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REVIEW ARTICLE

Recent Development of Small Molecule Glutaminase Inhibitors

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Abstract: Glutaminase (GLS), which is responsible for the conversion of glutamine to glutamate, plays a vital role in up-regulating cell metabolism for tumor cell growth and is considered to be a valuable therapeutic target for cancer treatment. Based on this important function of glutaminase in cancer, several GLS inhibitors have been developed in both academia and industry. Most importantly, Calithera Biosciences Inc. is actively developing the glutaminase inhibitor CB-839 for the treatment of various cancers, and it is currently being evaluated in phase 1 and 2 clinical trials. In this review, recent efforts to develop small molecule glutaminase inhibitors that target glutamine metabolism in both preclinical and clinical studies are discussed. In particular, more emphasis is placed on CB-839 because it is the only small molecule GLS inhibitor being studied in a clinical setting. The inhibition mechanism is also discussed based on X-ray structure studies of thiadiazole derivatives present in glutaminase inhibitor BPTES. Finally, recent medicinal chemistry efforts to develop a new class of GLS inhibitors are described in the hopes of providing useful information for the next generation of GLS inhibitors.

Keywords: Glutaminase, glutaminase inhibitors, cancer target, BPTES, CB-839, allosteric inhibitors.

1. INTRODUCTION

Following Kreb's pioneering study in 1935 that revealed the existence of glutaminase in mammalian tissues and the significance of glutamine metabolism in organismal homeostasis [1], the role of glutamine in cell growth and cancer cell biology has been extensively studied for over 80 years [2-4]. The essential role of glutamine in certain pathological conditions has been described in the past. For example, Lacey and Wilmore found that during stressed states, such as injury, sepsis, and inflammation, there was a dramatic increase in glutamine consumption by the gastrointestinal tract, immunologic cells, inflammatory tissues, and kidneys [5]. Glutamine is defined as a conditionally essential amino acid that is nonessential under healthy conditions but is required at greater than endogenous levels in certain stressed conditions. Due to the importance of glutamine in stressed states, glutamine metabolism has been a target for cancer therapy for decades. Bode *et al.* found that the rate of glutamine transport is higher in human hepatoma cell lines than in normal cells [6]. Pérez-Gómez *et al.* suggested that glutaminase activity correlates with the growth rates of human tumor cells, and kidney-type glutaminase (GLS1) is up-regulated with increased rates of proliferation, indicative that GLS1 provides the majority of glutaminase activity in these tumor cells [7]. Finally, Yuneva *et al.* discussed the dependence of growing cancer cells on glutamine and showed that the depletion of glutamine potently induced apoptosis in cells with ectopically activated MYC [8]. GLS1 expression was shown to be positively regulated by MYC and was identified as an important effector of Rho GTPase mediated transformation [9-11]. Proliferating cells, including cancer cells and activated lymphocytes, use glutamine as an energy-generating substrate [12-14].

Glutaminase (GLS) converts glutamine to stoichiometric amounts of ammonia and glutamate upon entry to mitochondria through the glutamine transporter. Glutamate is then deaminated, either by glutamate dehydrogenase (GLUD) or by aminotransferases, to yield α-ketoglutarate (α-KG). α-KG then enters the TCA cycle to produce ATP or is used for the synthesis of several amino acids and lipids, which are then used to fulfill bioenergetic and biosynthetic demands. Glutamine can therefore function as a primary carbon and nitrogen source for highly proliferating cells, such as cancer cells (Fig. **1**) [15].

Glutaminase is encoded by two genes in humans, kidney-type glutaminase (GLS1) and liver-type glutaminase (GLS2) [16]. The GLS1 enzyme is more broadly expressed in normal tissues, such as kidney and brain tissue and is considered to have an important role in cancers [17]. GLS1 is also known to regulate the acid-base balance during metabolic acidosis in the kidney [17, 18]. GLS2 is primarily expressed in the liver, where it provides nitrogen for the urea cycle. GLS1 has multiple transcripts, including the canonical splice variant 1 (kidney type glutaminase, KGA), a truncated and non-catalytically competent splice variant 2, and a C-terminal splice variant 3 (glutaminase C, GAC). GAC is identical to

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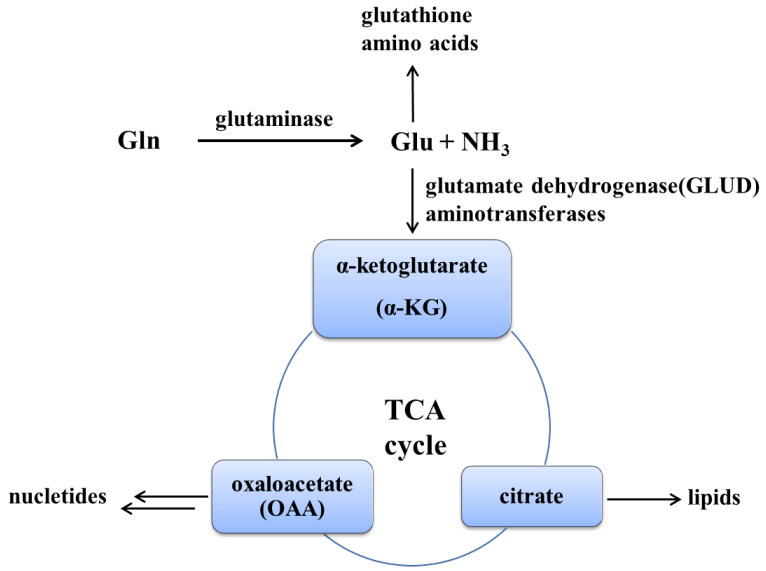


Fig. (1). Conversion of glutamine to glutamate by glutaminase, and its utilization in the TCA cycle.

KGA, with a variation at the C terminus [20]. GLS1 isoforms and GLS2 differ in their regulation and activity. For example, GLS1, especially GAC, shows much greater catalytic activity than GLS2 in the presence of inorganic phosphate as an enzyme activator. GAC is highly expressed in many primary tumors and tumor cell lines, but KGA and GLS2 expression appear to be relatively limited in cancer [21]. As GAC expression is greatly increased in several cancer cells, it is described as a "gate-keeper" enzyme for the elevated glutamine metabolism observed in these cancer cells. This also implies that GLS isoforms may have a significant role in the glutaminolytic flux in cancer [22]. Due to these vital roles of GLS1 in upregulating cell metabolism for tumor cell growth, targeting glutamine metabolism through the inhibition of glutaminase has gained considerable attention as a new therapeutic approach for the treatment of cancer. Several small molecule GLS inhibitors, such as DON, 968, BPTES, and CB-839, have been developed. In this review, the recent efforts toward developing small molecule glutaminase inhibitors targeting glutamine metabolism in preclinical and clinical studies are discussed. Particular focus is paid on CB-839 because it is the only small molecule GLS inhibitor being studied in the clinical setting. The inhibition mechanism is also discussed, based on X-ray structural studies of the thiadiazole derivatives BPTES and CB-839. Finally, recent medicinal chemistry efforts to develop a new class of GLS inhibitors are discussed in the hopes of providing useful information for forthcoming GLS inhibitors.

