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

Sarah C. Zimmermann,†,‡ Emily F. Wolf,† Andrew Luu,† Ajit G. Thomas,† Marigo Stathis,† Brad Poore,§ Christopher Nguyen,§ Anne Le,§ Camilo Rojas,∥ Barbara S. Slusher,†,‡ and Takashi Tsukamoto*,†,‡

†Johns Hopkins Drug Discovery Program, Johns Hopkins University, Baltimore, Maryland 21205, United States
‡Department of Neurology, Johns Hopkins University, Baltimore, Maryland 21205, United States
§Department of Pathology, Johns Hopkins University, Baltimore, Maryland 21231, United States
∥Department of Molecular and Comparative Pathobiology, Johns Hopkins University, Baltimore, Maryland 21205, United States

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]-benzenecacetamide, 2m, potently inhibited GLS with an IC50 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.

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-1-norleucine (DON, Figure 1)4 is one of the earliest glutaminase inhibitors, 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 benzophenanthridine 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

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

Letter

© 2016 American Chemical Society

DOI: 10.1021/acsmedchemlett.6b00060
ACS Med. Chem. Lett. 2016, 7, 520−524
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. Further, BPTES did not show any significant effects on body weight, blood chemistries, and hematology measurements.

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. 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) containing a pyridazine 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. 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 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.

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 S,S′-(butane-1,4-diyldi)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 S-(5-amino-1,3,4-thiadiazol-2-yl)pentanoate 5 as a starting material. The free amino group of 5 was coupled with 3-trifluoromethoxyphenylacetic acid 4r using HATU-mediated coupling method to give the corresponding carboxamide 6r. Preparation of 6s from 3-pyridylacetic acid 4s and S utilized propylphosphonic anhydride (T3P) 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(S-amino-1,3,4-thiadiazole) derivatives 8r−s by condensing with thiosemicarbazide in the presence of POCl₃. Coupling with 4-acetoxyphenylacetic acid provided 2r−s, which were further

---

**Scheme 1. Synthesis of 2a−o**

| Reaction | Product |
|----------|---------|
| 2a−l     | 2m−o    |

**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**

| Reaction | Product |
|----------|---------|
| 2p       | 2q      |

**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, T₃P, NEt₃, DMF, RT; (e) 2.5% LiOH, THF, RT; (f) NH₂CSNH₂, POCl₃, 90 °C; (g) DIPEA, HATU, DMF, 0 °C; (h) 2 N NaOH, THF, rt.

---

ACS Med. Chem. Lett. 2016, 7, 520–524

DOI: 10.1021/acsmedchemlett.6b00060

521
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-[3H]-glutamine as substrate and human kidney-type glutaminase (hKGA124–669). 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

| cmpd | R¹ | R² | IC_{50} (μM)± |
|------|----|----|--------------|
| 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-Me₂NPh | 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-MeO₂CPh | 13 ± 2     |
| 2l   | Ph | 4-BOCHNPh | 0.45 ± 0.04 |
| 2m   | Ph | 4-HOPh | 0.07 ± 0.01 |
| 2n   | Ph | 4-HO₂CPh | 0.11 ± 0.01 |
| 2o   | Ph | 4-H₂NPh | 2.3 ± 0.3 |
| 2p   | 4-AcOPh | 4-AcOPh | 1.2 ± 0.2 |
| 2q   | 4-HOPh | 4-HOPh | 0.12 ± 0.02 |
| 2r   | 3-CF₃OPh | 4-AcOPh | 2.0 ± 0.2 |
| 2s   | 3-pyridyl | 4-AcOPh | 0.12 ± 0.01 |
| 2t   | 3-CF₃OPh | 4-HOPh | 0.51 ± 0.06 |
| 2u   | 3-pyridyl | 4-HOPh | 0.14 ± 0.01 |

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-benzoyloxyphenylacetyl 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 2a) 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 hydroxyl. Indeed, the cocrystal structure of BPTES bound to GLS (3VOZ) 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. 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. 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
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 and presumably other elements such as BPTES, bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.6b00060.

