ACS Medicinal Chemistry Letters

Letter

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Allosteric Glutaminase Inhibitors Based on a 1,4-Di(5-amino-1,3,4-thiadiazol-2-yl)butane Scaffold

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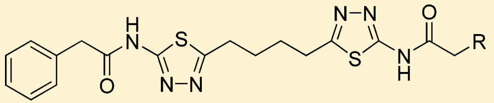
Ⓢ *Supporting Information*

ABSTRACT: A series of allosteric kidney-type glutaminase (GLS) inhibitors were designed and synthesized using 1,4-di(5-amino-1,3,4-thiadiazol-2-yl)-butane as a core scaffold. A variety of modified phenylacetyl groups were incorporated into the 5-amino group of the two thiadiazole rings in an attempt to facilitate additional binding interactions with the allosteric binding site of GLS. Among the newly synthesized compounds, 4-hydroxy-*N*-[5-[4-[5-[(2-phenylacetyl)amino]-1,3,4-thiadiazol-2-yl]butyl]-1,3,4-thiadiazol-2-yl]-benzeneacetamide, **2m**, potently inhibited GLS with an IC_{50} value of 70 nM, although it did not exhibit time-dependency as seen with CB-839. Antiproliferative effects of **2m** on human breast cancer lines will be also presented in comparison with those observed with CB-839.



2a (R = Ph): IC_{50} = 1.8 μM
2j (R = 4-AcOPh): IC_{50} = 0.54 μM
2m (R = 4-HOPh): IC_{50} = 0.07 μM

KEYWORDS: *Glutaminase, allosteric inhibition, cancer metabolism*

Glutaminolysis involves a series of biochemical reactions by which glutamine is utilized as a source of nitrogen atoms and carbon skeletons to create a variety of biologically important substances. The first step in glutaminolysis is the hydrolysis of glutamine to glutamate and ammonia, which is catalyzed by the phosphate-activated glutaminase. Mammalian tissues express two forms of glutaminase encoded by two paralogous genes located in distinct chromosomes.[1] Liver-type glutaminase (GLS2) is predominantly found in adult liver, whereas kidney-type glutaminase (GLS) is widely distributed throughout extra-hepatic tissues. Two splicing variants derived from the GLS gene are known as KGA and GAC, which share a common N-terminal sequence (1−550) but contain unique C-terminal segments (551−669 for KGA, 551−598 for GAC).[2] Although distinct molecular functions of the two splicing variants have not yet been clearly understood, it appears that the GAC form of GLS is predominantly upregulated in many proliferating cells, especially rapidly growing malignant cells.[3] Inhibition of GLS, therefore, has gained considerable attention as a new therapeutic approach for the treatment of cancer.

6-Diazo-5-oxo-L-norleucine (DON, Figure 1) is one of the earliest glutaminase inhibitors,[4] though its narrow therapeutic index highlighted the need for a new class of glutaminase inhibitors pharmacologically distinct from glutamine mimetics such as DON. In this regard, compound **968** (Figure 1)[5] is one of the promising new glutaminase inhibitors structurally dissimilar to glutamine. It remains to be seen whether successful structural optimization can be carried out using the benzophenanthridinone lead.[6] Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES, Figure 1)[7] is another glutaminase inhibitor structurally distinct from DON. Unlike DON, BPTES does not contain any reactive chemical group that might form a covalent bond and is unlikely to cause toxicity by irreversibly forming covalent adducts with endogenous proteins. In addition, while DON inhibits both GLS and GLS2, BPTES selectively inhibits GLS (both KGA and GAC isoenzymes) over GLS2.[8] Moreover, BPTES bears no

