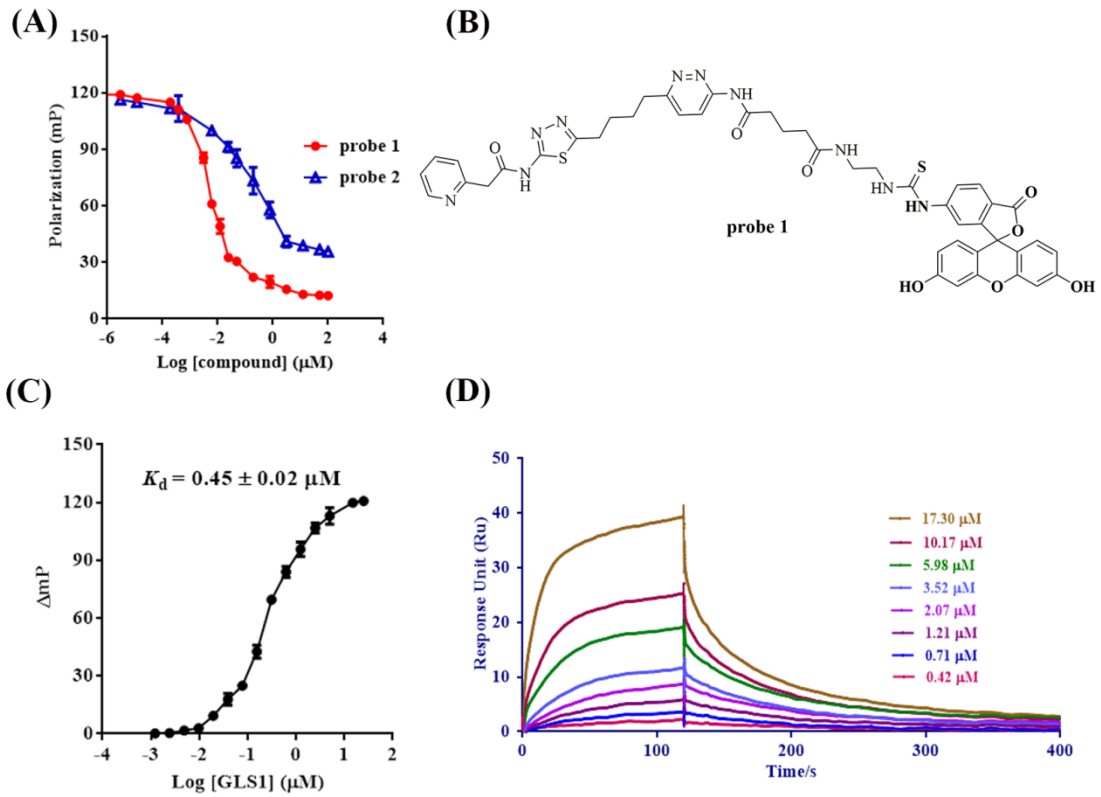


Figure 3. (**A**) Determination of the optimal concentration of free probe **1** and probe **2** for fluorescence polarization assay by varying the concentration of the free probes without GLS1 protein in assay buffer-1 with 10 mM Tris pH = 8.5 and 150 mM NaCl. (**B**) The structure of probe **1**. (**C**) Binding of the probe **1** (25 nM) to various concentrations of GLS1 protein. These assay buffer constants with 10 mM Tris pH = 8.5 and 150 mM NaCl. (**D**) SPR-based binding assay of probe **1** with GLS1. All data are mean values from three separate experiments.

To avoid the use of high amounts of probe but also to eliminate possible inconsistencies in concentration measurements, the concentration of probe **1** set as 25 nM appeared optimal for a suitable and reproducible FP effects. We next performed a saturation binding experiment by titrating a dilution series of GLS1 protein to probe **1**. FP signals were expressed as the change in millipolarization ΔmP, where ΔmP is the mP of the GLS1 mixture minus the mP of probe **1** only in assay buffer.[37] FP response signal (ΔmP) was gradually increased with the increased GLS1 protein concentration (**Figure 3C**). Moreover, it has been demonstrated that there was no discernible binding affinity between **FITC** and GLS1, thus confirming specificity of the probe **1**—GLS1 interaction. Concentration of GLS1 was plotted versus ΔmP, and the data were fit to the following equation (1)[38] using KaleidaGraph (v4.1.1, Synergy Software) to determine the K_d of probe **1**:

$$\Delta mP = [P_{min} + P_{max} \times (x/K_d)^n]/[1+(x/K_d)^n]) \qquad (1)^{38}$$

where P_{min} and P_{max} are the minimum and maximum observed ΔmP values, respectively, x is the GLS1 concentration, and n is the Hill coefficient of the binding curve. The K_d of probe **1** with GLS1 protein was calculated to be 0.45 μM (**Figure 3C**). Given the prominent potency of probe **1**, surface plasmon resonance (SPR) based binding assay was performed to quantitatively determine the binding affinity between probe **1** and GLS1. The results demonstrated that the interactions between probe **1** and GLS1 was dose-dependent and the equilibrium dissociation constant (K_d) is 0.61 μM (**Figure 3D**). The SPR measurement was consistent with FP assay. The dimethyl

sulfoxide (DMSO) tolerance in GLS1 FP assay was also evaluated. Different concentrations of DMSO was added into assay mixture. As shown in **Figure S5**, the assay window is reasonably stable in the presence of DMSO at least up to 8%. In addition, our GLS1 FP assay exhibited excellent signal-to-noise ratio (*S*/*N*) and an acceptable screening window coefficient (denoted Z'-factor). The Z' factor represents a characteristic parameter for the quality of the assay itself and is a measure of the reproducibility in the difference in signal between free and bound tracer controls across a large number of assay wells. Z' factor with more than 0.5 is considered to be acceptable for a good HTS assay.[33] In our GLS1 FP assay, the Z' factor is 0.96, indicating that the assay conditions are suitable for HTS (**Figure S6**).

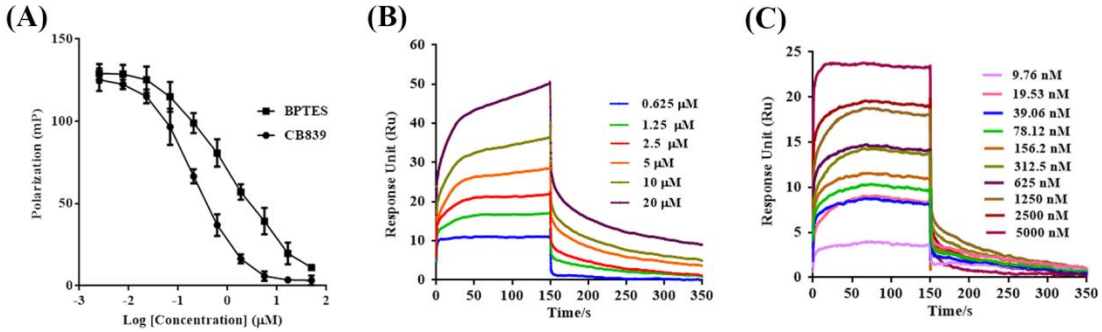


Figure 4. (**A**) Competition Binding effect of known allosteric site GLS1 inhibitors (**BPTES** and **CB839**) against probe **1**/GLS1 interaction. (**B**) SPR-based binding assay of **BPTES** with GLS1. (**C**) SPR-based binding assay of **CB839** with GLS1.

To evaluate whether the FP assay results correlate with a relevant biological parameter and the robustness *in vitro* screening for allosteric GLS1 inhibitors, we examined the binding affinities of known GLS1 inhibitors **BPTES** and **CB839** through FP assay using probe **1**. **Figure 4A** showed the results of **BPTES** and **CB839** with IC_{50} values at 2.9 μM and 0.11 μM, respectively, which were in good agreement with the values (**BPTES** K_d = 4.3 μM and **CB839** K_d = 92 nM) determined by SPR (**Figure 4B** and **4C**). These results supported the accuracy of FP assay to screen small-molecule compounds targeting the allosteric site of GLS1.

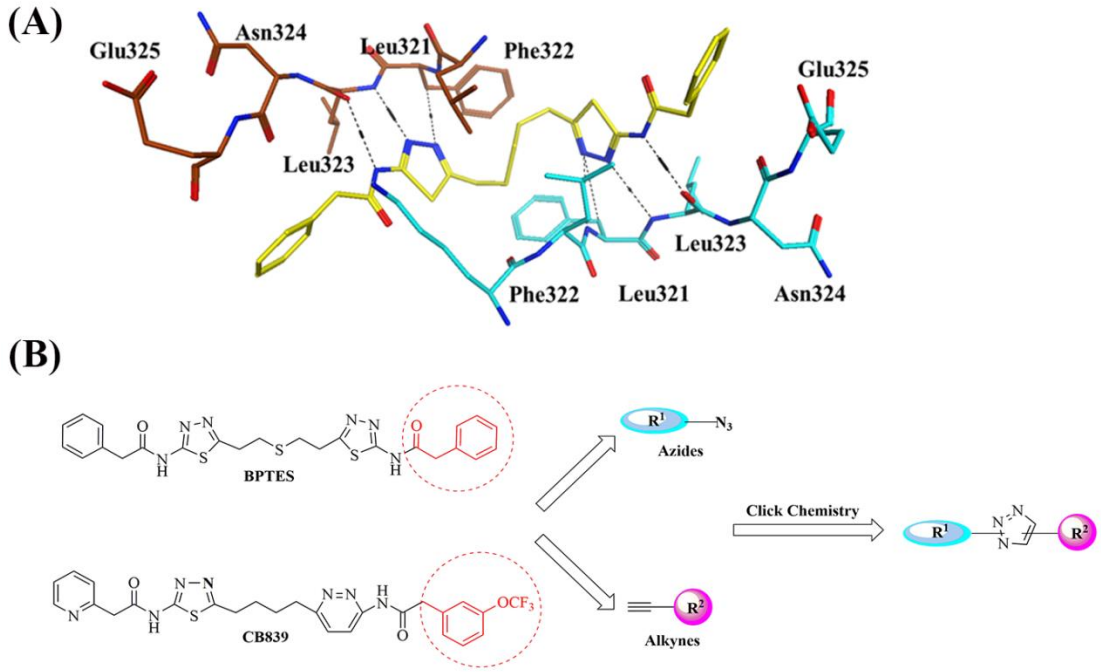


Figure 5. (**A**) The interactions of **BPTES** in the allosteric binding site of GLS1 (PDB: 3UO9). (**B**) The design of GLS1 inhibitors library by click chemistry.

GLS1 Inhibitors Library Generation by Click Chemistry.

To show the feasibility of the FP assay method to screen quantities of candidate ligands for binding to GLS1, we performed a screen of our in-house made GLS1 inhibitors library. According to structure-activity relationship (SAR) study and crystal structures analysis, one of the two phenyl groups of **BPTES** could be replaced due to the poor interaction with GLS1 (**Figure 5A**).[4,28] Thus, our in-house GLS1 inhibitors library were designed using a biosoteric replacement of the phenyl by 1,2,3-triazole core to improve the activity and drug-like property of **BPTES** (**Figure 5B**). A 200-member derivatives of **BPTES** were rapidly and efficient synthesized using "click chemistry" (**Figure S7** and **S8**). This library was used for primary screening by assuming a nearly 100% yield of the click reaction. These compounds were then evaluated at 10 μM in duplicates in the FP assay against GLS1. At 10 μM, all pure alkynes, azides and catalyst showed no or low disruption effect of probe **1**—GLS1 binding interaction with a range from 0% to 6%. In the primary screening, 36

members were considered as "hit" compounds with inhibition of >30% of the fluorescent probe **1** (25 nM) binding to the GLS1. Then, these compounds were selected for further synthesis (**Scheme S1**, **S2** and **S3**) and purification.

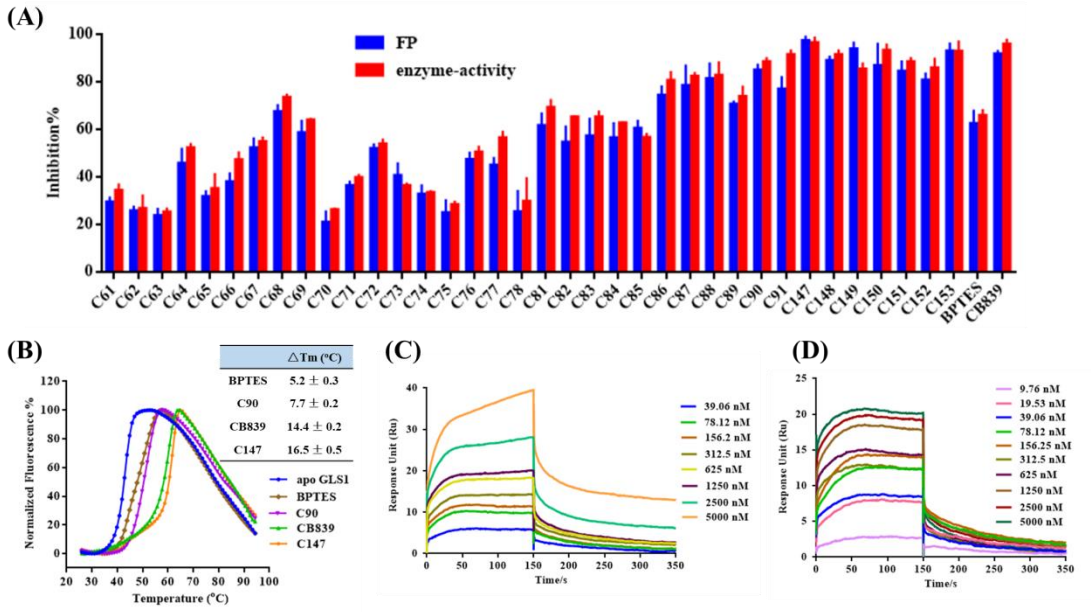
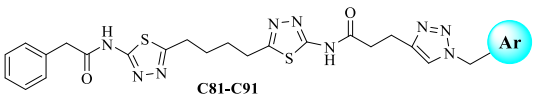


Figure 6. (**A**) Comparison of FP and enzyme-activity assay results from triazole-containing compounds against GLS1. These compounds were fully purified and characterized. And all target compounds had ≥ 95% purity. (**B**) Thermal shift profiles of GLS1 inhibitors interacting with GLS1. The melting temperature shifts compared with apo GLS1 are depicted in the insertion. (**C**) SPR-based binding assay of **C90** with GLS1. (**D**) SPR-based binding assay of **C147** with GLS1.