2. GLS INHIBITORS

To date, a number of different strategies using small molecule inhibitors have been devised to inhibit glutaminase. Among these, four GLS small molecule inhibitors, including 6-Diazo-5-oxo-L-norleucine (**DON**), 5-[3-Bromo-4-(dimethylamino)phenyl]-2,3,5,6-tetrahydro-2,2-dimethyl-benzo[a] phenanthridin-4(1H)-one (**968**), bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (**BPTES**), and **CB-839,** are worth discussing in more detail.

DON: 6-Diazo-5-oxo-L-norleucine (DON), an analog of the substrate glutamine, is a competitive inhibitor that binds covalently to the enzyme active site and consequently modifies the catalytic serine (S291). The nucleophilic attack of the Ser286 side chain on DON releases a stoichiometric amount of nitrogen from the inhibitor, resulting in the formation of an enzyme-inhibitor complex (Fig. **3**) [23]. DON is known to interfere with both nucleotide and protein synthetic pathways, where glutamine acts as a substrate, halting the formation of DNA and/or RNA in the tumor cell [24]. The potential anti-tumor activity of DON was observed in several animal tumor models, including the LI210 leukemia model (>50% increase in life span (ILS) over a dose range of 2.5 ~ 40 mg/kg, lethal at 80 mg/kg) and the colon 26 carcinoma model (70% ILS, a dose of 12.5 mg/kg). In xenograft experiments of LX1 lung cancer, significant tumor regression was observed [25]. However, a toxicology study using azotomycin, which contains two moles of DON, raised serious toxicity concerns, as it showed lethality with repeat doses of less than 1 mg/kg over 5 days [25]. In addition to toxicity concerns, the poor binding selectivity of DON compared to other glutamine-utilizing enzymes, such as the amidotransferases and glutamine synthetase, prohibited its progression to clinical studies [26].

968: 5-[3-Bromo-4-(dimethylamino)phenyl]-2,3,5,6-tetrahydro-2,2-dimethyl-benzo[a]phenanth-ridin-4(1H)-one, a class of bromo-benzophenanthridinone compounds, is known to be a non-competitive allosteric inhibitor of GAC (IC_{50} = ~2.5 μM), by interfering with the interaction of two GAC monomers to form a GAC dimer [27]. Wang *et al.* found that transformed fibroblasts and breast cancer cells exhibit elevated glutaminase activity that is dependent on Rho GTPases (Cdc42, Rac1, RhoC) and NF-kB activity, and the enzyme is blocked by the small molecule inhibitor **968**, by targeting the metabolic enzyme glutaminase [11]. Katt *et al.* provided the structure-activity relationship analysis of 968-derivatives. In the 968 derivatives, the 'hot-spot' ring of the molecule (H-ring, circled in blue) requires a large, anti-

periplanar group at the para position to retain inhibitory activity, and the naphthyl portion of the molecule (circled in red) can be significantly altered without affecting inhibitory activity (Fig. **4**) [28, 29].

BPTES: Unlike the catalytic site competitive inhibitor DON, bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES), introduced by Robinson *et al.,* is known to inhibit GAC through an allosteric mechanism that stabilizes an inactive tetrameric state of the enzyme upon binding [30]. BPTES has been shown to inhibit the growth of cancer cells in various tumor models, including non-small cell lung cancer (NSCLC) [31], renal cell carcinoma (RCC) [32], breast cancer [33], glioblastoma [34] and B cell lymphoma [35]. For example, in NSCLC, Lee *et al.* reported that the level of GLS1 is greatly increased, and this increased level of GLS1 is inversely correlated with the overall survival rate in NSCLC. The inhibition of GLS1, using BPTES, induced cell cycle arrest with a significant reduction in the ATP level, caused by glutamate reduction, which eventually led to cell death. When BPTES (10 mg/kg, p.o.) was concomitantly administered with the thymidylate synthase (TYMS) inhibitor 5-FU (20 mg/kg, p.o.) in A549 mouse xenograft experiment, significant inhibition of tumor growth was observed [31]. Shroff *et al.* found that the MYC oncogene and glutaminase are overexpressed in human renal cell carcinoma (RCC). The dependency of MYC-induced renal adenocarcinoma on the glutamine supply was confirmed using the MYC-induced renal carcinoma cell line E28. Decreased levels of glutamine transporters and glutaminolysis were observed upon MYC deactivation (MYC-OFF). The inhibition of glutaminase by BPTES halted the growth of MYC-induced RCC tumor progression, both *in vitro* and *in vivo*. The *in vitro* inhibitory activity of BPTES against E28 was observed in a dose-dependent fashion. The *in vivo* antitumor activity of BPTES, using an MYC-induced transgenic mouse model, was examined by using magnetic resonance imaging (MRI). Here, a significant reduction in tumor growth was observed after 14 days of BPTES treatment, compared to the DMSO control group (percent of tumor growth: BPTES/DMSO 49%/70%). The mean tumor weight of the kidney from the BPTES group was 32% less than that of the DMSO group [32]. Therefore, numerous studies support the idea that the inhibition of glutamine metabolism could be a potential therapeutic approach for the treatment of RCC associated with MYC overexpression. Overall, BPTES appeared to demonstrate therapeutic utility over a wide range of cancer types; however, its low solubility and moderate potency (IC_{50} ~3 μM) has limited its progress towards pharmacological applications [36]. X-ray structure studies on the interaction of BPTES with GAC are described in the structure study section below.

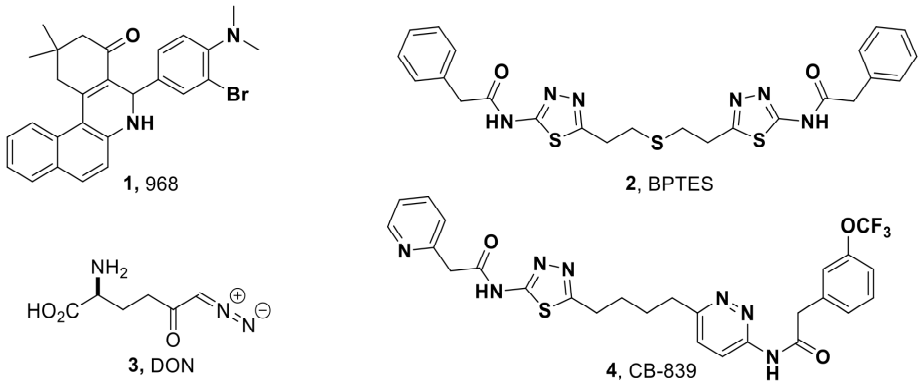


Fig. (2). Representative glutaminase inhibitors.