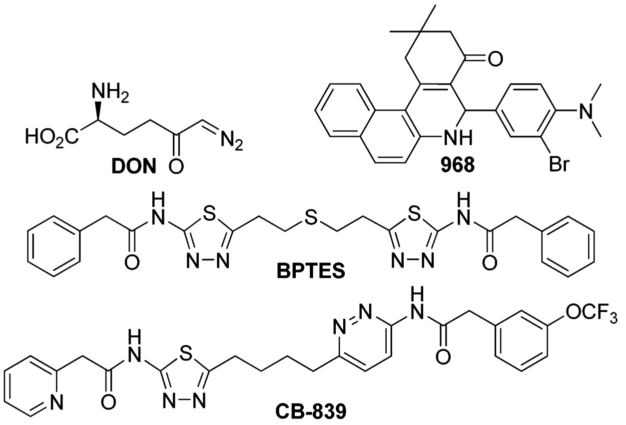


Figure 1. Representative glutaminase inhibitors.

Received: February 9, 2016
Accepted: March 13, 2016

DOI: 10.1021/acsmedchemlett.6b00060

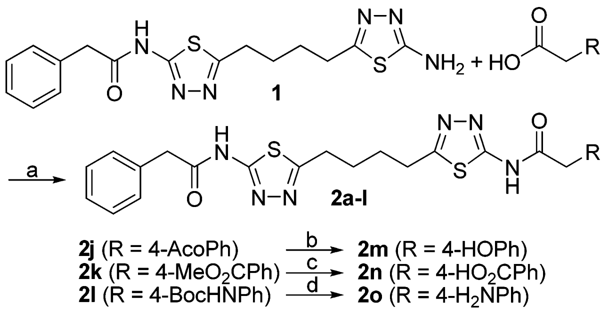
structural similarity to glutamine, minimizing off-target effects due to its interaction with other glutamine-related enzymes, transporters, or receptors. Indeed, preliminary toxicity studies of BPTES in mice showed no histopathologies in liver, heart, lung, skeletal muscle, kidney, and brain.[9] Further, BPTES did not show any significant effects on body weight, blood chemistries, and hematology measurements.[9]

BPTES is, however, limited as a therapeutic agent due to its poor aqueous solubility at any given pH, hindering its ability to be dosed at therapeutically relevant levels. Recently, we and other groups have begun exploring a new generation of GLS inhibitors based on BPTES. Our group has reported medicinal chemistry efforts on derivatives of BPTES with the primary objective of improving solubility as compared to BPTES.[10] While these analogues were successful in enhancing the solubility, a significant improvement in glutaminase inhibitory potency was not achieved. Meanwhile, Calithera Biosciences developed a BPTES derivative CB-839 (Figure 1)[11] containing a pryridazine ring, which is currently under clinical investigation in patients with cancer.

Recently published cocrystal structures of GLS in complex with BPTES uncovered the unique allosteric binding of BPTES to the dimer interface of GLS tetramer.[12,13] The cocrystal structures revealed that the two phenylacetyl groups at the edges of the molecule do not participate in any particular interactions with the allosteric binding site of GLS. The findings prompted us to modify the phenylacetyl moiety in an attempt to gain additional interactions with GLS. Herein we report the design, synthesis, and biological evaluation of allosteric glutaminase inhibitors based on a 1,4-di(5-amino-1,3,4-thiadiazol-2-yl)butane scaffold into which a variety of modified phenylacetyl groups were incorporated at the 5-amino group.