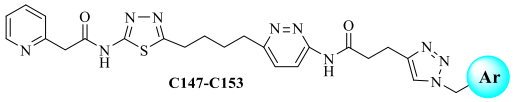
After obtaining these compounds, the enzymatic assay was employed to evaluate the GLS1 inhibitory activity and the results were consistent with the FP assay (**Figure 6A**), which further confirmed the validity and stability of the FP method using probe **1**. In general, compounds **C81-C91** and **C147-C153** at 10 μM displayed inhibition >50% of the probe **1** (25 nM) binding to the GLS1. And these compounds (**C81-C91**, **C147-C153**) are more potent than compounds **C61-C78** and **BPTES**. Next, the half maximal inhibitory concentration (IC_{50}) against GLS1 of **C81-C91** and **C147-C153** were further tested by FP and enzymatic assay (**Table 2**). Compared with **BPTES**, all of these compounds exhibited more potent GLS1 inhibitory activity. Among

them, compound **C147** showed the highest inhibitory potency against GLS1 with an IC_{50} value of 0.041 μM by FP assay and 0.027 μM by enzyme-activity assay, respectively, which were more potent than that of **CB839** with an IC_{50} value of 0.11 μM by FP assay and 0.038 μM by enzyme-activity assay, respectively. Representative compounds **C90** and **C147** were selected to validate the binding interactions with GLS1 by protein thermal shift (PTS) assay and surface plasmon resonance (SPR) analysis. **BPTES** and **CB839** were selected as positive references. Thermal shift assay (**Figure 6B**) showed that **C90** and **C147** gave rise to considerable stabilization of GLS1 protein with $\triangle T_m$ value being 7.7 ^oC and 16.5 ^oC, respectively. SPR analysis indicated a dissociation constant (K_d) of 0.35 μM and 0.068 μM (**Figure 6C** and **6D**). In addition, the antiproliferative activity *in vitro* of **C81-C91** and **C147-C153** were tested on HCT116 and MDA-MB-436 cells, which were reported to exhibit glutamine-dependent profiles.[19,26] Compound **C147** exhibited similar antiproliferative activity against HCT116 and MDA-MB-436 compared with **CB839** (HCT116 IC_{50}: 0.15 *vs* 0.06 μM; MDA-MB-436 IC_{50}: 0.18 *vs* 0.52 μM, respectively).

Table 2. GLS1 Inhibitory Activities and *in vitro* Antiproliferative Activities.



C81-C91



C147-C153

Comp[b].	Ar	GLS1 IC_{50} (μM)[a]		Antiproliferative activity IC_{50} (μM)[a]	
		FP	Enzyme-activity	HCT116	MDA-MB-436
C81	Ph	0.92 ± 0.11	0.90 ± 0.26	4.78 ± 0.58	1.16 ± 0.87
C82	4'-CN-Ph	0.93 ± 0.09	0.84 ± 0.31	5.14 ± 1.93	2.95 ± 0.57
C83	4'-NO_2-Ph	2.75 ± 0.24	2.50 ± 0.31	1.05 ± 0.44	10.67 ± 0.79
C84	4'-F-Ph	1.22 ± 0.30	1.11 ± 0.14	1.09 ± 0.65	0.35 ± 0.12
C85	4'-Cl-Ph	1.37 ± 0.16	1.25 ± 0.09	3.41 ± 0.28	1.14 ± 0.49
C86	4'-Br-Ph	1.08 ± 0.20	0.98 ± 0.10	1.85 ± 0.19	4.06 ± 0.42

C87	4'-CH_3-Ph	0.56 ± 0.08	0.42 ± 0.21	1.57 ± 0.73	1.79 ± 0.58
C88	4'-OCH_3-Ph	0.76 ± 0.10	0.51 ± 0.35	0.56 ± 0.29	5.49 ± 0.71
C89	3',4'-2OCH_3-Ph	0.85 ± 0.05	0.50 ± 0.29	0.45 ± 0.31	0.80 ± 0.28
C90	2'-CH_3-4'-CH_3-Ph	0.50 ± 0.13	0.28 ± 0.06	1.01 ± 0.62	0.43 ± 0.35
C91	2'-CH_3-6'-CH_3-Ph	0.78 ± 0.03	0.52 ± 0.09	0.74 ± 0.38	0.76 ± 0.13
C147	4'-CH_3-Ph	0.041 ± 0.02	0.027 ± 0.01	0.15 ± 0.03	0.18 ± 0.06
C148	4'-OCH_3-Ph	0.19 ± 0.11	0.15 ± 0.02	0.23 ± 0.10	0.63 ± 0.15
C149	3',4'-2OCH_3-Ph	0.40 ± 0.08	0.33 ± 0.05	0.38 ± 0.05	0.40 ± 0.19
C150	2'-CH_3-4'-CH_3-Ph	0.21 ± 0.13	0.11 ± 0.10	0.72 ± 0.17	0.20 ± 0.09
C151	2'-CH_3-6'-CH_3-Ph	0.46 ± 0.18	0.32 ± 0.09	0.27 ± 0.05	0.34 ± 0.21
C152	4'-OCF_3-Ph	0.39 ± 0.21	0.30 ± 0.11	0.25 ± 0.10	0.31 ± 0.19
C153	4'-CF_3-Ph	0.073 ± 0.03	0.044 ± 0.02	0.18 ± 0.05	0.21 ± 0.08
BPTES		2.90 ± 0.11	3.69 ± 0.26	1.02 ± 0.13	2.40 ± 0.79
CB839		0.11 ± 0.08	0.038 ± 0.01	0.06 ± 0.02	0.52 ± 0.20

[a]All data are mean values from triplicate experiments. [b]These compounds were fully purified and characterized. And all target compounds had ≥ 95% purity.

In order to confirm whether **C147** (**Figure 7B**) could block the hydrolysis of glutamine by inhibiting GLS1, its inhibition of cellular glutamate production was examined with HCT116 cells. Experimental results (**Figure 7A**) indicated that **C147** could inhibit the glutamate production in a dose-dependent manner in HCT116, similar with **CB839**. Moreover, it is well known that reactive oxygen species (ROS) is a key regulator of cancer cell growth, proliferation and glutamine metabolism plays a vital role for ROS homeostasis.[3,34] The intracellular ROS level was monitored using a ROS-sensitive fluorogenic dye (2',7'-dichlorodihydrofluorescein diacetate, DCFH-DA) by fluorescence microscopy and flow cytometry.[26] As shown in **Figure 7C** and **7D**, **C147** could potently increase the ROS levels in a dose-dependent manner in HCT116, which was similar with

that of **CB839**.

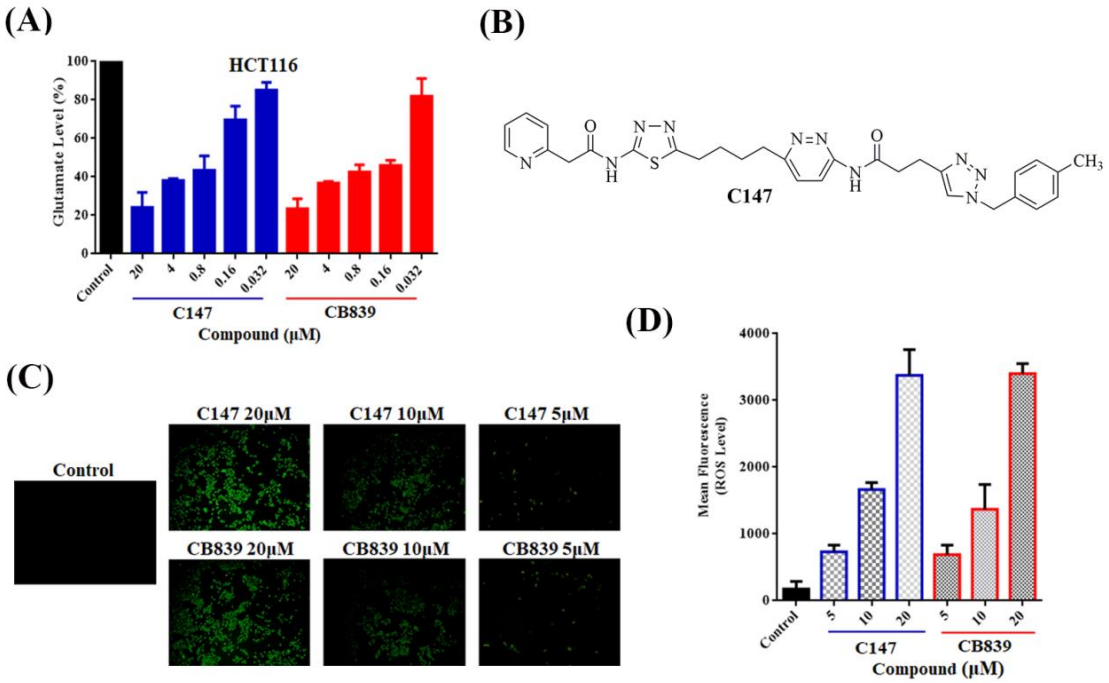


Figure 7. (**A**) Effect of compound **C147** and **CB839** on diminishing the cellular glutamate levels in HCT116 cell. (**B**) The chemical structure of **C147**. (**C**) Fluorescence microscopic images of intracellular ROS production by DCFH-DA staining (green) in HCT116 cells (**D**) Quantitatively analysis of ROS generation by the flow cytometry.

In addition, the preliminary physicochemical properties of the representative compound **C147** was also determined. Lipid-water distribution coefficient (LogD) and intrinsic aqueous solubility (**Table S1**) of compound **C147**, **BPTES** and **CB839** were determined by the LC-MS based shake-flask method.[32] Experimental results showed that **C147** displayed potent aqueous solubility for 20.8 μg/mL, nearly 9 times better than **CB839** and the LogD of **C147** was similar with that of **CB839** (1.29 *vs* 1.90). These results demonstrated that introduction of triazole could indeed improve the aqueous solubility. Further optimization and mechanism studies of compound **C147** are in progress in our lab.

CONCLUSIONS

In this study, using the potent small-molecule GLS1 inhibitor **CB839** as the structure template, we have synthesized two small-molecule probes **1** and **2** with FITC and NBD fluorescent group, respectively. Probe **1** exhibited desirable spectroscopic properties, such as a high quantum yield in PBS environments, high potency, selectivity and on-target action, which was successfully used to establish a reliable robust and customizable FP-based *in vitro* HTS for allosteric inhibitors of GLS1.

In addition, a compound library of GLS1 inhibitors containing triazole group was rapidly constructed by click chemistry. The inhibitory activity of these compounds against GLS1 were cross-screened by FP and enzyme activity assay. It has been demonstrated that the introduction of a suitable triazole moiety in the appropriate position of **BPTES** indeed could increase the GLS1 inhibitory activity and improve aqueous solubility. In summary, we propose that our probe and FP assay reported in this work are versatile and valuable tools for the *in vitro* evaluation of GLS1 characteristics, which will prove useful for the identification and profiling of new drug candidates targeting the allosteric binding site. In addition, the GLS1 potent inhibitor **C147** which was discovered through the high throughput screening method could be effective lead compound for further evaluation.

EXPERIMENTAL SECTION

General Chemistry Methods.

All reagents and solvents were from commercial sources and, unless otherwise noted, were used without further purification. All reactions were monitored by thin-layer chromatography (TLC) on

0.25 mm silica gel plates with fluorescent indicator (GF_{254}) and visualized under UV light. With tetramethylsilane (TMS) as internal standard, the ^{1}H and ^{13}C NMR spectra were recorded on Bruker AV-300 (300 and 75 MHz) apparatus at 25 ^{o}C. Samples were prepared as solutions in deuterated solvent. EI-MS was collected on shimadzu GCMS-2010 instruments. HR-MS spectral data was obtained on Agilent technologies 6520 Accurate-Mass Q-TOF LC/MS instruments. All target compounds were purified via silica gel (60 Å, 70-230 mesh) column chromatography. Melting points were measured by XT-4 melting point apparatus. Purity of all target compounds was ≥ 95%, as determined by HPLC (SHIMADZU Labsolutions) analysis on the Aglilent C18 column (4.6 × 150 mm, 5 μm) using gradient elution (Mobile Phase: A Phase = 0.1% formic acid in H_2O, B Phase = MeOH) at a flow rate of 0.5 mL/min.

Synthetic Procedures.

5-(But-3-yn-1-yl)-1,3,4-thiadiazol-2-amine (4)

To a solution of compound **3** (1.50 g, 0.015 mol) in $POCl_3$ was added thiosemicarbazide (1.39 g, 0.015 mol). The reaction mixture was stirred at 80 ℃ for 4 h. After cooling to the room temperature, the mixture was basified with NaOH to pH 9 and extracted with ethyl acetate. The reaction mixture was partitioned between ethyl acetate (15 mL) and water (20 mL). The water layer was extracted with ethyl acetate (15 mL × 3). The organic layer was combined, washed with saturated brine, dried over anhydrous Na_2SO_4 and concentrated in vacuo to afford **4** as a tawny solid, yield 88%. ^{1}H NMR (300 MHz, DMSO-d_6): δ 6.99 (s, 2H), 2.95 (t, J = 7.0 Hz, 2H), 2.83 (d, J = 2.4 Hz, 1H), 2.50 (dd, J = 7.1, 4.6 Hz, 2H) ppm. HRMS (ESI): m/z, calcd for $C_6H_8N_3S$ $[M + H]^+$, 154.0433; found: 154.0434.

_N_-(5-(but-3-yn-1-yl)-1,3,4-thiadiazol-2-yl)-2-(pyridin-2-yl)acetamide (5)

To a solution of compound **4** (3.00 g, 0.022 mol) in DMF was added 2-pyridine acetic acid hydrochloride (3.74 g, 0.022 mmol), HATU (8.80 g, 0.039 mmol) and DIPEA (7.50 g, 0.059 mmol). The reaction mixture was stirred at room temperature for 2 h. The reaction mixture was diluted in 50 mL water and was extracted with ethyl acetate (30 mL × 3). The organic layer was dried over sodium sulfate, filtered and concentrated in vacuo to afford **5**, yield 92%. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.71 (s, 1H), 8.50 (d, J = 4.8 Hz, 1H), 7.78 (td, J = 7.7, 1.7 Hz, 1H), 7.40 (d, J = 7.7 Hz, 1H), 7.29 (dd, J = 6.7, 5.0 Hz, 1H), 4.02 (s, 2H), 3.16 (t, J = 7.0 Hz, 2H), 2.88 (t, J = 2.6 Hz, 1H), 2.62 (td, J = 7.0, 2.6 Hz, 2H) ppm. HRMS (ESI): m/z, calcd for $C_{13}H_{13}N_4OS$ [M + H]$^+$, 273.0805; found: 273.0807.