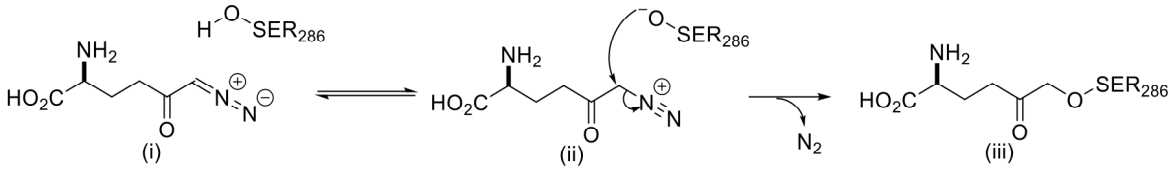


Fig. (3). Proposed active site inhibition mechanism of KGA. (i) Nucleophilic attack of Ser286 on DON; (ii) generation of a covalent linkage between Ser286 and DON and the subsequent release of N_2; (iii) formation of acetyl-inhibitor complex.

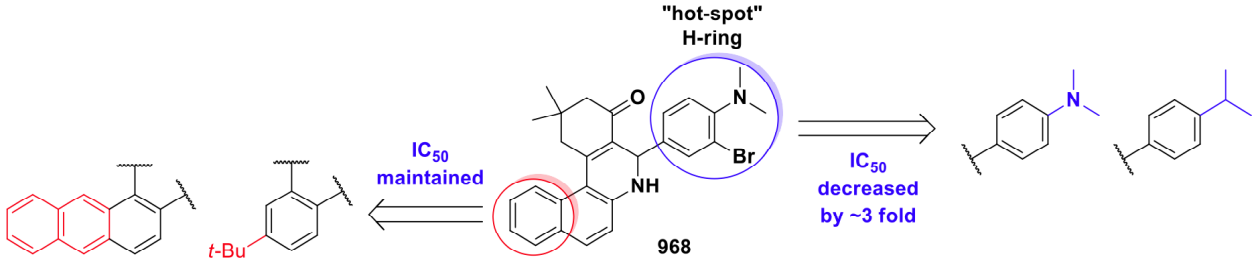


Fig. (4). The structure-activity relationship of 968-derivatives.

CB-839: CB-839 has been more extensively studied for its anti-tumor activity than the other glutaminase inhibitors discussed above. CB-839 is currently being used in several clinical studies in various stages for a broad range of cancers, including colorectal cancer, colon cancer, AML, clear cell renal cell carcinoma (ccRCC), non-small cell lung cancer (NSCLC), and triple negative breast cancer (TNBC) [37]. Detailed descriptions of the clinical studies using CB-839 are provided in the clinical study section below. More recent preclinical studies of CB-839 that are closely related to current clinical studies are discussed here. CB-839, a BPTES derivative, is a potent, selective, and orally bioavailable glutaminase inhibitor with an IC_{50} of ~ 30 nM for recombinant human GAC (rHu-GAC), which is approximately 100-fold lower than for BPTES (IC_{50} ~3 μM) [38]. CB-839 exhibits time-dependent and slow, reversible kinetics. CB-839 showed anti-proliferative activity in the triple-negative breast cancer (TNBC) cell lines HCC-1806 and MDA-MB-231, while no anti-proliferative activity was observed in an estrogen receptor–positive/HER2 negative ($ER^+/HER2^-$) cell line, T47D. Across a panel of breast cancer cell lines, CB-839 more greatly elevated glutamine levels and lowered glutamate levels in the majority of TNBC cell lines than in ER^+ cells. CB-839 also showed anti-tumor activity in xenograft models of TNBC and $HER2^+$ basal-like breast cancer. In a patient-derived TNBC xenograft model, CB-839 (200 mg/kg, p.o., BID) suppressed tumor growth by 61% relative to the vehicle control. In the mouse JIMT-1 xenograft model, CB-839 alone (200 mg/kg, p.o., BID) resulted in 54% tumor growth inhibition (TGI), while paclitaxel (10 mg/kg, p.o., QOD) alone showed an initial regression of the JIMT-1 tumor, but a rapid relapse followed, resulting in 73% TGI. When the combination of CB-839 (200 mg/kg, p.o.) with paclitaxel (10 mg/kg, p.o., QOD) was used, a synergistic effect was observed in the suppression of the tumor regrowth, resulting in a complete regression of tumor growth (TGI of 100%). The results from a phase 1 clinical study of CB-839 (≥ 600 mg, p.o., BID) + paclitaxel (80 mg, i.v., D1, 81, 15, Q28) for TNBC reported ~50% objective response rate (ORR) in taxane-refractory African American patients [39]. CB-839 is currently in phase 2 clinical study for advanced TNBC in combination with paclitaxel (NCT03057600). At the 2017 Keystone Symposium, Emberley *et al.,* from Calithera Biosciences, reported that CB-839 had anti-tumor activity as a treatment for RCC [40]. In contrast to other targeted therapies for the treatment of RCC, CB-839 targets glutaminase to inhibit the formation of glutamate, which is directly involved in multiple reactions that support cancer cell growth, including the TCA cycle, the synthesis of amino acids, and cellular oxidative stress. In this report, Emberley *et al.* showed the cytotoxic effect of CB-839 in most of the RCC cell lines tested, 18 out of 23 RCC cell lines. In these CB-839-sensitive RCC cell lines, decreases in mTOR signaling were observed, indicative of the direct influence of glutamate deprivation by CB-839 on the mTOR pathway. Synergistic effects between CB-839 and the signal transduction inhibitors cabozantinib and everolimus were examined. The CB-839 + cabozantinib combination showed enhanced reductions in signaling via AKT and ERK and in TCA cycle activity compared to single agent treatments. In the Caki-1 xenograft model, both CB-839 (200 mg/kg, p.o., BID) + cabozantinib (1 and 10 mg/kg each, p.o., QD) and CB-839 (200 mg/kg, p.o., BID) + everolimus (1 mg/kg each, p.o., QD) combination therapies showed enhanced tumor growth inhibition compared to either monotherapy. In the case of CB-839 + cabozantinib, a more severe relapse was observed within seven days than was observed for CB-839 + everolimus, which prolonged significant tumor regression over 28 days. From a phase 1 clinical study of CB-839 (100~800 mg, p.o., TID/BID) with everolimus (10 mg, p.o., QD), the combination therapy showed a 93% disease control rate (DCR) and a PFS of 8.5 months in ccRCC and papillary RCC. CB-839 is currently in phase 1/2 and phase 2 clinical investigations in combination therapies with nivolumab (NCT02771626) and everolimus (NCT03163667), respectively, for RCC. Myelodysplastic syndromes (MDS) are a group of cancers in which immature blood cells in the bone marrow do not mature or become healthy blood cells. Signs and symptoms of a myelodysplastic syndrome include shortness of breath, feeling tired, easy bleeding, or frequent infections. Some types may develop into acute myeloid leukemia (AML) [37]. CB-839 has been broadly studied for its activity in blood cancers and hematologic malignancies, such as MDS, AML [41], multiple myeloma (MM) [42], and lymphoma [43], in phase 1/2 clinical settings (Table **1**). In the preclinical stage, Gregory *et al.* reported that the combination of FMS-like tyrosine kinase 3 (FLT3) inhibitor AC220 with CB-839 may be an effective therapeutic strategy for the treatment of FLT3-mutated AML [44]. The FLT3 inhibitor AC220 inhibits glutamine flux into the antioxidant factor glutathione, due to defective glutamine import, and the glutaminase inhibitor CB-839 impairs glutathione production by blocking glutamine flux into glutamate. Taken together, the combination of AC220 with CB-839 synergistically leads to glutathione depletion, induces mitochondrial reactive oxygen species (ROS), and causes a loss of viability through apoptotic cell death. *In vivo*, the combination of CB-839 (200 mg/kg, p.o., BID) with AC220 (5 mg/kg, p.o., QD) enhanced leukemic cell elimination persistently and improved survival (~1 week compared to AC220 alone) significantly in a patient-derived xenograft AML mouse model (FLT3-ITD and NPM1 mutations). Another study of CB-839 for the treatment of proteasome inhibitor (PI) resistant MM was reported by Thomson *et al.* [45]. The treatment of multiple myeloma (MM) has been limited by the resistance of PI. PI-resistant cells exhibited increased mitochondrial respiration, driven by glutamine as the principle fuel source. CB-839 inhibited mitochondrial respiration and was more cytotoxic in PI-resistant cells as a single agent. When CB-839 was combined with the proteasome inhibitor carfilzomib (Crflz), the activity of Crflz was synergistically enhanced in a panel of genetically diverse PI sensitive and resistant MM cells. Mechanistically, CB-839 enhanced Crflz-induced endoplasmic reticulum (ER) stress and apoptosis, characterized by a robust induction of ATF4 and CHOP and the activation of caspases. A list of current clinical studies examining CB-839 for the treatment of various cancers is provided in Table **1**.