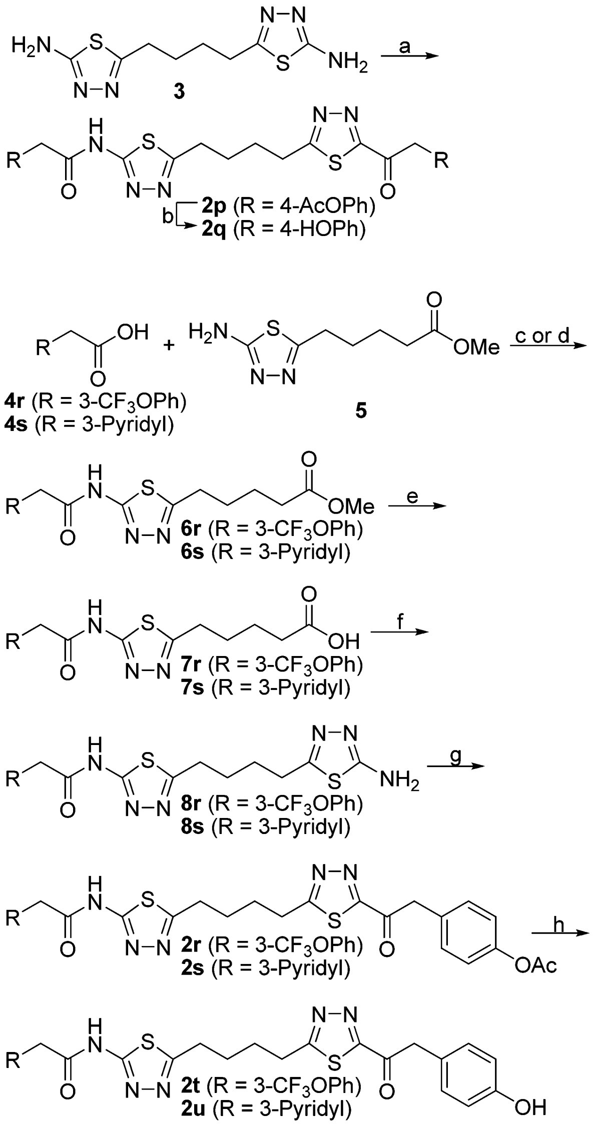
In the first series of compounds **2a−o**, we attached a phenylacetyl group to one end of the 1,4-di(5-amino-1,3,4-thiadiazol-2-yl)butane scaffold and incorporated various modified phenylacetyl groups into the other end of the scaffold. The general synthetic route from the common intermediate **1** is illustrated in Scheme 1. HATU-mediated coupling was found to be most effective in forming carboxamide at the 5-amino group of the thiadiazole ring of **1**. Compounds **2j−l** were further converted into **2m−o**, respectively.

Scheme 1. Synthesis of 2a−o[a]



[a]Conditions: (a) DIPEA, HATU, DMF, 0 °C; (b) 2 N NaOH, MeOH, rt; (c) 2 N NaOH, THF, rt; (d) 4 N HCl, 1,4-dioxane, rt.

Scheme 2. Synthesis of 2p−u[a]

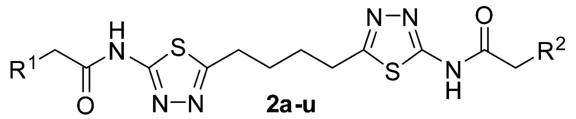


[a]Conditions: (a) DIPEA, HATU, DMF, 0 °C; (b) 2 N NaOH, THF, rt; (c) for **4r** to **6r**, DIPEA, HATU, DMF, 0 °C; (d) for **4s** to **6s**, T$_3$P, NEt$_3$, DMF, RT; (e) 2.5% LiOH, THF, RT; (f) NH$_2$CSN$_2$H$_3$, POCl$_3$, 90 °C; (g) DIPEA, HATU, DMF, 0 °C; (h) 2 N NaOH, THF, rt.

A second series of compounds contain modified phenylacetamide groups at the both ends of the 1,4-di(5-amino-1,3,4-thiadiazol-2-yl)butane scaffold. As shown in Scheme 2, bis(4-acetoxyphenylacetamide) derivative **2p** was prepared by coupling 4-acetoxyphenylacetic acid with 5,5′-(butane-1,4-diyl)bis(1,3,4-thiadiazol-2-amine) **3**. Compound **2p** was subsequently converted into bisphenol derivative **2q**. Additional derivatives **2r−u** containing two distinct modified phenylacetamide groups were synthesized using methyl 5-(5-amino-1,3,4-thiadiazol-2-yl)pentanoate **5** as a starting material. The free amino group of **5** was coupled with 3-trifuloromethyloxyphenylacetic acid **4r** using HATU-mediated coupling method to give the corresponding carboxamide **6r**. Preparation of **6s** from 3-pyridylacetic acid **4s** and **5** utilized propylphosphonic anhydride (T3P)[14] as a coupling reagent since HATU-mediated reaction resulted in the low yield of the desired product. Hydrolysis of the methyl ester of **6s−r** afforded **7s−r**, which was subsequently converted into bis(5-amino-1,3,4-thiadiazole) derivatives **8r−s** by condensing with thiosemicarbazide in the presence of POCl$_3$. Coupling with 4-acetoxyohenylacetic acid provided **2r−s**, which were further transformed into the corresponding phenol derivatives **2t–u** by base-mediated hydrolysis.