_N_-(5-(4-(6-aminopyridazin-3-yl)but-3-yn-1-yl)-1,3,4-thiadiazol-2-yl)-2-(pyridin-2-yl)acetamide (6)

To a solution of compound **5** (2.00 g, 7.40 mmol) in DMA was added 6-iodopyridazin-3-amine (1.35 g, 6.11 mmol), CuI (0.12 g, 0.61 mmol), TEA (3.10 g, 30.65 mmol) and (beta-4)-platinum (0.71 g, 0.61 mmol). The reaction mixture was stirred at room temperature for 3.5 h under nitrogen atmosphere. After cooling to the room temperature, the reaction mixture was then diluted in 30 mL isopropyl ether. After filtration, the reddish brown oily substance was collected, when add some water, we can get some gray solid. The residue was purified by column chromatography to afford the titled compound as a gray solid, yield 68%. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.69 (s, 1H), 8.49 (d, J = 4.0 Hz, 1H), 7.76 (t, J = 7.1 Hz, 1H), 7.39 (d, J = 7.8 Hz, 1H), 7.33-7.17 (m,

2H), 6.69 (d, *J* = 9.1 Hz, 1H), 6.63 (s, 2H), 4.01 (s, 2H), 3.31-3.23 (m, 2H), 2.90 (t, *J* = 6.8 Hz, 2H) ppm. HRMS (ESI): m/z, calcd for $C_{17}H_{16}N_7OS$ $[M + H]^+$, 366.1132; found: 366.1135.

_N_-(5-(4-(6-aminopyridazin-3-yl)butyl)-1,3,4-thiadiazol-2-yl)-2-(pyridin-2-yl)acetamide (7)

To a solution of compound **6** (1.00 g, 2.74 mmol) in methanol was added Raney Nickel (2 ml). The reaction mixture was stirred at room temperature for 12 h under hydrogen. The solution was filtered to remove the catalyst. The filtrate was concentrated to afford **7** as a yellow solid, yield 97%. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.67 (s, 1H), 8.50 (d, *J* = 4.9 Hz, 1H), 7.77 (td, *J* = 7.7, 1.8 Hz, 1H), 7.40 (d, *J* = 7.8 Hz, 1H), 7.29 (dd, *J* = 6.9, 5.3 Hz, 1H), 7.19 (d, *J* = 9.0 Hz, 1H), 6.76 (d, *J* = 9.1 Hz, 1H), 6.28 (s, 2H), 4.01 (s, 2H), 3.00 (t, *J* = 6.9 Hz, 2H), 2.70 (t, *J* = 6.9 Hz, 2H), 1.70 (s, 4H) ppm. HRMS (ESI): m/z, calcd for $C_{17}H_{20}N_7OS$ $[M + H]^+$, 370.1445; found: 370.1451.

5-Oxo-5-((6-(4-(5-(2-(pyridin-2-yl)acetamido)-1,3,4-thiadiazol-2-yl)butyl)pyridazin-3-yl)amino)pentanoic acid (8)

To a solution of compound **7** (0.50 g, 1.35 mmol) in DMF was added 1,5-pentanedioic acid (0.27 g, 2.01 mmol) and DMTMM (0.56 g, 2.03 mmol). The reaction mixture was stirred at 45 ℃ for 12 h. The reaction mixture was partitioned between DCM: MEOH = 10:1 (15 mL) and water (30 mL). The water layer was extracted with DCM/MEOH = 10:1 (15 mL × 3). The organic layer was combined, washed with saturated brine (10 mL × 3), dried over sodium sulfate, filtered and concentrated. The crude product was purified by column chromatography to afford the titled compound as a yellow solid, yield 45%. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.99 (s, 1H), 8.58 (s, 1H), 8.24 (d, *J* = 8.9 Hz, 1H), 7.92 (d, *J* = 8.1 Hz, 1H), 7.55 (dd, *J* = 15.2, 8.5 Hz, 2H), 7.43 (s,

1H), 4.09 (s, 2H), 3.02 (s, 2H), 2.89 (s, 2H), 2.45 (d, J = 6.4 Hz, 2H), 2.26 (d, J = 7.0 Hz, 2H), 1.85-1.70 (m, 6H) ppm. HRMS (ESI): m/z, calcd for $C_{22}H_{26}N_7O_4S$ $[M + H]^+$, 484.1761; found: 484.1761.

Tert-butyl(2-(5-oxo-5-((6-(4-(5-(2-(pyridin-2-yl)acetamido)-1,3,4-thiadiazol-2-yl)butyl)pyridazin-3-yl)amino)pentanamido)ethyl)carbamate (9)

To a solution of compound **8** (0.42 g, 0.86 mmol) in DMF was added *N*-boc-ethylenediamine (0.16 g, 1.05 mmol), HATU (0.67 mg, 1.77 mmol) and DIPEA (0.56 g, 4.36 mmol). The reaction mixture was stirred at room temperature for 2 h. The reaction mixture was partitioned between DCM/MEOH = 10:1 (15 mL) and water (30 mL). The water layer was extracted with DCM/MEOH = 10:1 (10 mL × 3). The organic layer was combined, washed with saturated NaCl solution (10 mL × 3), dried over sodium sulfate, filtered and concentrated. The residue was purified by column chromatography to afford the titled compound as a faint yellow solid, yield 69%. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.99 (s, 1H), 8.64 (d, J = 4.3 Hz, 1H), 8.25 (d, J = 9.1 Hz, 1H), 8.04 (d, J = 8.0 Hz, 1H), 7.81 (s, 1H), 7.67-7.49 (m, 3H), 6.76 (s, 1H), 4.15 (s, 2H), 3.10-2.83 (m, 8H), 2.41 (s, 2H), 2.14-2.03 (m, 2H), 1.85-1.68 (m, 6H), 1.35 (s, 9H) ppm. HRMS (ESI): m/z, calcd for $C_{29}H_{40}N_9O_5S$ $[M + H]^+$, 626.2868; found: 626.2868.

***N*^{1}-(2-aminoethyl)-*N*^{5}-(6-(4-(5-(2-(pyridin-2-yl)acetamido)-1,3,4-thiadiazol-2-yl)butyl)pyridazin-3-yl)glutaramide (10)**

To a solution of compound **9** (0.22 g, 0.35 mmol) in DCM was added trifluoroacetic acid (5 mL). The reaction mixture was stirred at room temperature for 1.5 h under nitrogen. The mixture was

basified with $NaHCO_3$ to pH 8. After filtration, the solid was purified by column chromatography to afford the titled compound as a yellow solid, yield 72%. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.99 (s, 1H), 8.61 (d, J = 5.4 Hz, 1H), 8.23 (d, J = 9.2 Hz, 1H), 8.02 (dd, J = 13.7, 6.6 Hz, 2H), 7.58 (d, J = 9.0 Hz, 2H), 7.53-7.46 (m, 1H), 4.13 (s, 2H), 3.27 (dd, J = 12.0, 6.3 Hz, 2H), 3.01 (s, 2H), 2.92-2.80 (m, 4H), 2.44 (t, J = 7.3 Hz, 2H), 2.15 (t, J = 7.6 Hz, 2H), 1.87-1.78 (m, 2H), 1.73 (s, 4H), 1.22 (s, 2H) ppm. HRMS (ESI): m/z, calcd for $C_{24}H_{32}N_9O_3S$ $[M + H]^+$, 526.2343; found: 526.2364.

N^1-(2-(3-(3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-6-yl)thioureido)ethyl)-N^5-(6-(4-(5-(2-(pyridin-2-yl)acetamido)-1,3,4-thiadiazol-2-yl)butyl)pyridazin-3-yl)glutaramide (probe 1)

To a solution of compound **10** (40 mg, 0.076 mmol) in THF was added FITC (20 mg, 0.051 mmol) and TEA (20 mg, 0.20 mmol). The reaction mixture was stirred at 35 ℃ for 2 h. The reaction mixture was concentrated in vacuum. The luminous yellow solid can be obtained by preparing high performance liquid phase, yield 35%. mp 179.2-180.1 ℃. ^{1}H NMR (300 MHz, MeOD-d_4): δ 8.69 (s, 1H), 8.39 (d, J = 8.9 Hz, 1H), 8.22 (d, J = 7.5 Hz, 1H), 8.13 (s, 1H), 7.80-7.65 (m, 4H), 7.18 (d, J = 7.9 Hz, 1H), 6.74 (d, J = 12.1 Hz, 4H), 6.58 (d, J = 8.8 Hz, 2H), 3.74 (dd, J = 13.1, 6.5 Hz, 6H), 3.05 (s, 2H), 2.95 (s, 2H), 2.53 (s, 2H), 2.31 (d, J = 6.9 Hz, 2H), 2.00 (s, 2H), 1.82 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 181.14, 172.97, 172.64, 168.97, 168.73, 164.46, 159.96, 159.88, 158.54, 154.77, 154.55, 152.33, 148.82, 147.14, 142.08, 138.19, 130.10, 129.50, 128.64, 126.78, 125.12, 123.02, 119.15, 113.03, 110.14, 102.68, 83.23, 54.59, 48.87, 44.02, 38.15, 35.80, 34.99, 34.65, 32.39, 29.48, 29.15, 29.03, 28.72, 27.30, 23.23, 21.32, 18.76, 17.15, 14.41 ppm.

HRMS (ESI): m/z, calcd for $C_{45}H_{43}N_{10}O_8S_2$ $[M + H]^+$, 915.2701; found: 915.2662. HPLC: t_R = 13.038 min, 97.5% purity.

Tert-butyl (2-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)ethyl)carbamate (12)

To a solution of compound **11** (0.50 g, 2.51 mmol) in DMF was added *N*-boc-ethylenediamine (0.40 g, 2.51 mmol), TEA (0.28 g, 2.77 mmol) in DCM. The reaction mixture was stirred at room temperature for 1 h. The reaction mixture was partitioned between ethyl acetate (10 mL) and water (30 mL). The water layer was extracted with ethyl acetate (10 mL × 3). The organic layer was combined, washed with saturated brine (10 mL × 3), dried over sodium sulfate, filtered and concentrated, yield 76%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.49 (d, J = 8.6 Hz, 1H), 7.69 (s, 1H), 6.18 (d, J = 8.6 Hz, 1H), 5.12 (s, 1H), 3.63 (s, 4H), 1.48 (s, 9H) ppm. HRMS (ESI): m/z, calcd for $C_{13}H_{18}N_5O_5$ $[M + H]^+$, 324.1302; found: 324.1292.

***N*^{1}-(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)ethane-1,2-diamine (13)**

To a solution of compound **12** (0.20 g, 0.62 mmol) in DCM was added 2, 2, 2-trifluoroacetic acid (5 mL). The reaction mixture was stirred at room temperature for 4 h under nitrogen. The mixture was basified with $NaHCO_3$ to pH 7. After filtration, the solid was purified by column chromatography to afford the titled compound as a black solid, yield 81%. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.58 (d, J = 8.8 Hz, 1H), 8.28 (s, 1H), 6.50 (d, J = 8.9 Hz, 1H), 3.78 (s, 2H), 3.18 (t, J = 6.0 Hz, 2H), 1.32 (d, J = 35.7 Hz, 1H) ppm. HRMS (ESI): m/z, calcd for $C_8H_8N_5O_3$ $[M - H]^-$, 222.0633; found: 222.0638.

N^1-(2-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)ethyl)-N^5-(6-(4-(5-(2-(pyridin-2-yl)aceta-mido)-1,3,4-thiadiazol-2-yl)butyl)pyridazin-3-yl)glutaramide (Probe 2)

To a solution of compound **8** (20 mg, 0.041 mmol) in DMF was added compound **13** (12 mg, 0.052 mmol), HATU (31 mg, 0.082 mmol) and DIPEA (31 mg, 0.24 mmol). The reaction mixture was stirred at 35 ℃ for 1 h. The reaction mixture was added some water, we can obtain some gray solid. The solid was purified by column chromatography to afford the titled compound as a yellow solid, yield 52%. m.p. 137.9-139.8 ℃. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.71 (s, 1H), 10.99 (s, 1H), 9.47 (s, 1H), 8.53 (d, J = 9.8 Hz, 2H), 8.26-8.04 (m, 2H), 7.81 (d, J = 7.1 Hz, 1H), 7.55 (d, J = 8.8 Hz, 1H), 7.46-7.26 (m, 2H), 6.44 (d, J = 9.4 Hz, 1H), 4.01 (s, 2H), 3.16 (s, 3H), 3.01 (s, 2H), 2.88 (s, 2H), 2.73 (s, 1H), 2.40 (s, 2H), 1.99 (d, J = 5.7 Hz, 2H), 1.75 (d, J = 15.5 Hz, 6H) ppm. ^{13}C NMR (101 MHz, DMSO-d_6): δ 172.93, 172.87, 168.97, 164.47, 159.93, 158.56, 155.36, 154.52, 149.57, 145.84, 144.93, 144.55, 138.42, 137.23, 128.52, 124.64, 122.67, 121.35, 118.97, 99.63, 44.54, 43.55, 37.67, 35.74, 34.99, 34.70, 29.05, 29.00, 28.73, 21.25 ppm. HRMS (ESI): m/z, calcd for $C_{30}H_{33}N_{12}O_6S$ $[M + H]^+$, 689.2361; found: 689.2371. HPLC: t_R = 11.910 min, 95.0% purity.

Ethyl 5-(5-amino-1,3,4-thiadiazol-2-yl)pentanoate (15)

To a solution of compound **14** (10 g, 57.40 mmol) in $POCl_3$ (30 mL) was added thiosemicarbazide (5.23 g, 57.40 mmol). The reaction mixture was stirred at 85 ℃ for 4 h. After cooling to the room temperature, 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 to get desired compound **15** as a white solid, yield 42%. **15** was ready for the next

step without the further purification. HRMS (ESI): m/z, calcd for $C_9H_{16}N_3O_2S$ $[M + H]^+$, 230.0958; found: 230.0962.

Ethyl 5-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)pentanoate (16)

The compound **15** (2.00 g, 8.73 mmol) was dissolved in THF (20 mL) and followed by addition of triethylamine (1.3 mL, 9.62 mmol) and dropwise addition of phenylacetyl chloride (1.35 g, 8.73 mmol). The reaction mixture was stirred at room temperature for 24 h. The solvent was removed under vacuum. The crude product was recrystallized using water to give compound **16** as a white solid, yield 92%. **16** was ready for the next step without the further purification. HRMS (ESI): m/z, calcd for $C_{17}H_{22}N_3O_3S$ $[M + H]^+$, 348.1376; found: 348.1371.

5-(5-(2-Phenylacetamido)-1,3,4-thiadiazol-2-yl)pentanoic acid (17)

The compound **16** (2.00 g) was dissolved in 4 N NaOH (15 mL) and MeOH (10 mL) followed by stirring for 3 h at room temperature. The solvent was removed under reduced pressure and the basic solution was acidified with HCl. The precipitate was filtered off to obtain compound **17** as a white solid, yield 90%. **17** was ready for the next step without the further purification. HRMS (ESI): m/z, calcd for $C_{15}H_{18}N_3O_3S$ $[M + H]^+$, 320.1063; found: 320.1064.

***N*-(5-(4-(5-amino-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)-2-phenylacetamide (18)**

Compound **17** (2.00 g, 6.20 mmol) and thiosemicarbazide (0.62 g, 6.80 mmol) were dissolved in $POCl_3$ (10 mL) and heated to 85 ℃ for 4 h. The reaction was cooled and the black viscous oil was poured onto ice. The solution was basified using NaOH to pH 10. The precipitate was filtered off

to afford the titled compound **18** as a black solid, yield 43%. **18** was ready for the next step without the further purification. HRMS (ESI): m/z, calcd for $C_{16}H_{19}N_6OS_2$ $[M + H]^+$, 375.1056; found: 375.1056.

