Inhibition of Human Glutaminase by 5-[3-bromo-4-(dimethylamino)phenyl]-2,3,5,6-tetrahydro-2,2-dimethylbenzo[a]phenanthridin-4(1H)-one

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Abstract:

The reliance of cancer cells on glutamine catabolism to provide biosynthetic precursors and sustain energy requirements has focused interest on glutaminase inhibition as a potential strategy for treating malignant tumors. "Compound 968", 5-[3-bromo-4-(dimethylamino)phenyl]-2,3,5,6-tetrahydro-2,2-dimethylbenzo[a] phenanthridin-4(1H)-one, was recently reported to selectively block the growth of human breast cancer and B lymphoma cells by inhibiting glutaminase. The present study was undertaken to characterize the kinetics of 968 inhibition of the GAC isoform of human glutaminase in order to determine its mechanism of action and to facilitate the design of more effective benzophenanthridinone derivatives. However, when assayed in the absence or presence of either low or high phosphate concentration, very little inhibition of recombinant GAC was observed with concentrations of 968 up to 50 μM. By contrast, the glutaminase activity in intact mitochondria was inhibited about 30% by 10 μM 968. Thus, the reported effects of this small molecule on the growth of transformed cells may involve a mechanism other than direct binding to glutaminase.

Introduction

Glutamine is the most abundant amino acid in the blood.[1] This distinction reflects the important role of glutamine in nitrogen transport as well as its ability to serve as an anaplerotic substrate for the citric acid cycle. The ability of glutamine to be catabolized to α-ketoglutarate is particularly important for cancer cells. Cancer cells are known to rapidly metabolize glucose, but favor an anaerobic process in which the pyruvate produced by glycolysis is reduced to lactic acid instead of entering the citric acid cycle as acetyl CoA.[2] To maintain the citric acid cycle, cancer cells depend upon the hydrolysis of glutamine to glutamate, which is subsequently oxidized to α-ketoglutarate.

Hydrolysis of glutamine is catalyzed by the mitochondrial enzyme glutaminase. Three glutaminase isoforms have been described in mammals: KGA and GAC, which are splice variants of the *GLS1* gene, and LGA, which is encoded by the *GLS2* gene. LGA is found only in the liver, while KGA occurs in a number of tissues, including kidney and brain, and GAC is most abundant in the pancreas and heart muscle.[3] Since it was first identified in 1999, GAC has attracted particular interest because it is the principal isoform of glutaminase in malignant cells such as human breast cancer cells. Its expression is also increased in human B lymphoma and in prostate cancer cells.[4] These observations suggest that selective inhibition of GAC could attenuate the growth of malignant cells.

A number of glutaminase inhibitors have been reported. Some, such as the KGA inhibitor L-2-amino-4-oxo-5-chloropentanoic acid (CK), are reactive analogues of glutamine that bind to a site that is specific for glutamate.[5] Another reactive analogue, 6-diazo-5-oxo-L-norleucine (DON), competes with glutamine for binding to the active site of KGA.[6] More recently, the compound bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES) was identified as a particularly potent uncompetitive inhibitor of KGA or GAC that functions by promoting the formation of an inactive tetramer of glutaminase.[7] This conclusion was recently confirmed by determination of the X-ray crystallographic structure of the GAC/BPTES complex.[8]

The benzophenanthridinone derivative, 5-[3-bromo-4-(dimethylamino)phenyl]-2,3,5,6-tetrahydro-2,2-dimethylbenzo[a] phenanthridin-4(1H)-one, or "compound 968", was first reported as a GAC inhibitor in 2010.[9] Similar to BPTES, 968 blocked the growth of transformed cells and was initially described as an allosteric inhibitor of glutaminase. However, the kinetic mechanism by which 968 inhibits GAC activity was not characterized. Therefore, the present study was designed to characterize the kinetics of 968 inhibition of GAC, with hopes that the resulting information would facilitate efforts to synthesize more effective analogues of 968.