Calithera Biosciences, Inc. is developing CB-839, a leading candidate from a program of human glutaminase inhibitors, for the potential oral treatment of multiple cancers, including colorectal cancer, MDS, ccRCC, TNBC, NSCLC, and other solid tumors, in various stages of clinical trials

Table 1. Current clinical studies of CB-839 for the treatment of various cancers.

Entry	Study title (NCT No.; status; period)	Conditions	Outcome measures
1	CB-839 +capecitabine in solid tumors and fluoropyrimidine resistant PIK3CA mutant colorectal cancer (NCT02861300; Phase 1/2; Aug. 2016 ~ Jan. 2020)	colorectal, colon, rectal cancer and other solid tumors	RP2D, RR (Ph 2)
2	CB-839 +azacitidine for treatment of myelodysplastic syndrome (MDS) (NCT03047993; Phase 1/2; Nov. 2017 ~ Nov. 2022)	myelodysplastic syndrome, other diseases of blood and blood-forming organs	MTD finding, AE, PK/PD testing, clinical activity
3	CB-839 w/ everolimus vs. placebo w/ everolimus in patients with RCC (NCT03163667; Phase 2; July 2017 ~ Mar. 2021)	clear cell renal cell carcinoma	PFS, OS
4	CB-839 in combination w/ paclitaxel in patients of African ancestry and non-African ancestry with advanced TNBC (NCT03057600; Phase 2; May 2017 ~ May 2020)	triple negative breast cancer	ORR, PFS, OS, DOR, CBR
5	CB-839 in combination w/nivolumab in patients with melanoma, ccRCC and NSCLC (NCT02771626; Phase 1/2; Aug. 2018 ~ June 2019)	clear cell renal cell carcinoma, melanoma, non-small cell lung cancer	AE, change in tumor size, RP2D, MPC
6	Novel PET/CT imaging biomarkers of CB-839 in combination w/ panitumumab and irinotecan in patients with metastatic and refractory RAS wildtype colorectal cancer (NCT03263429; phase 1/2; Aug. 2017 ~ Sept. 2020)	colorectal cancer (metastatic, RAS wildtype, and refractory)	MTD, RP2D, RR (Ph 2), OS, DCR, PFS, SUV_{max} of 18F-FSPG uptake (Ph 2), plasma exosomal content

RP2D: recommended phase 2 dose, RR: response rate, MTD; maximum tolerated dose, AE: adverse events, PK: pharmacokinetics, PD: pharmacodynamics, PFS: progression-free survival, OS: overall survival, ORR: objective response rate, DOR: duration of response, CBR: clinical benefit rate, MPC: maximum plasma concentration, DCR: disease control rate, SUV_{max}: maximum standardized uptake value, 18F-FSPG: F18 L-glutamate derivative BAY94-9392.

(Table **1**). As discussed above, CB-839 is used as a regulator of glutamine flux in combination with other known therapeutic agents. For most clinical studies, CB-839 is used at rather higher doses (for example, 600 mg, p.o. for the MDS trial, NCT03047993, and 800 mg, p.o. for the TNBC trial, NCT03057600) using a BID administration schedule, likely due to its short half-life and low plasma concentration. The pharmacokinetic properties of CB-839 are briefly discussed in a separate section.

3. EXCERPTS FROM CALITHERA BIOSCIENCES PATENTS ON CB-839

As previously indicated, CB-839 is being used at rather higher doses in a BID administration schedule. Curiosity drove the authors to locate original data from patents covering the *in vitro* ADME and *in vivo* PK properties of CB-839 to understand its effect in *in vivo* efficacy tests and its relation to human clinical trials. In the patents filed by Calithera Biosciences, CB-839 was assessed for its ability to inhibit the enzymatic activity of recombinant glutaminase 1 (GAC), using a biochemical assay that couples the production of glutamate (liberated by GAC) to glutamate dehydrogenase (GDH) and measures the change in absorbance to determine the reduction of NAD^+ to NADH. CB-839 had an IC_{50} (60 min preinc.) of 0.005 μM (5 nM) using this assay. For the cell proliferation assay, P493-6 (myc "on") cells were incubated with the test compound for 72 hrs at 37 °C prior to analysis. CB-839 had a cell proliferation IC_{50} (72 h preinc.) of 0.03 μM (30 nM) [46]. Caco-2 permeability and solubility data can also be found in the same patent. CB-839 (1 μM, 2 hrs) showed high permeability across the cell monolayer, with a high efflux ratio of 6.2. The solubility data of CB-839 in various solvents are provided below, and the best solubility achieved is 9.685 mg/mL in the solvent 20% HPBCD/50 mM citrate (Table **2**).

Pharmacokinetic properties of CB-839 in both rat and mouse, using a vehicle of 20% HPBCD/10 mM citrate, are reported by the same group [47]. The plasma concentrations of CB-839 and glutamine were measured at each dose level. Consistent increases in the CB-839 and glutamine levels in plasma were observed in a dose-dependent fashion with doses of 25, 80, 250, and 500 mg/kg in female SD rats (p.o.), using hydroxypropyl-β-cyclodextrin (HPBCD)/ citrate as the vehicle. It is noteworthy that the T_{max} value was relatively fast, with the exception of the 250 mg/kg case treatment (Table **3**).