2-Chloro-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide (19)

To a solution of compound **18** (3.00 g, 8.02 mmol) in DMF was added TEA (2.43 g, 24.06 mmol) and dropwise addition of chloroacetyl chloride (1.80 g, 16.04 mmol). The reaction mixture was stirred at room temperature for 12 h. The reaction was cooled and the reaction mixture was poured onto water. The precipitate was filtered off to obtain white solid, yield 84%. **19** was ready for the next step without the further purification. HRMS (ESI): m/z, calcd for $C_{18}H_{20}ClN_6O_2S_2$ $[M + H]^+$, 451.0772; found: 451.0785.

2-Azido-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide (A4)

To a solution of compound **19** (3.50 g, 7.78 mmol) in DMF was added NaN_3 (1.52 g, 23.30 mmol). The reaction mixture was stirred at room temperature for 12 h. The reaction mixture was then diluted in water ether. After the water layer was extracted with DCM (30 mL × 3). Finally the organic layer was dried over sodium sulfate, filtered and concentrated. And the crude product was purified by column chromatography to afford the titled compound as a white solid, yield 90%. HRMS (ESI): m/z, calcd for $C_{18}H_{20}N_9O_2S_2$ $[M + H]^+$, 458.1176; found: 458.1148.

2-(4-(2-Hydroxyethyl)-1*H*-1,2,3-triazol-1-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide (C61)

To a solution of compound **A4** (0.20 g, 0.44 mmol) in DMF was added CuI (16.70 mg, 0.088 mmol in water) and 3-butyn-1-ol **B1** (77.05 mg, 1.10 mmol). The reaction mixture was stirred in microwave 300 W at 100 ℃ for 0.5 h. The reaction mixture was then diluted in water. The precipitate was filtered off to obtain white solid. The residue was purified by column chromatography (DCM/MeOH = 20:1) to afford the titled compound **C61** as a white solid, yield 45%. m.p. 208-210 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.66 (s, 2H), 7.90 (s, 1H), 7.31 (s, 5H), 5.43 (s, 2H), 4.69 (s, 1H), 3.79 (s, 2H), 3.64 (s, 2H), 3.00 (s, 4H), 2.79 (s, 2H), 1.74 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.80, 165.77, 164.73, 164.28, 158.62, 144.89, 135.11, 129.71, 128.89, 127.34, 124.56, 60.79, 51.81, 42.01, 31.61, 29.56, 29.47, 29.14, 28.90, 28.84, 28.68, 22.54, 4.68 ppm. HRMS (ESI): m/z, calcd for $C_{22}H_{26}N_9O_3S_2$ $[M + H]^+$, 528.1595; found: 528.1577. HPLC: t_R = 21.625 min, 97.8% purity.

2-(4-(2-Hydroxypropan-2-yl)-1*H*-1,2,3-triazol-1-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide (C62)

Compound **C62** was prepared from **A4** as described for the preparation of **C61** except 2-methyl-3-butyn-2-ol **B2** was used in place of **B1**: white solid (39% yield). m.p. 210-212 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.93 (s, 1H), 12.67 (s, 1H), 7.92 (s, 1H), 7.32 (s, 5H), 5.44 (s, 2H), 5.15 (s, 1H), 3.80 (s, 2H), 3.01 (s, 4H), 1.75 (s, 4H), 1.48 (s, 6H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.84, 165.68, 164.80, 164.36, 158.72, 156.30, 135.09, 129.72, 128.91, 127.37, 122.66, 67.54, 51.83, 42.02, 31.16, 28.84, 28.68 ppm. HRMS (ESI): m/z, calcd for $C_{23}H_{28}N_9O_3S_2$

$[M + H]^+$, 542.1751; found: 542.1740. HPLC: t_R = 22.069 min, 96.8% purity.

2-(4-(2-Hydroxybutan-2-yl)-1*H*-1,2,3-triazol-1-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide (C63)

Compound **C63** was prepared from **A4** as described for the preparation of **C61** except 3-methylpent-1-yn-3-ol **B3** was used in place of **B1**: white solid (40% yield). m.p. 220-223 ^oC. ^1H NMR (300 MHz, DMSO-d_6): δ 12.93 (s, 1H), 12.65 (s, 1H), 7.90 (s, 1H), 7.32 (s, 5H), 5.45 (s, 2H), 5.00 (s, 1H), 3.80 (s, 2H), 3.02 (s, 4H), 1.75 (s, 6H), 1.44 (s, 3H), 0.76 (s, 3H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.81, 165.76, 164.27, 158.61, 155.25, 135.12, 129.72, 128.90, 127.35, 123.49, 70.24, 51.84, 42.02, 35.97, 29.46, 28.86, 28.70, 8.77 ppm. HRMS (ESI): m/z, calcd for $C_{24}H_{30}N_9O_3S_2$ $[M + H]^+$, 556.1908; found: 556.1891. HPLC: t_R = 22.457 min, 97.9% purity.

3-(1-(2-Oxo-2-((5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)amino)ethyl)-1*H*-1,2,3-triazol-4-yl)propanoic acid (C64)

Compound **C64** was prepared from **A4** as described for the preparation of **C61** except 4-pentynoicacid **B4** was used in place of **B1**: white solid (35% yield). m.p. 220-224 ^oC. ^1H NMR (300 MHz, DMSO-d_6): δ 12.96-12.86 (m, 1H), 12.64 (s, 2H), 7.89 (s, 1H), 7.32 (s, 5H), 5.43 (s, 2H), 3.79 (s, 2H), 3.00 (s, 6H), 2.58 (d, J = 7.3 Hz, 2H), 1.75 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.81, 164.18, 160.65, 139.27, 135.11, 131.28, 129.72, 128.89, 127.35, 61.07, 52.35, 42.02, 31.67, 28.84, 28.72, 14.63 ppm. HRMS (ESI): m/z, calcd for $C_{23}H_{26}N_9O_4S_2$ $[M + H]^+$, 556.1544; found: 556.1521. HPLC: t_R = 19.619 min, 98.9% purity.

Ethyl 1-(2-oxo-2-((5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)amino)ethyl)-1*H*-1,2,3-triazole-4-carboxylate (C65)

Compound **C65** was prepared from **A4** as described for the preparation of **C61** except ethyl propiolate **B5** was used in place of **B1**: white solid (37% yield). m.p. 204-208 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.61 (s, 1H), 8.76 (s, 1H), 7.31 (s, 5H), 5.57 (s, 2H), 4.32 (d, J = 7.1 Hz, 2H), 3.79 (s, 2H), 2.99 (s, 4H), 1.75 (s, 4H), 1.31 (t, J = 6.6 Hz, 3H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.83, 165.53, 160.66, 158.45, 139.29, 135.08, 131.25, 129.71, 128.90, 127.36, 61.11, 52.26, 42.00, 28.89, 28.82, 28.67, 14.61 ppm. HRMS (ESI): m/z, calcd for $C_{23}H_{26}N_9O_4S_2$ $[M + H]^+$, 556.1544; found: 556.1504. HPLC: t_R = 21.178 min, 95.7% purity.

2-(4-Cyclopropyl-1*H*-1,2,3-triazol-1-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl) butyl)-1,3,4-thiadiazol-2-yl)acetamide (C66)

Compound **C66** was prepared from **A4** as described for the preparation of **C61** except ethynylcyclopropane **B6** was used in place of **B1**: white solid (42% yield). m.p. 215-218 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.91 (s, 1H), 12.64 (s, 1H), 7.85 (s, 1H), 7.32 (s, 5H), 5.40 (s, 2H), 3.79 (s, 2H), 3.01 (s, 4H), 1.96 (s, 1H), 1.75 (s, 4H), 0.90 (s, 2H), 0.72 (s, 2H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.79, 165.72, 164.71, 164.23, 158.53, 149.30, 135.08, 129.70, 128.87, 127.33, 122.92, 51.83, 41.98, 28.81, 28.67, 8.07, 6.92 ppm. HRMS (ESI): m/z, calcd for $C_{23}H_{26}N_9O_2S_2$ $[M + H]^+$, 524.1645; found: 524.1633. HPLC: t_R = 21.443 min, 96.7% purity.

2-Phenyl-*N*-(5-(4-(5-(2-(4-phenyl-1*H*-1,2,3-triazol-1-yl)acetamido)-1,3,4-thiadiazol-2-yl)butyl

)-1,3,4-thiadiazol-2-yl)acetamide (C67)

Compound **C67** was prepared from **A4** as described for the preparation of **C61** except ethynylbenzene **B7** was used in place of **B1**: white solid (45% yield). m.p. 218-220 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.64 (s, 1H), 8.60 (s, 1H), 7.85 (s, 1H), 7.38 (d, J = 45.5 Hz, 9H), 5.55 (s, 2H), 3.78 (s, 2H), 3.00 (s, 4H), 1.74 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.29, 163.80, 158.16, 146.30, 134.61, 130.55, 129.20, 128.92, 128.38, 127.91, 126.84, 125.14, 123.01, 114.48, 51.66, 41.51, 33.62, 31.24, 31.11, 29.80, 28.96, 28.64, 28.34, 28.18, 22.04, 13.90 ppm. HRMS (ESI): m/z, calcd for $C_{26}H_{26}N_9O_2S_2$ $[M + H]^+$, 560.1645; found: 560.1617. HPLC: t_R = 20.846 min, 95.9% purity.

2-Phenyl-*N*-(5-(4-(5-(2-(4-(p-tolyl)-1*H*-1,2,3-triazol-1-yl)acetamido)-1,3,4-thiadiazol-2-yl)but-yl)-1,3,4-thiadiazol-2-yl)acetamide (C68)

Compound **C68** was prepared from **A4** as described for the preparation of **C61** except 1-ethynyl-4-methylbenzene **B8** was used in place of **B1**: white solid (33% yield). m.p. 237-242 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.99 (s, 1H), 12.66 (s, 1H), 8.55 (s, 1H), 7.75 (s, 2H), 7.33 (s, 2H), 7.29 (s, 5H), 5.54 (s, 2H), 3.79 (d, J = 12.5 Hz, 2H), 3.01 (s, 4H), 2.34 (d, J = 13.4 Hz, 3H), 1.74 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.80, 165.07, 158.84, 146.89, 137.71, 135.11, 129.96, 129.72, 128.89, 128.29, 127.34, 125.58, 123.10, 52.12, 42.02, 28.85, 28.70, 21.30 ppm. HRMS (ESI): m/z, calcd for $C_{27}H_{28}N_9O_2S_2$ $[M + H]^+$, 574.1802; found: 574.1806. HPLC: t_R = 23.368 min, 99.3% purity.

2-Phenyl-*N*-(5-(4-(5-(2-(4-(4-(trifluoromethyl)phenyl)-1*H*-1,2,3-triazol-1-yl)acetamido)-1,3,4-

thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide (C69)

Compound **C69** was prepared from **A4** as described for the preparation of **C61** except 1-ethynyl-4-(trifluoromethyl)benzene **B9** was used in place of **B1**: white solid (40% yield). m.p. 109-111 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.68 (s, 1H), 8.79 (s, 1H), 8.10 (d, J = 7.7 Hz, 2H), 7.83 (d, J = 7.9 Hz, 2H), 7.34-7.24 (m, 5H), 5.60 (s, 2H), 3.79 (s, 2H), 3.00 (s, 4H), 1.75 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.80, 164.28, 158.78, 145.45, 135.11, 135.02, 129.72, 128.88, 128.40, 127.34, 126.44, 126.19, 124.83, 123.37, 52.46, 42.02, 28.85, 28.71 ppm. HRMS (ESI): m/z, calcd for $C_{27}H_{25}F_3N_9O_2S_2$ $[M + H]^+$, 628.1519; found: 628.1528. HPLC: t_R = 23.557 min, 99.2% purity.

2-(4-(4-Fluorophenyl)-1*H*-1,2,3-triazol-1-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide (C70)

Compound **C70** was prepared from **A4** as described for the preparation of **C61** except 1-ethynyl-4-fluorobenzene **B10** was used in place of **B1**: white solid (41% yield). m.p. 249-250 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.59 (s, 1H), 8.51 (s, 1H), 7.91-7.76 (m, 2H), 7.23 (s, 5H), 7.18 (d, J = 8.3 Hz, 2H), 5.46 (s, 2H), 3.71 (s, 2H), 2.92 (s, 4H), 1.67 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.81, 164.29, 163.89, 160.65, 158.68, 153.86, 149.65, 145.96, 135.10, 129.70, 128.88, 127.73, 127.63, 127.33, 123.42, 116.48, 116.19, 42.01, 31.73, 29.46, 29.02, 28.84, 28.71, 27.01, 22.54, 14.39 ppm. HRMS (ESI): m/z, calcd for $C_{26}H_{25}FN_9O_2S_2$ $[M + H]^+$, 578.1551; found: 578.1533. HPLC: t_R = 22.877 min, 95.1% purity.

2-(4-(4-Chlorophenyl)-1*H*-1,2,3-triazol-1-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol

-2-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide (C71)

Compound **C71** was prepared from **A4** as described for the preparation of **C61** except 1-chloro-4-ethynylbenzene **B11** was used in place of **B1**: white solid (43% yield). m.p. 232-240 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.64 (s, 1H), 8.63 (s, 1H), 7.88 (d, J = 8.1 Hz, 2H), 7.51 (d, J = 8.2 Hz, 2H), 7.27 (d, J = 14.2 Hz, 5H), 5.55 (s, 2H), 3.78 (s, 2H), 2.99 (s, 4H), 1.74 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.80, 166.26, 164.33, 160.03, 158.66, 145.71, 135.09, 132.82, 129.93, 129.71, 129.48, 128.88, 127.33, 123.87, 41.99, 28.69 ppm. HRMS (ESI): m/z, calcd for $C_{26}H_{25}ClN_9O_2S_2$ $[M + H]^+$, 594.1256; found: 594.1243. HPLC: t_R = 22.605 min, 95.1% purity.

2-(4-(4-Bromophenyl)-1*H*-1,2,3-triazol-1-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide (C72)

Compound **C72** was prepared from **A4** as described for the preparation of **C61** except 1-bromo-4-ethynylbenzene **B12** was used in place of **B1**: white solid (30% yield). m.p. 235-240 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.96 (s, 1H), 12.62 (s, 1H), 8.63 (s, 1H), 7.82 (d, J = 8.0 Hz, 2H), 7.65 (d, J = 8.2 Hz, 2H), 7.38-7.24 (m, 5H), 5.55 (s, 2H), 3.78 (s, 2H), 2.99 (s, 4H), 1.74 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.80, 164.73, 158.47, 145.79, 135.10, 132.38, 130.30, 129.72, 128.89, 127.63, 127.34, 123.94, 121.39, 42.01, 28.86 ppm. HRMS (ESI): m/z, calcd for $C_{26}H_{25}BrN_9O_2S_2$ $[M + H]^+$, 638.0751; found: 638.0739. HPLC: t_R = 22.038 min, 98.5% purity.