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

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., R01CA1938501 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-norleucine

REFERENCES
(1) Curthoys, N. P.; Watford, M. Regulation of glutaminase activity and glutamine metabolism. *Annu. Rev. Nutr.* 1995, 15, 133–59.
(2) Mates, J. M.; Segura, J. A.; Martin-Rufian, M.; Campos-Sandoval, J. A.; Alonso, F. J.; Marquez, J. Glutaminase isoenzymes as key regulators in metabolic and oxidative stress against cancer. *Curr. Mol. Med.* 2013, 13, 514–34.
(3) van den Heuvel, A. P.; Jing, J.; Wooster, R. F.; Bachman, K. E. Analysis of glutamine dependency in non-small cell lung cancer: GLS1 splice variant GAC is essential for cancer cell growth. *Cancer Biol. Ther.* 2012, 13, 1185–94.
(4) Abluwalia, G. S.; Grem, J. L.; Hao, Z.; Cooney, D. A. Metabolism and action of amino acid analog anti-cancer agents. *Pharmacol. Ther.* 1990, 46, 243–71.
(5) Wang, J. B.; Erickson, J. W.; Fuji, R.; Ramachandran, S.; Gao, P.; Dinavahi, R.; Wilson, K. F.; Ambrosio, A. L.; Dias, S. M.; Dang, C. V.; Cerione, R. A. Targeting mitochondrial glutaminase activity inhibits oncogenic transformation. *Cancer Cell* 2010, 18, 207–19.
(6) Katt, W. P.; Ramachandran, S.; Erickson, J. W.; Cerione, R. A. Dibenzophenanthridines as inhibitors of phosphate-activated glutaminase C and cancer cell proliferation. *Mol. Cancer Ther.* 2012, 11, 1269–79.
(7) Newcomb, R. W. Selective inhibition of glutaminase by bis-thiadiazoles. US 6,451,828 B1, 2002.
(8) Robinson, M. M.; Mc Bryan, 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, 407–14.
(9) Xiang, Y.; Stine, Z. E.; Xia, J.; Lu, Y.; O’Connor, R. S.; Altman, B. J.; Hsieh, A. L.; Gouw, A. M.; Thomas, A. G.; Gao, P.; Sun, L.; Song, L.; Yan, B.; Slusher, B. S.; Zhuo, J.; Ooi, L. L.; Lee, C. G.; Mancuso, A.; McCallion, A. S.; Le, A.; Milone, M. C.; Rayport, S.; Felsher, D. W.; Dang, C. V. Targeted inhibition of tumor-specific glutaminase...
diminishes cell-autonomous tumorigenesis. *J. Clin. Invest.* 2015, 125, 2293–306.

(10) Shukla, K.; Ferraris, D. V.; Thomas, A. G.; Stathis, M.; Duvall, B.; Delahanty, G.; Ali, 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, 10551–63.

(11) Gross, M. I.; Demo, S. D.; Dennison, J. B.; Chen, L.; Chernov-Rogan, 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. Y.; Bennett, M. K. Antitumor Activity of the Glutaminase Inhibitor CB-839 in Triple-Negative Breast Cancer. *Mol. Cancer Ther.* 2014, 13, 890–901.

(12) DeLaBarre, B.; Gross, S.; Fang, C.; Gao, Y.; Jha, A.; Jiang, F.; Song, J. J.; Wei, W.; Hurov, J. B. Full-length human glutaminase in complex with an allosteric inhibitor. *Biochemistry* 2011, 50, 10764–70.

(13) 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.

(14) Sukalovic, V.; Ignjatovic, D.; Tovilovic, G.; Andric, D.; Shakib, K.; Kostic-Rajacic, S.; Sosic, V. Interactions of N-([2-(4-phenylpiperazin-1-yl)-ethyl]-2-aryl-2-y1-acetamides and 1-[[2-(4-phenylpiperazin-1-yl)-ethyl]-phenyl]-3-aryl-2-y1-ureas with dopamine D2 and 5-hydroxytryptamine SHT(1A) receptors. *Bioorg. Med. Chem. Lett.* 2012, 22, 3967–72.