All new synthetic compounds were tested for their ability to inhibit GLS using L-[^{3}H]-glutamine as substrate and human kidney-type glutaminase (hKGA124–669).[10] In this assay, BPTES and CB-839 inhibited GLS with IC_{50} values of 3.3 and 0.06 μM, respectively. GLS inhibitory data of the new analogues **2a–u** are summarized in Table 1.

Table 1. Inhibition of GLS by Compounds 2a–u



2a-u

cmpd	R^1	R^2	IC_{50} (μM)[a]
2a	Ph	Ph	1.8 ± 0.1
2b	Ph	4-MeOPh	0.21 ± 0.04
2c	Ph	4-PhOPh	0.38 ± 0.03
2d	Ph	4-BnOPh	>25
2e	Ph	4-Me_2NPh	3.0 ± 0.3
2f	Ph	3-HOPh	0.12 ± 0.01
2g	Ph	4-bromo-2-pyridyl	0.17 ± 0.02
2h	Ph	2-pyridyl	0.07 ± 0.01
2i	Ph	3-pyridyl	0.07 ± 0.00
2j	Ph	4-AcOPh	0.54 ± 0.04
2k	Ph	4-MeO_2CPh	13 ± 2
2l	Ph	4-BocHNPh	0.45 ± 0.04
2m	Ph	4-HOPh	0.07 ± 0.01
2n	Ph	4-HO_2CPh	0.11 ± 0.01
2o	Ph	4-H_2NPh	2.3 ± 0.3
2p	4-AcOPh	4-AcOPh	1.2 ± 0.2
2q	4-HOPh	4-HOPh	0.12 ± 0.02
2r	3-CF_3OPh	4-AcOPh	2.0 ± 0.2
2s	3-pyridyl	4-AcOPh	0.12 ± 0.01
2t	3-CF_3OPh	4-HOPh	0.51 ± 0.06
2u	3-pyridyl	4-HOPh	0.14 ± 0.01

[a]Values are mean ± SD of at least four experiments.

Modifications at one of the two phenylacetyl groups of **2a** (compounds **2b–o**) had varied effects on GLS inhibitory potency. Among them, four compounds displayed IC_{50} values higher than that of **2a**. Incorporation of a 4-benzyloxyphenylacetyl group (compound **2d**) resulted in a significant loss of inhibitory activity, underscoring the steric limits of the pockets at the edges of the allosteric binding site. Incorporation of positively charged substituents such as dimethylamino (compound **2e**) and amino (compound **2o**) groups also led to inhibitors with slightly higher IC_{50} values, while a more substantial loss of inhibitory potency was seen as a result of introducing a 4-methoxycarbonyl group (compound **2k**). Many derivatives within this series exhibited improved GLS inhibitory potency compared to **2a**. It is worth noting that most of these inhibitors possess substituents capable of serving as a hydrogen bond acceptor, which may be contributing to the stronger intermolecular interaction with GLS. Compounds **2h**, **2i**, and **2m** displayed IC_{50} values below 100 nM. Compound **2m** is particularly interesting as its phenolic group can possibly act as both a hydrogen bond acceptor and a donor. In fact, nearly 8-fold increase in potency from the corresponding acetoxy derivative **2j** suggests the potentially important role played by the phenolic hydrogen. Indeed, the cocrystal structure of BPTES bound to GLS (3VOZ)[13] presents Arg317 from one GLS unit and Glu325 from a diagonally opposite GLS unit in the vicinity of the phenylacetyl groups of BPTES. Given the flexible side chain of these residues, it is possible that the phenolic group of **2m** serves as a hydrogen bond acceptor from the guanidinium group of Arg317 and a donor to the carboxylate group of Glu325, contributing to the stronger affinity of **2m** to GLS. The phenolic group also appears to improve the aqueous solubility of **2m** (17 μg/mL) as compared to BPTES (0.14 μg/mL).