2-(4-(4-Methoxyphenyl)-1*H*-1,2,3-triazol-1-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide (C73)

Compound **C73** was prepared from **A4** as described for the preparation of **C61** except 1-ethynyl-4-methoxybenzene **B13** was used in place of **B1**: white solid (42% yield). m.p. 215-216 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 13.02 (s, 1H), 12.66 (s, 1H), 8.48 (s, 1H), 7.79 (d, J = 8.1 Hz, 2H), 7.29 (dd, J = 14.4, 3.7 Hz, 5H), 7.03 (d, J = 8.3 Hz, 2H), 5.54 (s, 2H), 3.79 (s, 5H), 3.00 (s, 4H), 1.75 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.85, 165.67, 159.52, 146.80, 135.08, 129.71, 128.90, 127.36, 127.02, 123.58, 122.56, 114.82, 55.61, 52.10, 42.02, 28.89, 28.68 ppm. HRMS (ESI): m/z, calcd for $C_{27}H_{28}N_9O_3S_2$ $[M + H]^+$, 590.1751; found: 590.1754. HPLC: t_R = 23.280 min, 99.5% purity.

2-(4-(3-Methoxyphenyl)-1*H*-1,2,3-triazol-1-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide (C74)

Compound **C74** was prepared from **A4** as described for the preparation of **C61** except 1-ethynyl-3-methoxybenzene **B14** was used in place of **B1**: white solid (33% yield). m.p. 227-229 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 13.00 (s, 1H), 12.65 (s, 1H), 8.62 (s, 1H), 7.47-7.36 (m, 3H), 7.29 (d, J = 14.5 Hz, 5H), 6.92 (d, J = 7.2 Hz, 1H), 5.55 (s, 2H), 3.80 (d, J = 8.9 Hz, 5H), 3.00 (s, 4H), 1.75 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.29, 159.67, 146.22, 134.59, 131.87, 130.06, 129.20, 128.37, 126.83, 123.25, 117.48, 113.68, 110.32, 55.09, 51.75, 41.50, 28.33, 28.18 ppm. HRMS (ESI): m/z, calcd for $C_{27}H_{28}N_9O_3S_2$ $[M + H]^+$, 590.1751; found: 590.1741. HPLC: t_R = 22.789 min, 95.3% purity.

2-(4-(3-Hydroxyphenyl)-1*H*-1,2,3-triazol-1-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide (C75)

Compound **C75** was prepared from **A4** as described for the preparation of **C61** except 3-ethynylphenol **B15** was used in place of **B1**: white solid (38% yield). m.p. 235-239 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 13.02-12.90 (m, 1H), 12.63 (s, 1H), 9.55 (s, 1H), 8.50 (s, 1H), 7.27 (d, J = 16.6 Hz, 8H), 6.73 (s, 1H), 5.52 (s, 2H), 3.78 (s, 2H), 2.99 (s, 4H), 1.74 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 221.75, 216.77, 204.35, 174.90, 169.82, 166.04, 164.93, 158.23, 146.91, 135.10, 132.21, 130.46, 129.71, 128.89, 127.35, 118.92, 116.53, 115.46, 112.34, 42.01, 31.60, 30.29, 29.45, 28.83, 22.53 ppm. HRMS (ESI): m/z, calcd for $C_{26}H_{26}N_9O_3S_2$ $[M + H]^+$, 576.1595; found: 576.1576. HPLC: t_R = 22.896 min, 97.4% purity.

2-(4-(3-Aminophenyl)-1*H*-1,2,3-triazol-1-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide (C76)

Compound **C76** was prepared from **A4** as described for the preparation of **C61** except 3-ethynylaniline **B16** was used in place of **B1**: white solid (42% yield). m.p. 232-235 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.65 (s, 2H), 8.42 (s, 1H), 7.31 (s, 9H), 5.51 (s, 2H), 4.15 (s, 1H), 3.77 (s, 3H), 2.99 (s, 4H), 1.73 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.81, 165.71, 158.78, 147.51, 135.10, 129.72, 128.89, 127.35, 123.06, 52.31, 42.02, 29.48, 29.16, 28.84, 28.70 ppm. HRMS (ESI): m/z, calcd for $C_{26}H_{27}N_{10}O_2S_2$ $[M + H]^+$, 575.1754; found: 575.1741. HPLC: t_R = 23.246 min, 98.5% purity.

2-(4-(4-Aminophenyl)-1*H*-1,2,3-triazol-1-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide (C77)

Compound **C77** was prepared from **A4** as described for the preparation of **C61** except

4-ethynylaniline **B17** was used in place of **B1**: white solid (40% yield). m.p. 201-204 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.66 (s, 1H), 8.28 (s, 1H), 7.52 (d, J = 6.5 Hz, 2H), 7.31 (s, 5H), 7.27 (d, J = 4.0 Hz, 2H), 6.61 (s, 2H), 5.49 (s, 2H), 3.79 (s, 2H), 3.00 (s, 4H), 1.74 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.81, 165.71, 158.48, 147.55, 135.11, 129.72, 128.89, 127.35, 126.56, 126.25, 121.30, 114.62, 52.01, 42.02, 28.85, 28.70 ppm. HRMS (ESI): m/z, calcd for $C_{26}H_{27}N_{10}O_2S_2$ $[M + H]^+$, 575.1754; found: 575.1759. HPLC: t_R = 23.684 min, 98.2% purity.

2-Phenyl-*N*-(5-(4-(5-(2-(4-(pyridin-2-yl)-1*H*-1,2,3-triazol-1-yl)acetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide (C78)

Compound **C78** was prepared from **A4** as described for the preparation of **C61** except 2-ethynylpyridine **B18** was used in place of **B1**: white solid (38% yield). m.p. 223-227 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.62 (s, 2H), 8.67 (s, 2H), 7.95 (s, 2H), 7.29 (s, 5H), 7.25 (s, 1H), 5.59 (s, 2H), 3.77 (s, 2H), 2.98 (s, 4H), 1.73 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.80, 164.24, 158.65, 135.09, 129.71, 128.88, 129.34, 41.99, 28.82, 28.69 ppm. HRMS (ESI): m/z, calcd for $C_{25}H_{25}N_{10}O_2S_2$ $[M + H]^+$, 561.1598; found: 561.1597. HPLC: t_R = 22.756 min, 96.6% purity.

3-(1-Benzyl-1*H*-1,2,3-triazol-4-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)propanamide (C81)

To a solution of compound **D1** (0.10 g, 0.20 mmol) in DMF was added CuI (7.78 mg, 0.041 mmol) in water and (azidomethyl)benzene **E1** (63.30 mg, 0.47 mmol). The reaction mixture was stirred in microwave 300 W at 100 ℃ for 0.5 h. The reaction mixture was then diluted in water. Then the

precipitate was filtered off to obtain white solid. The residue was purified by column chromatography (DCM/MeOH = 20:1) to afford the titled compound **C81** as a white solid, yield 38%. m.p. 213-216 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.90 (s, 1H), 7.30 (t, J = 11.1 Hz, 10H), 5.54 (s, 2H), 3.77 (s, 2H), 3.04-2.92 (m, 6H), 2.82 (d, J = 6.9 Hz, 2H), 1.75 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 171.02, 170.17, 163.92, 159.34, 158.83, 146.19, 136.69, 135.41, 129.72, 129.12, 128.84, 128.44, 128.17, 127.24, 122.71, 53.07, 42.31, 34.86, 28.92, 28.75, 20.99 ppm. HRMS (ESI): m/z, calcd for $C_{28}H_{30}N_9O_2S_2$ $[M + H]^+$, 588.1958; found: 588.1960. HPLC: t_R = 23.538 min, 95.3% purity.

3-(1-(4-Cyanobenzyl)-1*H*-1,2,3-triazol-4-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)propanamide (C82)

Compound **C82** was prepared from compound **D1** as described for the preparation of **C81** except 4-(azidomethyl)benzonitrile **E2** was used in place of **E1**: white solid (43% yield). m.p. 220-224 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.67 (s, 1H), 12.44 (s, 1H), 7.95 (s, 1H), 7.80 (s, 2H), 7.32 (s, 7H), 5.67 (s, 2H), 3.80 (s, 2H), 3.00 (s, 6H), 2.84 (s, 2H), 1.75 (s, 4H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 170.86, 169.84, 164.23, 164.07, 158.57, 146.33, 142.21, 135.16, 133.12, 129.73, 128.93, 128.89, 127.92, 127.34, 123.13, 119.00, 96.23, 111.26, 52.47, 42.04, 34.76, 29.49, 29.17, 28.89, 28.75, 22.56, 20.97, 14.41 ppm. HRMS (ESI): m/z, calcd for $C_{29}H_{29}N_{10}O_2S_2$ $[M + H]^+$, 613.1911; found: 613.1903. HPLC: t_R = 23.022 min, 97.4% purity.

3-(1-(4-Nitrobenzyl)-1*H*-1,2,3-triazol-4-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)propanamide (C83)

Compound **C83** was prepared from **D1** as described for the preparation of **C81** except 1-(azidomethyl)-4-nitrobenzene **E3** was used in place of **E1**: white solid (40% yield). m.p. 218-220 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.65 (s, 1H), 12.45 (s, 1H), 8.19 (d, J = 8.6 Hz, 2H), 7.97 (s, 1H), 7.45 (d, J = 8.6 Hz, 2H), 7.31 (t, J = 6.6 Hz, 5H), 5.73 (s, 2H), 3.80 (s, 2H), 3.03-2.95 (m, 6H), 2.84 (t, J = 6.9 Hz, 2H), 1.75 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 170.85, 169.80, 164.26, 164.08, 158.67, 158.53, 147.60, 146.34, 144.17, 135.13, 129.73, 129.25, 128.89, 127.34, 124.30, 123.19, 52.98, 52.21, 42.03, 34.73, 32.00, 31.61, 29.89, 29.46, 28.88, 28.72, 22.57, 20.97 ppm. HRMS (ESI): m/z, calcd for $C_{28}H_{29}N_{10}O_4S_2$ $[M + H]^+$, 633.1809; found: 633.1808. HPLC: t_R = 23.403 min, 95.1% purity.

3-(1-(4-Fluorobenzyl)-1*H*-1,2,3-triazol-4-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)propanamide (C84)

Compound **C84** was prepared from **D1** as described for the preparation of **C81** except 1-(azidomethyl)-4-fluorobenzene **E4** was used in place of **E1**: white solid (34% yield). m.p. 203-207 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.65 (s, 1H), 12.40 (s, 1H), 7.87 (s, 1H), 7.30 (s, 5H), 7.14 (t, J = 7.8 Hz, 3H), 5.51 (s, 2H), 3.78 (s, 2H), 2.98 (s, 6H), 2.80 (s, 2H), 1.73 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.30, 163.37, 160.13, 134.61, 132.42, 130.07, 129.96, 129.21, 128.38, 126.83, 122.23, 119.86, 118.62, 115.60, 115.31, 63.99, 58.41, 56.52, 51.81, 41.53, 38.64, 34.22, 28.37, 28.21, 20.44 ppm. HRMS (ESI): m/z, calcd for $C_{28}H_{29}FN_9O_2S_2$ $[M + H]^+$, 606.1864; found: 606.1878. HPLC: t_R = 22.991 min, 95.1% purity.

3-(1-(4-Chlorobenzyl)-1*H*-1,2,3-triazol-4-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-

2-yl)butyl)-1,3,4-thiadiazol-2-yl)propanamide (C85)

Compound **C85** was prepared from **D1** as described for the preparation of **C81** except 1-(azidomethyl)-4-chlorobenzene **E5** was used in place of **E1**: white solid (39% yield). m.p. 203-204 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.70 (s, 1H), 12.45 (s, 1H), 7.94 (s, 1H), 7.39 (dd, J = 18.3, 6.0 Hz, 5H), 7.29 (d, J = 7.9 Hz, 4H), 5.58 (s, 2H), 3.84 (s, 2H), 3.02 (d, J = 13.0 Hz, 6H), 2.87 (d, J = 6.5 Hz, 2H), 1.79 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 215.01, 170.35, 169.29, 159.33, 151.51, 135.17, 134.69, 134.61, 132.68, 129.61, 129.20, 128.62, 128.38, 126.83, 122.37, 51.80, 41.52, 38.66, 34.24, 28.37, 28.20, 20.45, 10.18 ppm. HRMS (ESI): m/z, calcd for $C_{28}H_{29}ClN_9O_2S_2$ $[M + H]^+$, 622.1569; found: 622.1528. HPLC: t_R = 23.672 min, 95.5% purity.

3-(1-(4-Bromobenzyl)-1*H*-1,2,3-triazol-4-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)propanamide (C86)

Compound **C86** was prepared from **D1** as described for the preparation of **C81** except 1-(azidomethyl)-4-bromobenzene **E6** was used in place of **E1**: white solid (36% yield). m.p. 217-219 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.69 (s, 1H), 12.44 (s, 1H), 7.93 (s, 1H), 7.56 (d, J = 8.2 Hz, 2H), 7.42-7.29 (m, 5H), 7.23 (d, J = 8.2 Hz, 2H), 5.57 (s, 2H), 3.84 (s, 2H), 3.02 (d, J = 14.2 Hz, 6H), 2.86 (s, 2H), 1.80 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 206.66, 170.86, 169.81, 164.30, 136.10, 135.11, 132.06, 130.42, 129.73, 128.91, 127.36, 122.95, 121.73, 102.34, 52.36, 42.02, 34.72, 29.49, 28.88, 28.72, 20.96 ppm. HRMS (ESI): m/z, calcd for $C_{28}H_{29}BrN_9O_2S_2$ $[M + H]^+$, 666.1064; found: 666.1059. HPLC: t_R = 23.796 min, 95.5% purity.

3-(1-(4-Methylbenzyl)-1*H*-1,2,3-triazol-4-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-

2-yl)butyl)-1,3,4-thiadiazol-2-yl)propanamide (C87)

Compound **C87** was prepared from **D1** as described for the preparation of **C81** except 1-(azidomethyl)-4-methylbenzene **E7** was used in place of **E1**: white solid (44% yield). m.p. 216-217 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.67 (s, 1H), 12.41 (s, 1H), 7.85 (s, 1H), 7.30 (dd, J = 14.6, 3.9 Hz, 5H), 7.13 (s, 4H), 5.47 (s, 2H), 3.80 (s, 2H), 3.06-2.91 (m, 6H), 2.83 (d, J = 6.2 Hz, 2H), 2.25 (s, 3H), 1.75 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 170.87, 169.80, 164.20, 162.78, 158.83, 137.79, 135.12, 133.66, 129.73, 129.66, 128.90, 128.26, 127.35, 122.61, 52.92, 42.03, 36.25, 34.74, 31.76, 31.24, 29.50, 28.88, 28.74, 25.59, 24.48, 22.57, 21.14, 20.96 ppm. HRMS (ESI): m/z, calcd for $C_{29}H_{32}N_9O_2S_2$ $[M + H]^+$, 602.2115; found: 602.2143. HPLC: t_R = 22.74 min, 99.1% purity.