Based on these PK properties, CB-839 was subjected to various *in vivo* xenograft studies including lung adenocarcinoma (female SCID/beige mice, H2122 cells, CB-839 200 mg/kg, BID, p.o., 23 days), triple negative breast cancer

Table 2. Solubility data of CB-839 in various solvents.

Solvent/Solubility (mg/mL)				
Water	**0.9% NaCl**	**0.1 M HCl**	**50 mM cit. pH 2.3**	**50 mM cit. pH 3.3**
0.007	<0.002	0.005	0.066	0.003
EtOH	labrasol	PBS	50 mM cit. pH 4.4	50 mM cit. pH 5.4
0.964	5.024	<0.002	<0.002	<0.002
canola oil	PG	20% HPBCD/ 50 mM cit.	10% CrEL/ 50 mM cit.	20% SBECD/ 50 mM cit.
0.012	2.569	9.685	0.458	5.256
capryol 90	capryol PGMC	PEG400	0.1 M NaOH	10% PS80/ 50 mM cit.
1.974	1.519	9.901	0.227	1.204

Table 3. Pharmacokinetic properties of CB-839 in rat and mouse.

Species	Dose (mg/kg)	Vehicle	C_{max} (μM)	T_{max} (h)
rat (female SD)	25	20% HPBCD/10 mM citrate pH 2.0	4.4	1
	80	65% HPBCD/10 mM citrate pH 2.6	12.5	1
	250	40% HPBCD/10 mM citrate pH 2.2	9.9	24
	500	40% HPBCD/10 mM citrate pH 2.2	15.3	2
mouse (female CD-1)	100	25% HPBCD/10 mM citrate pH 2.2	5.75	1
	200	25% HPBCD/10 mM citrate pH 2.2	5.05	1

(CB.17 SCID mice, JIMT-1 cells; CB-839 200 mg/kg, p.o., BID x 35 days; paclitaxel 10 mg/kg, i.v., pod x 5 days; and CB-839 [(200 mg/kg, p.o., BID x 35 days) + paclitaxel (10 mg/kg, i.v., QOD x 5 days)], and multiple myeloma (female CB.17 SCID mice, RPMI-8226 cells, CB-839 200 mg/kg, p.o., BID, 28 days). CB-839 showed minimal to medium efficacy in the lung adenocarcinoma xenograft model and in the multiple myeloma xenograft model when it was administered as a single therapy of 200 mg/kg, p.o., BID. However, in the triple negative breast cancer xenograft study (CB.17 SCID mice, JIMT-1 cells), a combination therapy of CB-839 and paclitaxel resulted in the complete regression of the tumor after 35 days of treatment. Moreover, CB-839 was further applied for the treatment of multiple myeloma cell lines, including RPMI-8226 and MM1S cells [48]. The cell viability of CB-839, lenalidomide [LEN], and pomalidomide [POM] for MM1S and RPMI-8226 cells were reported as IC_{50} values of 0.104, 0.041, 0.022 μM for MM1S and 0.1, 0.392, 57.032 μM for RPMI-8226, respectively. Additionally, when the multiple myeloma cells MM1S & RPMI-8226 were treated with a mixture of CB-839 and pomalidomide [POM] or lenalidomide [LEN], a synergistic effect of the combination therapy was observed in an antiproliferation assay, in a dose-dependent manner. This synergistic effect of the combination therapy was confirmed by an *in vivo* efficacy study using an RPMI-8226 myeloma xenograft model (female SCID/beige mice). When a combination of CB-839 (200 mg/kg, p.o., BID x 24 days) and lenalidomide (30 mg/kg, QD, p.o. x 24 days) or pomalidomide (1 mg/kg, QD, p.o.) was used, complete tumor regression was observed over the whole treatment period of over 20 days. Finally, Calithera also filed a separate patent describing a combination therapy of CB-839 with the check-point inhibitor PD-L1/PD-1. In a colon cancer xenograft model (balb/c mice, CT26 cells), CB-839 (200 mg/kg, p.o., BID, starting day 2) together with α-PD-L1 (5 mg/kg, ip, QOD, days 5, 7, 9, 11, 13, 15) as a combination therapy resulted in rapid tumor regression [49].

4. X-RAY CRYSTAL STUDY

A number of X-ray crystal structures have been reported to describe the interactions between inhibitors and GAC [2, 50]. The identification of the new allosteric inhibitory site occupied by the BPTES molecule in the GAC tetramer represents a new opportunity to inhibit GLS1 in a specific manner, potentially providing an avenue to avoid the toxicity observed with other glutaminase inhibitors. Robinson *et al.* first described the inhibition mechanism of rat KGA by the small molecule inhibitor BPTES [bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide] [30]. A kinetic study demonstrated that BPTES had a highly effective affinity for KGA, with a Ki of 3 μM for both the free enzyme and the

enzyme-substrate complex. It was also suggested that BPTES inhibits the allosteric activation caused by phosphate binding and promotes the formation of a highly stable inactive tetramer, regardless of the phosphate concentration. The resulting data established that BPTES selectively inhibits the allosteric activation caused by phosphate binding and promotes the formation of a highly stable inactive tetramer. The first full-length crystal structure of human glutaminase, both with and without BPTES, was reported by DeLaBarre *et al.* [51]. Coordinates and structure factors have been deposited in the Protein Data Bank as entries 3UNW and 3UO9. Both the BPTES bound and unbound GLS1 (GAC) was comprised of residues 71-598, arranged as a tetramer with both long and short interfaces. For both the bound and unbound tetramer structures, each of the four monomers possessed a glutamate in the active site with identical conformations, with slight variations in the loops at the short dimer interface (Fig. **5**). In the BPTES bound GAC, it was found that BPTES binds to each of the two short dimer−dimer interfaces, so that there is one BPTES per GLS1 dimer. BPTES interacts with the loop region, residues 320−327, and the interface helix α-13, residues 386−399. The loop region undergoes a large rearrangement upon BPTES binding. F322 swings toward Y394 to form a hydrophobic "π-basket", which accommodates the central thioether segment of BPTES. Each of two thiadiazolyl amide moieties of the symmetrical BPTES molecule forms three major hydrogen bonds with the loop region: N_4 with the backbone amide nitrogen of F322, and N_3 and N_6 with the backbone amide nitrogen and carbonyl oxygen of L323, respectively. In addition, it was suggested that the terminal phenyl ring of BPTES extends into the solvent region. This selective binding was confirmed by using BPTES resistant mutant forms of GAC (a F318Y/F322S double mutant and a Y394L single mutant), where nearly no inhibitory activity was observed upon treatment with BPTES (IC_{50} > 100 μM, compared to an IC_{50} of 80 nM against wild-type GAC). Finally, it has been suggested that the glutamate-bound structure of glutaminase represent the inactive enzyme-product complex that occurs after releasing ammonia from the active site. Between the BPTES-bound and unbound forms of GAC, no conformational change was observed in the active site, suggesting that the glutamate-bound GAC structure represents an inactive conformation of the tetramer and that the BPTES molecule serves to stabilize the glutamate-bound tetramer.