Compounds **2p–u** possess modified phenylacetamide groups at both ends of the 1,4-di(5-amino-1,3,4-thiadiazol-2-yl)butane scaffold. Although compound **2p** bearing two 4-acetoxyphenylacetyl groups was only as potent as compound **2a**, the bis(4-hydroxyphenylacetyl) derivative **2q** exhibited 10-fold higher GLS inhibitory potency. While this is consistent with the improvement seen in the 4-hydroxyphenylacetyl derivative **2m** as compared to 4-acetoxyphenylacetyl derivative **2j**, there appears to be little synergistic effects between the two phenol moieties of **2q**, which would make the compound more potent than **2m**. Nevertheless, compounds containing a 4-hydroxyphenylacetyl group (**2q**, **2t**, and **2u**) still showed reasonably potent GLS inhibitory activity, demonstrating the key role played by the 4-hydroxyphenylacetyl moiety in binding to the GLS allosteric site.

In order to further characterize the mode of inhibition of compound **2m**, we examined the time dependency of GLS inhibitory potency. As shown in Figure 2, compound **2m** showed no time-dependent increase in GLS inhibitory potency. In sharp contrast to compound **2m**, CB-839 displayed increasing potency toward GLS over time as previously reported.[11] The precise structural features that produce time-dependent GLS inhibition have not been clearly elucidated though it is conceivable that the pyridazine ring unique to CB-839 may play a critical role in its mode of GLS inhibition.

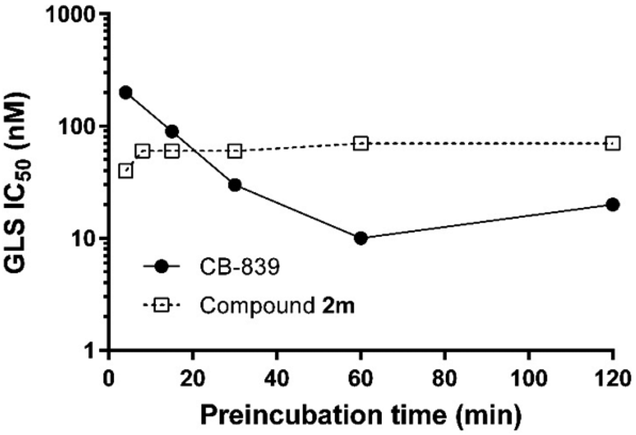


Figure 2. Effect of preincubation time on GLS inhibitory potency of CB-839 and compound **2m**.

Antiproliferative effects of **2m** were compared to BPTES and CB-839 in triple negative breast cancer cell lines, MDA-MB-231 and HCC1806. As shown in Figure 3, the three compounds exhibited various degrees of antiproliferative activity in both cell lines. At 72 h of incubation, compound **2m** was least effective in both cell lines and displayed statistically significant growth inhibition only in MDA-MB-231 at the higher concentration (1000 nM). The superior effects of CB-839 may be attributed to its time-dependent