3-(1-(4-Methoxybenzyl)-1*H*-1,2,3-triazol-4-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)propanamide (C88)

Compound **C88** was prepared from **D1** as described for the preparation of **C81** except 1-(azidomethyl)-4-methoxybenzene **E8** was used in place of **E1**: white solid (46% yield). m.p. 237-240 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.67 (s, 1H), 12.42 (s, 1H), 7.84 (s, 1H), 7.32 (s, 5H), 7.22 (d, J = 8.3 Hz, 3H), 6.88 (d, J = 8.2 Hz, 2H), 5.45 (s, 2H), 3.80 (s, 2H), 3.72 (s, 3H), 2.97 (d, J = 18.2 Hz, 6H), 2.82 (s, 2H), 1.75 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 203.20, 170.88, 169.80, 164.27, 159.50, 143.09, 135.12, 129.86, 129.73, 128.90, 128.57, 127.36, 122.41, 114.51, 55.58, 52.66, 42.02, 35.75, 34.74, 28.87, 28.74, 26.25, 20.95 ppm. HRMS (ESI): m/z, calcd for $C_{29}H_{32}N_9O_3S_2$ $[M + H]^+$, 618.2064; found: 618.2070. HPLC: t_R = 22.325 min, 96.1% purity.

3-(1-(3,4-Dimethoxybenzyl)-1*H*-1,2,3-triazol-4-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)propanamide (C89)

Compound **C89** was prepared from **D1** as described for the preparation of **C81** except 4-(azidomethyl)-1,2-dimethoxybenzene **E9** was used in place of **E1**: white solid (48% yield). m.p. 232-236 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.66 (s, 1H), 12.41 (s, 1H), 7.84 (s, 1H), 7.30 (t, J = 7.8 Hz, 5H), 6.95 (s, 1H), 6.88 (d, J = 8.1 Hz, 1H), 6.79 (d, J = 8.3 Hz, 1H), 5.43 (s, 2H), 3.80 (s, 2H), 3.74-3.67 (m, 6H), 3.06-2.90 (m, 6H), 2.83 (d, J = 6.8 Hz, 2H), 1.75 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 170.88, 169.80, 164.29, 164.09, 149.19, 158.68, 158.52, 149.18, 149.10, 146.09, 143.52, 135.13, 129.73, 128.90, 128.81, 127.36, 122.38, 120.89, 112.34, 112.25, 55.97, 55.92, 53.03, 44.75, 42.02, 34.74, 28.87, 28.74, 20.93 ppm. HRMS (ESI): m/z, calcd for $C_{30}H_{34}N_9O_4S_2$ $[M + H]^+$, 648.2170; found: 648.2167. HPLC: t_R = 21.819 min, 96.1% purity.

3-(1-(2,4-Dimethylbenzyl)-1*H*-1,2,3-triazol-4-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)propanamide (C90)

Compound **C90** was prepared from **D1** as described for the preparation of **C81** except 1-(azidomethyl)-2,4-dimethylbenzene **E10** was used in place of (azidomethyl)benzene **E1**: white solid (30% yield). m.p. 228-231 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.67 (s, 1H), 12.41 (s, 1H), 7.72 (s, 1H), 7.32 (s, 5H), 6.96 (d, J = 17.0 Hz, 3H), 5.48 (s, 2H), 3.80 (s, 2H), 3.01 (s, 6H), 2.81 (s, 2H), 2.21 (d, J = 8.2 Hz, 6H), 1.75 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 207.48, 171.13, 170.87, 169.82, 158.85, 137.95, 136.53, 135.10, 131.64, 131.50, 129.72, 129.20, 128.90, 127.36, 127.14, 72.01, 51.14, 42.02, 37.67, 34.74, 30.01, 29.32, 28.87, 28.73, 21.04, 20.98, 18.95

ppm. HRMS (ESI): m/z, calcd for $C_{30}H_{34}N_9O_2S_2$ $[M + H]^+$, 616.2271; found: 616.2278. HPLC: t_R = 22.681 min, 96.5% purity.

3-(1-(2,6-Dimethylbenzyl)-1*H*-1,2,3-triazol-4-yl)-*N*-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)propanamide (C91)

Compound **C91** was prepared from **D1** as described for the preparation of **C81** except 2-(azidomethyl)-1,3-dimethylbenzene **E11** was used in place of **E1**: white solid (38% yield). m.p. 205-210 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.66 (s, 1H), 12.37 (s, 1H), 7.57 (s, 1H), 7.30 (s, 5H), 7.15-7.08 (m, 1H), 7.02 (d, J = 7.2 Hz, 2H), 5.50 (s, 2H), 3.78 (s, 2H), 2.95 (d, J = 22.9 Hz, 6H), 2.77 (s, 2H), 2.26 (s, 6H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 196.72, 170.88, 169.80, 138.21, 135.12, 132.22, 129.73, 128.90, 128.84, 127.35, 125.40, 122.23, 47.97, 42.02, 40.62, 34.77, 28.87, 28.75, 20.95, 19.87 ppm. HRMS (ESI): m/z, calcd for $C_{30}H_{34}N_9O_2S_2$ $[M + H]^+$, 616.2271; found: 616.2288. HPLC: t_R = 23.199 min, 96.2% purity.

3-(1-(4-Methylbenzyl)-1*H*-1,2,3-triazol-4-yl)-*N*-(6-(4-(5-(2-(pyridin-2-yl)acetamido)-1,3,4-thiadiazol-2-yl)butyl)pyridazin-3-yl)propanamide (C147)

To a solution of compound **D4** (0.12 g, 0.20 mmol) in DMF was added CuI (7.78 mg, 0.041 mmol) in water and 1-(azidomethyl)-4-methylbenzene **E7** (69.10 mg, 0.47 mmol). The reaction mixture was stirred in microwave 300 W at 100 ℃ for 0.5 h. The reaction mixture was then diluted in water. Then the precipitate was filtered off to obtain white solid. The crude product was purified by column chromatography (DCM/MeOH = 20:1) to afford the titled compound **C147** as a white solid, yield 36%. m.p. 160-164 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.69 (s, 1H), 11.04 (s, 1H),

8.50 (s, 1H), 8.22 (d, 1H), 7.86 (s, 1H), 7.78 (s, 1H), 7.57 (s, 1H), 7.41 (d, 1H), 7.29 (s, 1H), 7.14 (d, 4H), 5.48 (s, 2H), 4.01 (s, 2H), 3.02-2.74 (m, 8H), 2.26 (s, 3H), 1.75 (s, 2H), 1.24 (s, 2H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 172.32, 137.78, 133.63, 129.62, 128.26, 122.79, 119.09, 52.90, 34.80, 30.31, 28.92, 21.15, 20.96 ppm. HRMS (ESI): m/z, calcd for $C_{30}H_{33}N_{10}O_2S$ $[M + H]^+$, 597.2503; found: 597.2487. HPLC: t_R = 21.812 min, 95.7% purity.

3-(1-(4-Methoxybenzyl)-1*H*-1,2,3-triazol-4-yl)-*N*-(6-(4-(5-(2-(pyridin-2-yl)acetamido)-1,3,4-thiadiazol-2-yl)butyl)pyridazin-3-yl)propanamide (C148)

Compound **C148** was prepared from **D4** as described for the preparation of **C147** except 1-(azidomethyl)-4-methoxybenzene **E8** was used in place of **E7**: white solid (yield 34%). m.p. 115-120 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.68 (s, 1H), 11.03 (s, 1H), 8.50 (s, 1H), 8.21 (d, 1H), 7.96-7.77 (m, 3H), 7.56 (d, 1H), 7.41 (s, 1H), 7.24 (d, 2H), 6.88 (d, 2H), 5.45 (s, 2H), 4.01 (s, 2H), 3.72 (s, 3H), 3.01-2.75 (m, 8H), 1.74 (s, 2H), 1.23 (s, 2H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 172.30, 162.78, 159.49, 157.25, 154.45, 129.88, 128.58, 122.38, 118.99, 116.16, 114.50, 55.57, 52.66, 36.24, 35.86, 34.72, 31.23, 29.45, 29.01, 28.76, 21.15, 20.93 ppm. HRMS (ESI): m/z, calcd for $C_{30}H_{33}N_{10}O_3S$ $[M + H]^+$, 613.2452; found: 613.2439. HPLC: t_R = 21.543 min, 97.2% purity.

3-(1-(3,4-Dimethoxybenzyl)-1*H*-1,2,3-triazol-4-yl)-*N*-(6-(4-(5-(2-(pyridin-2-yl)acetamido)-1,3,4-thiadiazol-2-yl)butyl)pyridazin-3-yl)propanamide (C149)

Compound **C149** was prepared from **D4** as described for the preparation of **C147** except 4-(azidomethyl)-1,2-dimethoxybenzene **E9** was used in place of **E7**: white solid (yield 32%). m.p.

110-115 °C. ^{1}H NMR (300 MHz, DMSO-d_6 + D_2O): δ 8.51 (s, 1H), 8.19 (t, 1H), 7.95-7.77 (m, 2H), 7.56 (d, 1H), 7.43 (d, 1H), 7.30 (s, 1H), 6.98-6.81 (m, 3H), 5.43 (s, 2H), 3.70 (s, 6H), 3.00-2.73 (m, 8H), 1.73 (s, 2H), 1.23 (s, 2H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 172.31, 149.16, 149.07, 146.66, 141.17, 128.84, 128.55, 122.36, 120.90, 112.32, 112.22, 111.67, 108.72, 73.54, 55.94, 55.88, 52.98, 35.85, 29.04, 21.11 ppm. HRMS (ESI): m/z, calcd for $C_{31}H_{35}N_{10}O_4S$ $[M + H]^+$, 643.2558; found: 643.2574. HPLC: t_R = 21.521 min, 95.5% purity.

3-(1-(2,4-Dimethylbenzyl)-1*H*-1,2,3-triazol-4-yl)-*N*-(6-(4-(5-(2-(pyridin-2-yl)acetamido)-1,3,4-thiadiazol-2-yl)butyl)pyridazin-3-yl)propanamide (C150)

Compound **C150** was prepared from **D4** as described for the preparation of **C147** except 1-(azidomethyl)-2,4-dimethylbenzene **E10** was used in place of **E7**: white solid (yield 33%). m.p. 110-111 °C. ^{1}H NMR (300 MHz, DMSO-d_6 + D_2O): δ 8.49 (s, 1H), 8.16 (s, 1H), 7.71 (s, 2H), 7.56 (d, 1H), 7.40 (s, 1H), 7.29 (s, 1H), 7.02-6.91 (m, 3H), 5.47 (s, 2H), 3.00-2.89 (m, 6H), 2.79 (s, 2H), 2.20 (t, 6H), 1.72 (s, 2H), 1.22 (s, 2H) ppm. HRMS (ESI): m/z, calcd for $C_{31}H_{35}N_{10}O_2S$ $[M + H]^+$, 611.2660; found: 611.2672. HPLC: t_R = 21.752 min, 96.4% purity.

3-(1-(2,6-Dimethylbenzyl)-1*H*-1,2,3-triazol-4-yl)-*N*-(6-(4-(5-(2-(pyridin-2-yl)acetamido)-1,3,4-thiadiazol-2-yl)butyl)pyridazin-3-yl)propanamide (C151)

Compound **C151** was prepared from **D4** as described for the preparation of **C147** except 2-(azidomethyl)-1,3-dimethylbenzene **E11** was used in place of **E7**: white solid (yield 38%). m.p. 210-215 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.99 (s, 2H), 7.71 (s, 2H), 7.64 (s, 2H), 7.43 (d, 2H), 7.14 (s, 2H), 5.53 (s, 2H), 4.00 (s, 1H), 3.02 (s, 1H), 2.89 (s, 3H), 2.77-2.73 (m, 3H), 2.33 (s,

6H), 1.74 (s, 2H), 1.23 (s, 2H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 172.31, 170.89, 159.95, 154.47, 145.94, 138.19, 132.24, 128.83, 122.15, 118.88, 111.67, 108.65, 88.14, 73.33, 47.94, 35.96, 34.72, 29.03, 19.87 ppm. HRMS (ESI): m/z, calcd for $C_{31}H_{35}N_{10}O_2S$ $[M + H]^+$, 611.2660; found: 611.2655. HPLC: t_R = 21.464 min, 95.1% purity.

_N_-(6-(4-(5-(2-(Pyridin-2-yl)acetamido)-1,3,4-thiadiazol-2-yl)butyl)pyridazin-3-yl)-3-(1-(4-(trifluoromethoxy)benzyl)-1_H_-1,2,3-triazol-4-yl)propanamide (C152)

Compound **C152** was prepared from **D4** as described for the preparation of **C147** except 1-(azidomethyl)-4-(trifluoromethoxy)benzene **E12** was used in place of **E7**: white solid (yield 37%). m.p. 210-215 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.66 (s, 1H), 11.02 (s, 1H), 8.50 (s, 1H), 8.22 (d, 1H), 7.93 (s, 1H), 7.77 (t, 1H), 7.57 (d, 1H), 7.37-7.31 (m, 6H), 5.60 (s, 2H), 4.01 (s, 2H), 3.02 (s, 4H), 2.93 (t, 2H), 2.82 (d, 2H), 1.75 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 172.28, 168.91, 164.44, 158.55, 155.23, 154.47, 149.40, 148.38, 146.59, 137.41, 136.18, 130.21, 128.56, 122.82, 121.75, 119.01, 111.65, 85.28, 73.44, 52.17, 44.42, 35.81, 34.69, 31.15, 29.00, 28.71, 21.13 ppm. HRMS (ESI): m/z, calcd for $C_{30}H_{30}F_3N_{10}O_3S$ $[M + H]^+$, 667.2170; found: 667.2208. HPLC: t_R = 21.957 min, 95.2% purity.