In a related study, Stalnecker *et al.* developed a fluorescence resonance energy transfer (FRET) assay to measure

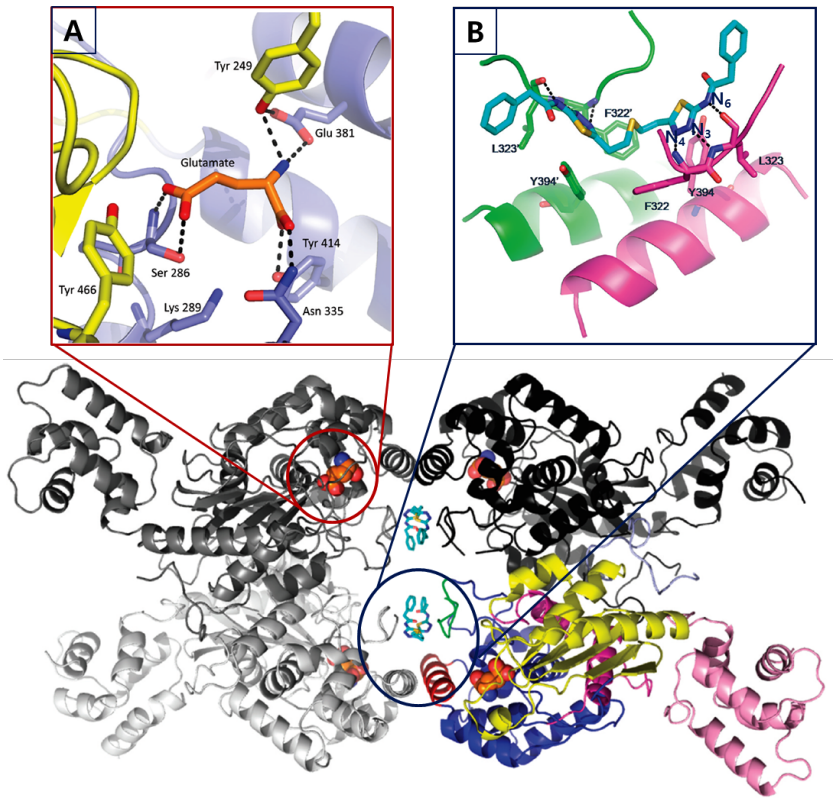


Fig. (5). GAC tetramer with BPTES. One monomer has the N-terminal domain colored pink, the α/β domain colored yellow, the extended helical domain colored blue, the short dimer−dimer helix interaction colored red, and the BPTES binding loop colored green. The three remaining tetramers are shown as shades of gray. The BPTES molecule is shown as a cyan stick figure, and the glutamate molecules are shown as orange spheres. **(A)** Glutamate binding pocket. The bound glutamate is shown as an orange stick. Key amino acids forming H-bonds are shown as sticks. **(B)** BPTES binding pocket interactions. The BPTES molecule is shown as a stick and is colored by atom type, with cyan carbons, yellow sulfurs, blue nitrogens, and red oxygens. The short dimer interface helices and BPTES binding loops are shown with monomers colored green and maroon. Key hydrogen bond interactions to peptide backbone atoms are represented as dashed lines, and the residues contributing to the hydrophobic π-basket (Y394 and F322) are shown. This research was originally published in *Biochemistry* **2011**, *50*, 10764-10770. Figures 2, 3 & 4. © The American Society for Biochemistry. The image was edited for repurposing.

the effects of allosteric activators, inorganic phosphate (HPO_4^{2-}) and sulfate (SO_4^{2-}), and the inhibitors BPTES and CB-839 on GAC tetramer formation in real time [52]. FRET signal was increased by the addition of both the allosteric activators and the inhibitors, indicating the formation of tetramers. However, upon the addition of excess unlabeled GAC subunits that could compete with the GAC FRET pairs, a decrease in the FRET signal was observed only for the activator-bound GAC tetramers, implying that the inhibitors better stabilize the tetrameric state (Fig. **6**). The binding site of BPTES within the GAC tetramer was examined using the known X-ray crystal structures of the KGA/GAC, and large conformational changes in the orientation of the activation loop were found in the GAC structure between the active site when bound to the inhibitor DON and when bound to the allosteric inhibitor BPTES (Fig. **7**). Focused on this large conformational change in the activation loop, a GAC(F327W) mutant, where tryptophan was used as a fluorescent reporter, was utilized to examine the conformational transition caused by the binding of allosteric inhibitors and activators in real time. BPTES was incorporated into GAC tetramers in an orientation of the activation loop that prohibits catalysis, which is indicated by a decrease in GAC(F327W) fluorescence. However, the binding of inorganic activators, such as phosphate or sulfate, increased the intrinsic GAC(F327W) fluorescence in proportion to the enzyme activity, which supports the idea that allosteric activators bind at the dimer-dimer interface, changing the conformation and environment of the loop to promote activated GAC tetramers.

5. MEDICINAL CHEMISTRY EFFORTS

Several groups have attempted to improve the physicochemical properties of BPTES and develop a novel class of GLS inhibitors. Studies focused on thiadiazole derivatives are discussed below.

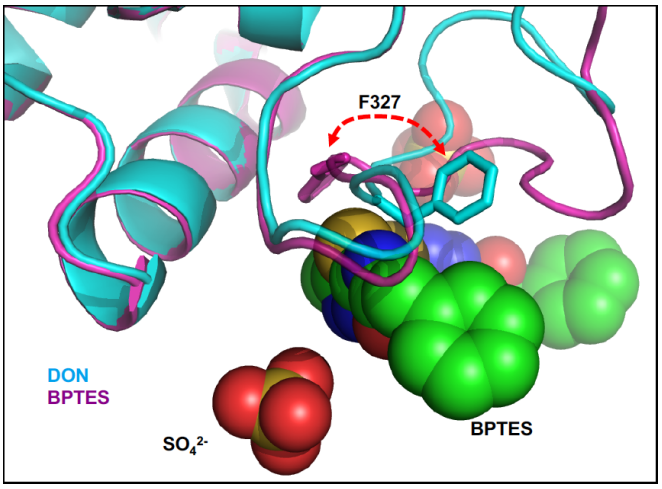


Fig. (7). Comparison of the activation loop of the GAC tetramer when bound with DON (4O7D), BPTES, and sulfate (SO_4^{2-}) (aligned from 3VOZ). BPTES and sulfate bind proximal to the activation loop, and DON binds within the active site. The DON-bound GAC structure is in cyan, and the BPTES/SO_4^{2-} bound GAC is in magenta. The ~180° rotation of the F327 residue with and without bound BPTES is highlighted (red dashed line). This research was originally published in *Journal of Biological Chemistry* (**2017**), *292*(15), 6095-6107. Figure 3B. © The American Society for Biochemistry and Molecular Biology. The image was edited for repurposing.