DOI: 10.1021/acsmedchemlett.6b00060
ACS Med. Chem. Lett. XXXX, XXX, XXX–XXX

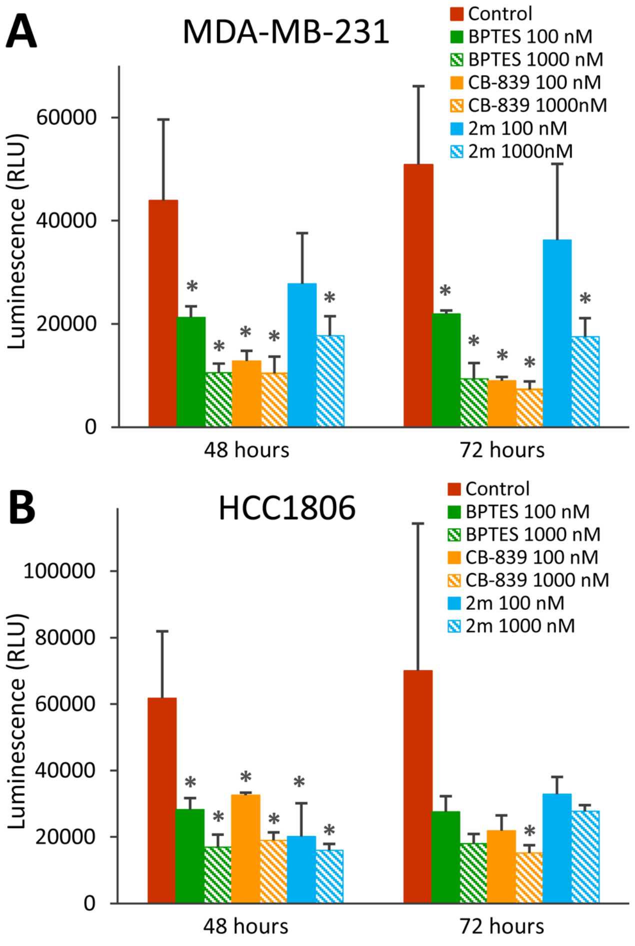


Figure 3. Effects of BPTES, CB-839, and compound **2m** on (A) MDA-MB-231 and (B) HCC1806 cell growth. Cell viability assays were performed in quadruplicate, and error bars represent standard deviation. Live cell viability was determined using CellTiter-Glo Luminescent Cell Viability Assay daily. $^*P < 0.05$ compared to the controls.

inhibition of GLS, which should result in a substantially higher degree of GLS inhibition over time. Another possibility is the decreased cell permeability of **2m** due to the presence of the polar phenolic group. Indeed, despite its weaker GLS inhibitory potency, BPTES also appears more effective in attenuating cell growth, possibly due to its greater cell permeability.

The unique allosteric mode of inhibition of GLS by BPTES and presumably its derivatives has presented a renewed opportunity to explore this key metabolic enzyme as a therapeutic target. Initiation of clinical studies with CB-839 undoubtedly represents an important milestone in the effort to translate GLS inhibitors into novel therapeutics. Further optimization will likely benefit from structure-based drug design focused on gaining additional binding interaction(s) with the allosteric site of GLS. Our SAR studies reported herein indicate that the terminal phenylacetyl groups can be exploited to improve potency of GLS inhibitors based on a 1,4-di(5-amino-1,3,4-thiadiazol-2-yl)butane scaffold. While this work underscores one aspect of the possible structural optimization strategies that can be taken forward, other elements such as understanding of the molecular basis of the time-dependent inhibition can be built upon our findings in order to identify new GLS inhibitors with therapeutic potential.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchem-lett.6b00060.

> Description of synthetic procedures and identification of compounds; experimental protocols for glutaminase and antiproliferative assays (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding

This work was supported by NIH grants (R21NS074151 to T.T., R21CA169757 to A.L., P30MH075673 to B.S.S., R01CA19389501 to B.S.S., and F32CA200275 to S.C.Z.) and a Maryland Innovation Initiative grant (MII 90062191 to A.L.).

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

BPTES, bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide; T3P, propylphosphonic anhydride; DON, 6-diazo-5-oxo-L-norlucine

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DOI: 10.1021/acsmedchemlett.6b00060