_N_-(6-(4-(5-(2-(pyridin-2-yl)acetamido)-1,3,4-thiadiazol-2-yl)butyl)pyridazin-3-yl)-3-(1-(4-(trifluoromethyl)benzyl)-1_H_-1,2,3-triazol-4-yl)propanamide (C153)

Compound **C153** was prepared from **D4** as described for the preparation of **C147** except 1-(azidomethyl)-4-(trifluoromethyl)benzene **E13** was used in place of **E7**: white solid (yield 45%). m.p. 210-215 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.67 (s, 1H), 11.03 (s, 1H), 8.50 (s, 1H),

8.20 (s, 1H), 7.96 (s, 1H), 7.68 (s, 3H), 7.42 (s, 4H), 7.28 (d, 1H), 5.68 (s, 2H), 4.02 (s, 2H), 2.97 (s, 4H), 2.88 (d, 4H), 1.74 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ 172.29, 168.90, 159.97, 154.46, 146.65, 141.35, 129.19, 128.85, 128.77, 128.57, 126.31, 126.06, 126.01, 123.06, 122.70, 119.09, 85.05, 73.32, 52.44, 35.81, 34.69, 31.16, 28.99, 28.70, 21.14 ppm. HRMS (ESI): m/z, calcd for $C_{30}H_{30}F_3N_{10}O_2S$ $[M + H]^+$, 651.2221; found: 651.2188. HPLC: t_R = 21.801 min, 95.1% purity.

_N_-(5-(4-(5-(2-phenylacetamido)-1,3,4-thiadiazol-2-yl)butyl)-1,3,4-thiadiazol-2-yl)pent-4-ynamide (D1)

To a solution of compound **18** (3.00 g, 8.02 mmol) in DMF was added 4-pentynoicacid (0.78 g, 8.02 mmol), HATU (4.56 g, 12.03 mmol) and DIPEA (4.20 g, 32.08 mmol). The reaction mixture was stirred at room temperature for 12 h. The reaction mixture was then diluted in water. The precipitate was filtered off to obtain white solid, yield 94.5%. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.67 (s, 1H), 12.47 (s, 1H), 7.29 (m, 5H), 3.80 (s, 2H), 3.02 (s, 4H), 2.83 (s, 1H), 2.67 (t, 2H), 1.76 (s, 4H), 1.24 (s, 2H) ppm. HRMS (ESI): m/z, calcd for $C_{21}H_{23}N_6O_2S_2$ $[M + H]^+$, 455.1318; found: 455.1310.

_N_-(6-(4-(5-(2-(pyridin-2-yl)acetamido)-1,3,4-thiadiazol-2-yl)butyl)pyridazin-3-yl)pentyn-4-amide (D4)

To a solution of compound **7** (2.96 g, 8.02 mmol) in DMF was added 4-pentynoicacid (0.78 g, 8.02 mmol), HATU (4.56 g, 12.03 mmol) and DIPEA (4.20 g, 32.08 mmol). The reaction mixture was stirred at room temperature for 12 h. The reaction mixture was then diluted in water. The

precipitate was filtered off to obtain white solid, yield 92%. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.65 (s, 1H), 11.05 (s, 1H), 8.47 (s, 1H), 8.22 (d, 1H), 7.75 (s, 1H), 7.56 (d, 1H), 7.38 (d, 2H), 3.98 (s, 2H), 2.99 (s, 3H), 2.87 (s, 3H), 2.77 (t, 1H), 2.63 (s, 2H), 1.72 (s, 4H) ppm. HRMS (ESI): m/z, calcd for $C_{22}H_{24}N_7O_2S$ $[M + H]^+$, 450.1707; found: 450.1721.

Construction of a 200-Member Triazole Library (C1-C200).

To a solution of an alkyne (0.20 mM, 20 μL) and an azide (0.20 mM, 20 μL) in DMF was added an aqueous solution of CuI (0.04 mM, 25 μL). The reaction mixture was stirred in microwave 300 W at 100 ℃ for 0.5 h. The reaction was monitored by TLC and LCMS. When the reaction was completed, the reaction solution was transferred to a 96-well plate. Then DMSO (150 μL) was added to the mixture, in which the concentration of the triazole is assumed to be 0.20 mM. The crude triazoles were diluted to a desired concentration for FP or enzyme assays.

Bioassays

Fluorescence Spectroscopy.

The probes were dissolved in DMSO completely to get 10 mM stock solution. The stock solution was diluted by different solvent to acquired 2.5 μM, 5 μM and 10 μM solutions. The fluorescent property of the probes were obtained on the SpectraMax® Paradigm® Muti-Mode detection platform (Molecular Devices).

In addition, the quantum yield of the probe was calculated under PBS solution (pH = 7.4) by comparison with fluorescein in 0.1 M NaOH (Φ_{ST} = 0.92) which was considered as the reference using the following equation:

$$\Phi_X = \Phi_{ST} (A_{ST}/A_X) (F_X/F_{ST}) (\eta_X/\eta_{ST})^2$$

Where the subscripts ST and X denote standard and test respectively, Φ is the quantum yield, F is the integrated area under the fluorescence spectra, A is the absorbance, η is the refractive index of the solvent.

Cell Viability Assay.

The cytotoxicity effect of compounds were determined by the 3-[4,5-dimethylthiazol]-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay, using HCT116, A549, MCF-7, HepG-2 and MDA-MB-436 cells. Briefly, cells were seeded into 96-well plates to a density of 4 x 10^3/100 μL/well. After 24 h of growth to allow attachment to the wells, compound was added at various concentrations (from 0.01 to 100 μM). After 72 h, 20 μL of MTT (5 mg/mL) was added to each well, and cells were incubated in the dark for 4 h (37 ℃, 5% CO_2). When dark crystals appeared at the bottom of the well, the solution was then gently aspirated from each well, and the formazan crystals were dissolved with 100 μL of DMSO, yielding a purple solution. Optical densities were read at 490 nm using a Multiskan Sky (Thermo Scientific).

Cloning, Expression, and Purification of Glutaminases.

The human GAC was prepared as described.[36] The human GAC gene was amplified from a cDNA library derived from MCF7 cells. Briefly, total RNAs were isolated from MCF7 cells using TRIzol reagent (Invitrogen, Inc.) and converted into cDNAs using the SuperScript III transcriptor first-strand cDNA synthesis system for RT-PCR (Invitrogen, Inc.) according to the manufacturer. A cDNA fragment of 1428 bp, which encodes a human GAC polypeptide of residues Leu123 to

Ser598, was obtained by PCR against the obtained MCF7 cDNAs library with primers of 5'-ATACGCGGATCCCTGGTGGCCTCAGGTGAAAA-3' (containing a BamHI restriction site) and 5'-GCAAGGAAAAAAGCGGCCGCAGCGTTAGCTTTTCTCTCCCAGACTTTCCA-3' (containing a Notl restriction site). This PCR amplified cDNA fragment and the protein expression vector pET28a(+) (Novagen) were separately digested with BamHI and NotI. The desired DNA fragments were cleaned and purified prior to being ligated into a His-tagged GAC protein expression plasmid. The resultant His-tagged GAC-expression plasmid, pET28a(+)-GAC, was transformed into Escherichia coli strain BL21(DE3)pLysS and then cultured in Luria-Bertani medium in the presence of kanamycin. Once the Escherichia coli culture reached an absorbance at 600 nm of 0.6-0.8, isopropyl β-D-1-thiogalactopyranoside (1 mM) was added to induce the His-tagged GAC protein expression for 12 h at 16 ℃. The resultant bacteria were harvested by centrifugation at 10,000 rpm for 15 min at 4 ^{o}C and then resuspended in a lysis buffer (50 mM $NaH_2PO4·H_2O$, 300 mM NaCl, 5 mM Tris-HCl, pH 8.5, 5% glycerol, 0.1% Triton X-100, 10 mM β-ME, and 50 mM Imidazole) prior to breaking bacteria by sonication. The supernatants from sonication treatment were collected for further protein purification at cold room with a Ni^{2+} charged resin (GE Healthcare) column, and the His-tagged GAC bound resin column was washed with the lysis buffer containing 50 mM and 75 mM imidazole. His-tagged GAC was eluted with the lysis buffer containing 500 mM imidazole, and the buffer was then exchanged to 50 mM $NaH_2PO_4·H_2O$, 50 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, and 50% glycerol by using PD-10 desalting column (GE Healthcare).

GLS1 enzyme-activity inhibition assay.

Human glutaminase activity was measured using a two-step assay as described previously.[15,20] Typically, 10 μL of compound, which was dissolved in DMSO, was added to 80 μl of an initial assay mix (0.1 μM GAC, 0.2 mM EDTA and 50 mM Tris/acetate, pH 8.6). Samples were incubated at 25 °C for 10 min and then 10 μL of 200 mM glutamine were added to the reaction mix. After adding glutamine, the reactions were incubated at 37 ℃ for 60 min and then quenched by the addition of 10 μL of 0.6 M HCl. Subsequently, 100 μL of a second reaction mixture (3.7 units of purifed bovine liver glutamate dehydrogenase, 160 mM Tris/acetate [pH 9.4], 400 mM hydrazine, 5 mM ADP and 2 mM NAD^+) was added to the stopped first reaction mixture and incubated for 30 min at 25 ℃. The absorbance at 340 nm was measured using a Multiskan Sky (Thermo Scientific), fitted with a 340 nm excitation filter. Sample absorbance was measured against a blank, containing no human glutaminase.

Fluorescence polarization binding assay.

The fluorescence polarization assay was performed on a SpectraMax multi-mode microplate reader (Molecular Devices) using the excitation and emission filters. The plates used for the FP measurements were the black nonbinding surface Corning #3676 384-well plates loaded with 40 μL of assay solution per well, consisting 20 μL various concentrations probe **1** and 20 μL Tris buffer to measure the mP value of the probe **1**. As for the determination of the equilibrium dissociation constant (K_d) of probe **1** with GLS1, the plates loaded with 40 μL of assay solution per well, consisting 20 μL Tris buffer, 10 μL probe **1** (25 nM), and 10 μL GLS1 at varying concentrations. For fluorescein, 485 nm excitation and 535 nm emission filters were used. Concentration of GLS1 was plotted versus ΔmP, and the data were fit to the following equation

(1)[38] using KaleidaGraph (v4.1.1, Synergy Software) to determine the K_d of probe **1**:

$$\Delta mP = [P_{min} + P_{max} \times (x/K_d)^n]/[1+(x/K_d)^n]) \qquad (1)^{38}$$

where P_{min} and P_{max} are the minimum and maximum observed ΔmP values, respectively, x is the GLS1 concentration, and n is the Hill coefficient of the binding curve.

Fluorescence polarization competitive binding assay.

The fluorescence polarization assay was performed on a SpectraMax multi-mode microplate reader (Molecular Devices) using the excitation and emission filters. The plates used for the FP measurements were the black nonbinding surface Corning #3676 384-well plates loaded with 40 μL of assay solution per well. Two-fold serial dilutions of various compounds were prepared as 40 × solutions in DMSO. First, 20 μL Tris buffer, 10 μL probe **1** (25 nM), and 10 μL GLS1 (30 nM) were added to the experimental wells. Then, 1 μL of various compound solutions was added and the plate was incubated at room temperature for 30 min. FP signals were recorded three times and averaged.

GLS1 protein thermal shift assay.

The protein thermal shift assay was performed on a QuantStudio™ 6 Flex real-time PCR machine (Applied Biosystems) according to a protocol which gathers fluorescence signals with the slope of 2.5 ℃ min^{-1} from 25 ℃ to 95 ℃. The reaction solution contains 2 μM GLS1 proteins, 5 × SYPRO® Orange (Molecular Probes) and testing compound in 20 μL of thermal shift assay buffer (10 mM Tris pH 8.5 and 150 mM NaCl). The change in the fluorescence intensities of SYPRO® Orange was monitored as a function of the temperature. The melting temperature was

calculated using the software Protein Thermal Shift™ v1.0 (Life Technologies), and curves were plotted in Graphpad Prism 5.0.

Surface plasmon resonance (SPR) based binding assay.

The SPR binding assays were performed on a Biacore T200 instrument (GE Healthcare). GLS1 protein was covalently coupled on CM5 chip according to standard procedure.[35] The whole system was equilibrated overnight first with assay buffer (10 mM Tris pH 8.5, 150 mM NaCl, 0.05% (v/v) surfactant P20, and 0.25% (v/v) dimethyl sulfoxide). Compounds were diluted with assay buffer and then injected at a flow of 30 mL/min for 60 s to contact, followed by disassociation for 400 s. The equilibrium dissociation constants of the compounds were determined by Biacore T200 evaluation software (GE Healthcare).

Determination of Intracellular Glutamate Levels.

HCT116 cells were seeded at 5,000 cells/well in 96-well plates and incubated in a CO_2 incubator at 37 ℃ for 24 h. The cells were treated with either the vehicle control (1% DMSO) or 0.032-20 μM GLS1 inhibitors as indicated for an additional 4 h. After a 4 h treatment, the medium was removed and cells per well were washed twice with 1× PBS, and 50 μL of nonidet-P40 lysis buffer was added per well at 4 °C for 20 min. Aliquots (each 10 μL) of the resultant cell lysates were subjected to Amplex red glutamic acid assay (Invitrogen) followed by manufacturer's recommendations.

ROS Assay.

The fluorescent imaging of ROS production was performed on HCT116 cells. Cells were seeded at 1×10^6 cells/well in a 6-well plate and were incubated at 37 ℃ under a 5% CO_2 atmosphere for 24 h. Cells were then treated with compounds at the indicated time (12 h) and dosages, incubated with 10 μM DCFH-DA (Beyotime), DHE (Beyotime) for 30 min at 37 ℃. Finally, the fluorescence images of differently treated cells were observed by fluorescence microscopy after washed twice with PBS. At an excitation wavelength of 488 nm, DCF was observed emitting the green fluorescence. Also, the production of ROS in cells was quantitatively detected by the flow cytometry. Using the same method as above, cells were cultured with samples and treated with DCFH-DA (10 μM). After digestion, the medium containing cells were centrifuged in the centrifuge tube (1.5 mL) under the conditions of 4 ℃ and 1000 g for 3 min. The centrifuged cells were redispersed in PBS (0.5 mL). Finally, flow cytometric analysis was utilized to detect the fluorescence intensity of DCF.

Physicochemical Properties.

The distribution coefficients (Log D 7.4) and intrinsic aqueous solubility were determined according to modified shake-flask method.[32] 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 ℃. 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

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.xxxxxxx.

Additional figures and schemes; General methods for fluorescence polarization, dimethyl sulfoxide tolerance, assay accuracy and precision; Analytical Data (^{1}H NMR, ^{13}C NMR and HRMS) for representative target compounds; HPLC assessment of purity for target compounds (PDF)

Molecular formula strings (CSV)

AUTHOR INFORMATION

Corresponding Authors

*E-mail: zhiyuli@cpu.edu.cn (Z. Li); bianjl@cpu.edu.cn (J. Bian).

Address: China pharmaceutical University, Nanjing.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We gratefully acknowledge financial support from the National Natural Science Foundation of China (no. 81703347, 21672260, and 21372260), the National Natural Science Foundation of Jiangsu Province of China (no. BK20170743 and BK20171393), and the “Double First-class” University Project (CPU2018GY07), the State Key Laboratory of Drug Research (SIMM1903KF-03) and Postgraduate Research & Practice Innovation Program of Jiangsu Province (KYCX18_0770).