Although considerable attention has been paid to glutaminase as a potential therapeutic target for the treatment of cancers and inflammatory neurological disorders, the limited progress on the direct inhibition of the glutaminase active site can be attributed to the poor potency and selectivity of the inhibitors, such as 6-diazo-5-oxo-L-norleucine (DON) [23, 24, 53]. In 2002, a potent and selective inhibitor of kidney-type glutaminase (KGA), BPTES, targeting an allosteric site was reported by Newcomb [54]. One of a series of com-

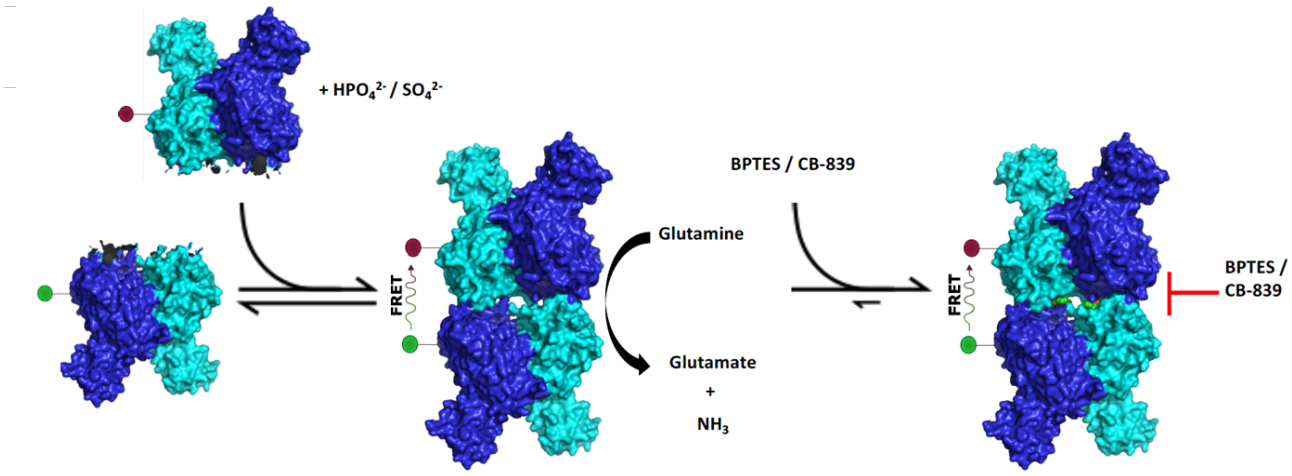


Fig. (6). The transition from a GAC dimer to a tetramer, where the tetramer species gives rise to the FRET signal. Both allosteric activators and BPTES-class inhibitors induce GAC tetramer formation. The allosteric activators inorganic phosphate (HPO_4^{2-}) and sulfate (SO_4^{2-}) promote the dimer to tetramer transition to emphasize their ability to activate GAC, whereas inhibitors BPTES and CB-839 bind to the FRET pairs at the dimer-dimer interface, forming a stable BPTES or CB-839-bound inactive GAC tetramer (BPTES is shown as green spheres). This research was originally published in *Journal of Biological Chemistry* (**2017**), *292*(15), 6095-6107. Figure 1D. © The American Society for Biochemistry and Molecular Biology. The image was edited for repurposing.

pounds that were synthesized in a symmetrical fashion with various substituents on amides, bis-2-(5-phenylacetamide-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES) demonstrated the most potent inhibitory activity against KGA. The advent of BPTES accelerated the development of glutaminase inhibitors for cancer treatment and attempts to develop a better GLS inhibitor have been pursued further. For example, Shukla *et al.* found that the removal of one or both of the two phenylacetyl groups and the central –S-CH_2-unit from the symmetrical BPTES did not sacrifice inhibitory activity against KGA, with pronounced increases in solubility and metabolic stability (Fig. **8**) [36]. Subsequently, Zimmermann *et al.* also showed that attaching a phenolic moiety on the terminus can act as both a hydrogen bond acceptor and donor, which resulted in a significant increase in the inhibitory activity against KGA and in the aqueous solubility (17 μg/mL vs. 0.14 μg/mL of BPTES) [55]. Consequently, based on an SAR analysis using BPTES derivatives, it was concluded that one of the two terminal substituents on symmetrical BPTES could be eliminated or replaced by phenol to achieve significant improvements in activity and solubility. These SAR studies could provide the groundwork for the identification of GLS inhibitors with improved physicochemical properties.

McDermott *et al.* desymmetrized BPTES by focusing on flexible middle linker [56]. Rotation-restricted saturated ring systems bearing other-than-sulfur heteroatoms were used as surrogates to mimic the flexible chain observed on BPTES bound to glutaminase in the X-ray structure [57]. The biochemical evaluation of the analogs was performed using an assay against recombinant kidney glutaminase isoform C (GAC) and in the MDA-MB-231 cancer cell line. In general, analogs with 5 or 6 membered ring systems as surrogates for the flexible chain showed 6- to 10-fold increases in potency against GAC. In addition, the cLogP, which was maintained as low as possible in the derivatives, as shown in Table **4**, correlated well with greatly improved stability in the human microsomal stability (HLMS) assay. Among the various BPTES analogs listed in Table **3**, compound **8** had the most potent nanomolar activity against GAC and MDA-MB-231, with decent microsomal stability.

Calithera Biosciences pursued independent research to circumvent the poor physicochemical properties of BPTES and disclosed BPTES analogs (709 compounds), including CB-839, in 2013 [58]. They adopted an approach that was utilized by Shukla *et al.* in an attempt to obtain better solubility and metabolic stability for their analogs by attaching polar soluble groups or by removing sulfide units. Surprisingly, replacing one of the two thiadiazoles with pyrazine resulted in dramatic increases in both enzymatic and cellular potencies (Fig. **9**). It is presumed that the terminal electron-withdrawing CF_3 group of CB-839 deactivates the aromatic ring system and increase the electronegativity of the pyridazinyl nitrogen atoms, thus strengthening the hydrogen bonding interactions. In addition, the trifluoromethoxy group increases the lipophilicity of CB-839 [59]. CB-839 possess the most potent *in vitro* activity to date and is orally available with good selectivity. The *in vivo* antitumor activities were also evaluated in xenograft models of various tumor types that are associated with glutamate metabolism. With all these results, CB-839 has been advanced to various cancer clinical trials.

Finally, an interesting class of GLS inhibitors, thiazolidin-2,4-dione derivatives, was reported by Yeh *et al.* [60]. Unlike the thiadiazole class of GLS inhibitors, such as BPTES or CB-839, mentioned above, it was predicted that thiazolidine-2,4-dione **9** and **10** bind to the substrate binding pocket of KGA and not to the dimer-dimer interface. A kinetic study revealed that **9** inhibits KGA in a mixed mode of competitive and partially noncompetitive inhibition. The combination therapy of two GLS inhibitors with different binding sites or with doxorubicin, a stabilizer of the topoisomerase-DNA complex, resulted in synergistic effects prohibiting carcinoma cell proliferation *in vitro* and *in vivo*. However, poor solubility and pharmacokinetic properties, such as the bioavailability of these compounds, limited their progress to therapeutic use. Nonetheless, the fact that the combination of two GLS inhibitors, for example, **10** with BPTES, showed synergistic effects on tumor growth inhibition is interesting because the synergistic effect was derived by targeting different binding sites on the same enzyme using two different classes of GLS inhibitors, which is rare.