ABBREVIATIONS USED

BPTES, bis-2-(5-phenylacetamido-1,2,4-thiadiazol)ethyl sulfide; BSA, bovine-serum albumin; DCM, dichloromethane; DIPEA, N,N-diisopropylethylamine; DMA, N,N-dimethylacetamide; DMF, dimethyl formamide; DMSO, dimethyl sulfoxide; DMTMM, 4-(4,6-dimethoxy-1,3,5,-triazin-2-yl)-4-methyl morpholinium chloride; DON, 6-Diazo-5-oxo-L-norleucine; FITC, fluorescein isothiocyanate; FP, fluorescence polarization; GAC, glutaminase C; GLS, glutaminase; HATU, 2-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate; HTS, high-throughput screening; KGA, kidney glutaminase; α-KG, α-ketoglutarate; K_d, dissociation constant; LGA, liver glutamiase; IC_{50}, The half maximal inhibitory concentration; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; NBD, 7-nitro-1,2,3-benzoxadiazole; PBS, phosphate buffered saline; PTS, protein thermal shift; Φ, quantum yield; ROS, reactive oxygen species; SAR, Structure-activity relationship; S/N, signal-to-noise ratio; SPR, surface plasmon resonance; SS, Stocks shift; TCA, tricarboxylic acid; TEA, triethylamine; THF, tetrahydrofuran; Z'-factor, screening window coefficient.

REFERENCES

(1) Warburg, O.; On the Origin of Cancer Cells. *Science* **1956**, *123*, 309-314.

(2) Schulze, A.; Harris, A. L. How Cancer Metabolism Is Tuned for Proliferation and Vulnerable to Disruption. *Nature* **2012**, *491*, 364-373.

(3) Brian J. A.; Zachary E. S.; Chi V. D. From Krebs to Clinic: Glutamine Metabolism to Cancer Therapy. *Nat. Rev. Cancer* **2016**, *16*, 619-634.

(4) Xu, X.; Meng, Y.; Li, L.; Xu, P. F.; Wang, J. B.; Li, Z. Y.; Bian. J. L. Overview of the

Development of Glutaminase Inhibitors: Achievements and Future Directions. *J. Med. Chem*. **2019**, *62*, 1096-1115.

(5) Seltzer, M. J.; Bennett, B. D.; Joshi, A. D.; Gao, P.; Thomas, A. G.; Ferraris, D. V.; Tsukamoto, T.; Rojas, C. J.; Slusher, B. S.; Rabinowitz, J. D.; Dang, C. V.; Riggins, G. J. Inhibition of Glutaminase Preferentially Slows Growth of Glioma Cells with Mutant IDH1. *Cancer Res*. **2010**, *70* , 8981-8987.

(6) Gao, P.; Tchernyshyov, I.; Chang, T. C.; Lee, Y. S.; Kita, K.; Ochi, T.; Zeller, K. I.; De Marzo, A. M.; Van Eyk, J. E.; Mendell, J. T.; Dang, C. V. C-Myc Suppression of Mir-23a/B Enhances Mitochondrial Glutaminase Expression and Glutamine Metabolism. *Nature* **2009**, *458*, 762-765.

(7) Wise, D. R.; Thompson, C. B. Glutamine Addiction: A New Therapeutic Target in Cancer. *Trends Biochem. Sci*. **2010**, *35*, 427-433.

(8) Rubin, A. L. Suppression of Transformation by and Growth Adaptation to Low Concentrations of Glutamine in NIH-3T3 Cells. *Cancer Res*. **1990**, *50*, 2832-2839.

(9) Yuneva, M.; Zamboni, N.; Oefner, P.; Sachidanandam, R.; Lazebnik, Y. Deficiency In Glutamine but not Glucose Induces MYC Dependent Apoptosis In Human Cells. *J. Cell Biol*. **2007**, *178*, 93-105.

(10) Cervantes-Madrid, D.; Romero, Y.; Duenas-Gonzalez, A. Reviving Lonidamine and 6-Diazo-5-Oxo-L-Norleucine To Be Used In Combination For Metabolic Cancer Therapy. *BioMed Res. Int*. **2015**, *2015*, 690492.

(11) Ahluwalia, G. S.; Grem, J. L.; Hao, Z.; Cooney, D. A. Metabolism and Action of Amino Acid Analog Anti-Cancer Agents. *Pharmacol. Ther*. **1990**, *46*, 243-271.

(12) Grayzel, A. I.; Seegmiller, J. E.; Love, E. Suppression of Uric Acid Synthesis in the Gouty

Human by the Use of 6-Diazo-5-Oxo-L-Norleucine. *J. Clin. Invest*. **1960**, *39*, 447-454.

(13) Barclay, R. K.; Phillipps, M. A. Effects of 6-Diazo-5-Oxo-L-Norleucine and Other Tumor Inhibitors On the Biosynthesis of Nicotinamide Adenine Dinucleotide in Mice. *Cancer Res*. **1966**, *26*, 282-286.

(14) Hofer, A.; Steverding, D.; Chabes, A.; Brun, R.; Thelander, L. Trypanosoma Brucei CTP Synthetase: A Target for the Treatment of African Sleeping Sickness. *Proc. Natl. Acad. Sci. U S A*. **2001**, *98*, 6412-6416.

(15) Robinson, M. M.; McBryant, S. J.; Tsukamoto, T.; Rojas, C.; Ferraris, D. V.; Hamilton, S. K.; Hansen, J. C.; Curthoys, N. P. Novel Mechanism of Inhibition of Rat Kidney-Type Glutaminase by Bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl Sulfide (BPTES). *Biochem. J*. **2007**, *406*, 407-414.

(16) Shukla, K.; Ferraris, D. V.; Thomas, A. G.; Stathis, M.; Duvall, B.; Delahanty, G.; Alt, J.; Rais, R.; Rojas, C.; Gao, P.; Xiang, Y.; Dang, C. V.; Slusher, B. S.; Tsukamoto, T. Design, Synthesis, and Pharmacological Evaluation of Bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl Sulfide 3 (BPTES) Analogs as Glutaminase Inhibitors. *J. Med. Chem*. **2012**, *55*, 10551-10563.

(17) Zimmermann, S. C.; Wolf, E. F.; Luu, A.; Thomas, A. G.; Stathis, M.; Poore, B.; Nguyen, C.; Le, A.; Rojas, C.; Slusher, B. S.; Tsukamoto, T. Allosteric Glutaminase Inhibitors Based on A 1,4-Di(5-amino-1,3,4-thiadiazol-2-yl)butane Scaffold. A*CS Med. Chem. Lett*. **2016**, *7*, 520-524.

(18) McDermott, L. A.; Iyer, P.; Vernetti, L.; Rimer, S.; Sun, J. R.; Boby, M.; Yang, T. Y.; Fioravanti, M.; O'Neill, J.; Wang, L. W.; Drakes, D.; Katt, W.; Huang, Q. Q.; Cerione, R. Design and Evaluation of Novel Glutaminase Inhibitors. *Bioorg. Med. Chem*. **2016**, *24*, 1819-1839.

(19) Gross, M. I.; Demo, S. D.; Dennison, J. B.; Chen, L.; ChernovRogan, T.; Goyal, B.; Janes, J.

R.; Laidig, G. J.; Lewis, E. R.; Li, J.; Mackinnon, A. L.; Parlati, F.; Rodriguez, M. L.; Shwonek, P. J.; Sjogren, E. B.; Stanton, T. F.; Wang, T.; Yang, J.; Zhao, F.; Bennett, M. K. Antitumor Activity of the Glutaminase Inhibitor CB-839 in Triple Negative Breast Cancer. *Mol. Cancer Ther*. **2014**, *13*, 890-901.

(20) Kenny, J.; Bao, Y.; Hamm, B.; Taylor, L.; Toth, A.; Wagers, B.; Curthoys, N. P. Bacterial Expression, Purification, and Characterization of Rat Kidney-type Mitochondrial Glutaminase. *Protein Expression Purif*. **2003**, *31*, 140-148.

(21) Ramachandran, S.; Pan, C. Q.; Zimmermann, S. C.; Duvall, B.; Tsukamoto, T.; Low, B. C.; Sivaraman, J. Structural Basis For Exploring the Allosteric Inhibition of Human Kidney Type Glutaminase. *Oncotarget* **2016**, *7*, 57943-57954.

(22) Lu, M. C.; Zhou, H. S.; You, Q. D.; Jiang, Z. Y. Design, Synthesis, and Initial Evaluation of Affinity-Based Small Molecule Probes for Fluorescent Visualization and Specific Detection of Keap1. *J. Med. Chem*. **2016**, *59*, 7305-7310.

(23) Anders H. H.; Eugenia S. K.; Pandey, Brian D. H.; Elisabeth C.; Graeme M.; Trond U. Development and Characterization of a Fluorescent Tracer for the Free Fatty Acid Receptor 2 (FFA2/GPR43). *J. Med. Chem*. **2017**, *60*, 5638-5645.

(24) Newcomb, R. W.; Newocmb, M. Selective Inhibition of Glutaminase by Bisthiadiazoles. US6451828B1, Sep 17, 2002.

(25) Jim, L.; Chen, L. J.; Goyal, B.; Guy, L.; Friend, S. T.; Brian, S. E. Heterocyclic Inhibitors of Glutaminase. WO2013078123, May 30, 2013.

(26) Chen, Z.; Li, D.; Xu, N.; Fang, J. Z.; Yu, Y.; Hou, W.; Ruan, H. Q.; Zhu, P. P.; Ma, R. C.; Lu, S. Y.; Cao, D. H.; Wu, R.; Ni, M. W.; Zhang, W.; Su, W. K.; Ruan, B. H. Novel

1,3,4-Selenadiazole-Containing Kidney-Type Glutaminase Inhibitors Showed Improved Cellular Uptake and Antitumor Activity. *J. Med. Chem*. **2019**, *62*, 589-603.

(27) Kim, J.; Felts, S.; Llauger, L.; He, H.; Huezo, H.; Rosen, N.; Chiosis, G. Development of A Fluorescence Polarization Assay for the Molecular Chaperone Hsp90, *J. Biomol. Screen* **2004**, *9*, 375-381.

(28) Thangavelu, K.; Pan, C. Q.; Karlberg, T.; Balaji, G.; Uttamchandani, M.; Suresh, V.; Schuler, H.; Low, B. C.; Sivaraman, J. Structural Basis for the Allosteric Inhibitory Mechanism of Human Kidney-Type Glutaminase (KGA) and Its Regulation by Raf-Mek-Erk Signaling in Cancer Cell Metabolism. *Proc. Natl. Acad. Sci. U S A*. **2012**, *109*, 7705-7710.

(29) Wurz, R. P.; Dellamaggiore, K.; Dou, H.; Javier, N.; Lo, M. C.; McCarter, J. D.; Mohl, D.; Sastri, C.; Lipford, J. R.; Cee, V. J. A "Click Chemistry Platform" for the Rapid Synthesis of Bispecific Molecules for Inducing Protein Degradation. *J. Med. Chem*. **2018**, *61*, 453-461.

(30) Suzuki, T.; Ota, Y.; Ri, M.; Bando, M.; Gotoh, A.; Itoh, Y.; Tsumoto, H.; Tatum, P. R.; Mizukami, T.; Nakagawa, H.; Iida, S.; Ueda, R.; Shirahige, K.; Miyata, N. Rapid Discovery of Highly Potent and Selective Inhibitors of Histone Deacetylase 8 Using Click Chemistry to Generate Candidate Libraries. *J. Med. Chem*. **2012**, *55*, 9562-9575.

(31) Wu, Y.; Jiang, Z. S.; Li, Z. H.; Gu, J.; You, Q. D.; Zhang X. J. Click Chemistry-Based Discovery of [3-Hydroxy-5-(1H-1,2,3-triazol-4-yl)picolinoyl]glycines as Orally Active Hypoxia-Inducing Factor Prolyl Hydroxylase Inhibitors with Favorable Safety Profiles for the Treatment of Anemia. *J. Med. Chem.* **2018**, *61*, 5332-5349.

(32) Zhou, L.; Yang, L.; Tilton, S.; Wang, J. Development of A High Throughput Equilibrium Solubility Assay Using Miniaturized Shake-flask Method in Early Drug Discovery. *J. Pharm. Sci*.

2007, *96*, 3052-3071.

(33) Zhang, J. H.; Chung, T. D.; Oldenburg, K. R. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J. Biomol. Screening* **1999**, *4*, 67-73.

(34) Weinberg, F.; Hamanaka, R.; Wheaton, W. W.; Weinberg, S.; Joseph, J.; Lopez, M.; Kalyanaraman, B.; Mutlu, G. M.; Scott Budinger, G. R.; Chandel, N. S. Mitochondrial Metabolism and ROS Generation are Essential for Kras-Mediated Tumorigenicity. *Proc. Natl. Acad. Sci. U S A.* **2010**, *107*, 8788-8793.

(35) Chen, S. J.; Wang, Y. L.; Zhou, W.; Li, S. S.; Peng, J. L.; Shi, Z.; Hu, J. C.; Liu, Y. C.; Ding, H.; Lin, Y. Y.; Li, L. J.; Cheng, S. F.; Liu, J. Q.; Lu, T.; Jiang, H. L.; Liu, B.; Zheng, M. Y.; Luo, C. Identifying Novel Selective Non-nucleoside DNA Methyltransferase 1 Inhibitors Through Docking-based Virtual Screening. *J. Med. Chem*. **2014**, *57*, 9028-9041.

(36) Yeh, T. K.; Kuo, C. C.; Lee, Y. Z.; Ke, Y. Y.; Chu, K. F.; Hsu, H.Y.; Chang, H.Y.; Liu, Y. W.; Song, J. S.; Yang, C. W.; Lin, L. M.; Sun, M.; Wu, S. H.; Kuo, P. C.; Shih, C.; Chen, C. T.; Tsou, L. K.; Lee. S. J. Design, Synthesis, and Evaluation of Thiazolidine-2,4-dione Derivatives as a Novel Class of Glutaminase Inhibitors. *J. Med. Chem*. **2017**, *60*, 5599-5612.

(37) Roehrl, M. H.; Wang, J. Y.; Wagner, G. A General Framework for Development and Data Analysis of Competitive High-Throughput Screens for Small-Molecule Inhibitors of Protein-Protein Interactions by Fluorescence Polarization. *Biochemistry*. **2004**, *43*, 16056-16066.

(38) Marholz, L. J.; Wang, W.; Zheng, Y.; Wang, X. A Fluorescence Polarization Biophysical Assay for the Naegleria DNA Hydroxylase Tet1. *ACS Med. Chem. Lett*. **2016**, *7*, 167-171.

(39) Lei, Y.; Hu, T.; Wu, X.; Wu, Y.; Bao, Q.; Zhang, L.; Xia, H.; Sun, H.; You, Q.; Zhang, X.

Affinity-Based Fluorescence Polarization Assay for High-Throughput Screening of Prolyl Hydroxylase 2 Inhibitors. *ACS Med. Chem. Lett.* **2015**, *6*, 1236-1240.

Table of Contents graphic.