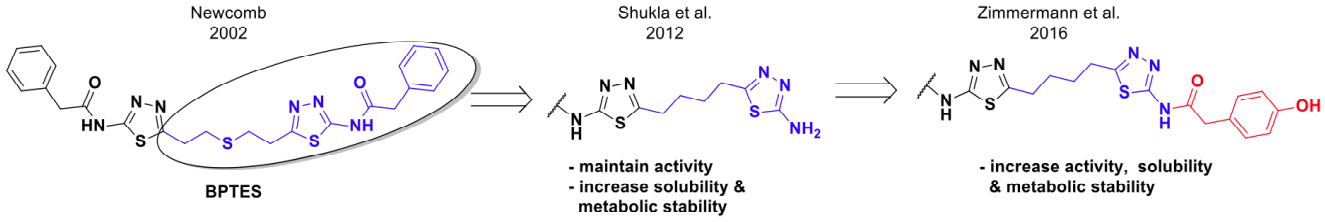
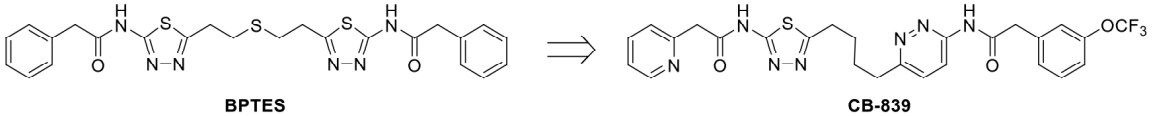


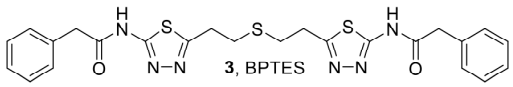
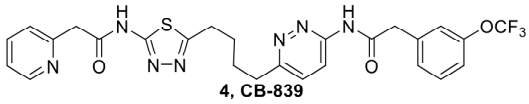
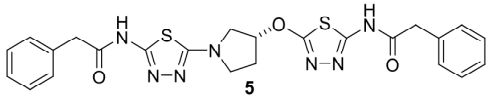
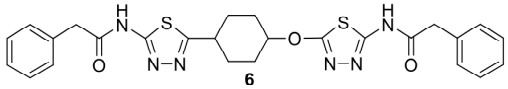
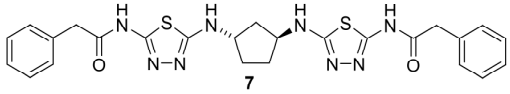
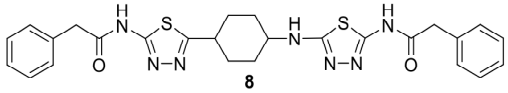
Fig. (8). Inhibitory activity and solubility of BPTES analogs.



	BPTES	CB-839
GAC IC_{50} (nM, 60m preinc.)	100	5
Cell Prolif. IC_{50} (nM, 72h, P493)	470	30

Fig. (9). Evolution of BPTES to derive CB-839 and their *in vitro* activities.

Table 4. Inhibitory activity and microsomal stability of BPTES analogs.

Entry	Structure	GAC IC_{50} (nM)	MDA-MB-231 IC_{50} (nM)	CLogP	HLMS (%, 30m)
1	 **3**, BPTES	371	2,610	4.15	56
2	 **4, CB-839**	180/25	33	4.74	25
3	 **5**	36	420	4.10	76
4	 **6**	30	140	4.16	78
5	 **7**	50	320	3.74	100
6	 **8**	29	70	3.80	100

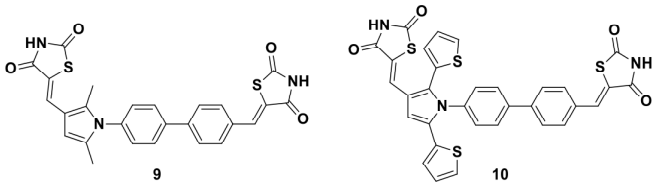


Fig. (10). Thiazolidine-2,4-dione class of GLS inhibitors.

CONCLUSION

Many tumors rely on the catabolism of glutamine to produce metabolic intermediates that fuel bioenergetic and biosynthetic demands [61]. The mitochondrial enzyme glutaminase (GLS) initiates this process by converting glutamine to glutamate, which is subsequently used in multiple reactions that support tumor cell growth and survival, including the generation of energy (TCA cycle), the synthesis of amino acids, and the production of glutathione. The activity of glutaminase correlates with the growth rates of human tumor cells, and kidney-type glutaminase (GLS1) is upregulated with increased rates of proliferation. The inhibition of GLS1 with siRNA knockdown or with small molecule inhibitors results in antiproliferative effects in tumor cells, both *in vitro* and *in vivo* [35]. Due to these vital roles of GLS1 in upregulating cell metabolism for tumor cell growth, targeting glutamine metabolism through the inhibition of glutaminase has gained considerable attention as a promising therapeutic approach for the treatment of cancer. CB-839 appears to be the most plausible drug candidate for cancers among many known GLS small molecule inhibitors in the literature. While Katt *et al.* discussed other GLS inhibitors developed by Astra Zeneca and Agios pharmaceuticals [29], a Cortellis database analysis revealed that no small molecule GLS inhibitors are being actively developed except CB-839. In this regard, CB-839 is clearly positioned to be a pioneer in the GLS inhibitor arena, and it is significant as a new therapeutic option for cancer for the same reason. Studies are still being performed to develop the next generation of GLS small molecule inhibitors. As Ramachandran *et al.* mentioned in their study [59], there may be room to investigate and further develop a newer class of GLS inhibitors that are linear and flexible thiadiazole derivatives of CB-839 and BPTES. For example, applying rigidity to the central linker of CB-839 could be a viable option to consider. It is also noted by the same authors that the terminal atoms of CB-839 do not engage in key binding interactions with cKGA, therefore chemical modifications can be targeted near this region to improve the solubility of new inhibitors. Another aspect includes the prospective potential of combination therapy using multiple GLS inhibitors, including CB-839. Calithera recently announced that CB-839 showed positive results in a phase 1 study in combination with cabozantinib to treat advanced ccRCC by providing an ORR of 40% and a DCR of 100%. It is planned to begin phase 2 in 2018, 2Q. Another example of CB-839 in combination with Opdivo® (nivolumab) for melanoma, renal cell carcinoma and non-small cell lung cancer was reported in late 2017, achieving 67% and 75% stable disease in NSCLC and RCC, respectively. These results clearly suggest the combination therapy can be a considerable option for developing future GLS inhibitors.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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