Experimental Procedures

$hGAC_{\Delta 1}$ Expression

E. coli containing the $hGAC_{\Delta 1}$ plasmid, which encodes a truncated form of human GAC that lacks the N-terminal mitochondrial targeting signal, were grown in LB media.[10] The plasmid was isolated using a Qiagen Plasmid Maxi kit and it exhibited a 260/280-absorbance ratio of 1.6. BamH1 and Nde1 restriction digests were analyzed to confirm the plasmid. BL-21(DE3) competent E. coli cells were transformed with the purified $hGAC_{\Delta 1}$ plasmid and plated on agar/LB medium containing 0.25% ampicillin and 0.125% chloramphenicol to select for transformed cells. Single colonies from the plate were cultured for seventeen hours at 37oC in three separate tubes of 2xYT medium containing 0.01% ampicillin and 0.0025% chloramphenicol. Aliquots (500 μL) of each culture were transferred to three 1-L Erlenmeyer flasks containing 500 mL of 2xYT containing 0.01% ampicillin. Cell growth was promoted by shaking at 37oC and monitored by recording the absorbance

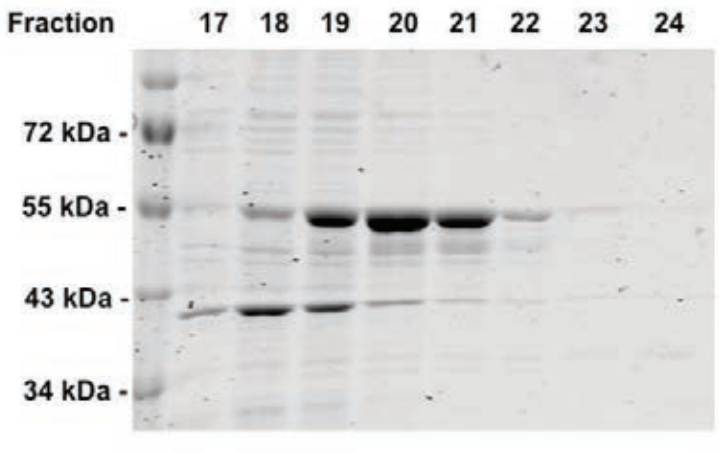

Figure 1: FLPC elution of hGACΔ1 from the Nickel-affinity column. Fractions 17-24 were separated on an SDS-10% polyacrylamide gel and stained with Coomassie-Blue. Fractions 20 and 21 were combined and used for the kinetic assays.

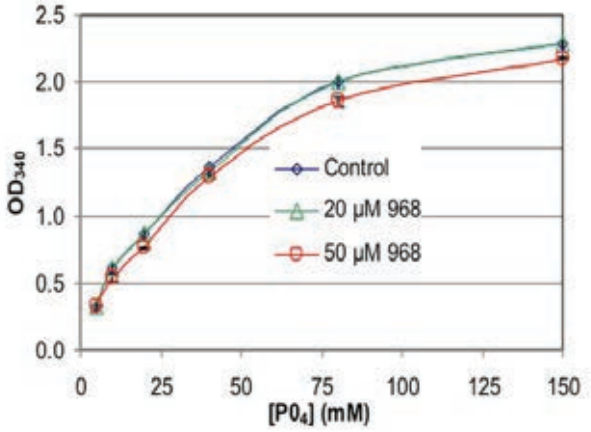

Figure: 2: Phosphate activation profiles of hGACΔ1. The hGACΔ1 activity was measured in the presence of increasing phosphate concentrations (2.5 to 150 mM) and in the absence and presence of 968. The data are the mean of three measurements for the control and 20 μM 968 and of two measurements for 50 μM 968.

at 600 nm. The cell cultures reached an OD_{600} value of 1.3 after five hours. At this point, the cells were cooled to 18oC and induced by the addition of 250 μL of 100 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After 18 hours of shaking at 18oC, the cells were pelleted by centrifugation at 2000 x g for fifteen minutes at 4oC and then stored at -80oC.

$hGAC_{\Delta 1}$ Purification

Cells containing the expressed $hGAC_{\Delta 1}$ were thawed and re-suspended in 20 mL of a lysis buffer consisting of 300 mM potassium chloride, 10 mM Tris chloride (pH 8), 10 mM potassium phosphate (pH 8), 10 mM imidazole, 10% v/v glycerol, and 1 tablet of complete Protease Inhibitor Cocktail (Roche Diagnostics) per 50 mL of buffer. The combined cell suspensions were subjected to four 30-second disruption cycles using an ultrasonic probe set to a 70% duty cycle and an output setting of 7. The cell suspensions were cooled in ice water during the sonication, with 1-minute pauses between cycles. Cell debris was pelleted by ultracentrifugation at 128,000 x g for thirty minutes at 4oC, and the supernatant was filtered through 0.22 μm syringe filters. Fractionation of His_6-tagged $hGAC_{\Delta 1}$ was performed by FPLC on a 5-mL HiTrap Chelating HP nickel-resin column using gradient elution with "Buffer A" (300 mM potassium chloride, 10 mM trishydroxyethylammonium chloride (pH 8), 10 mM potassium phosphate (pH 8), and 10% v/v glycerol), and "Buffer B" ("Buffer A" containing 1M imidazole). Tris-(2-carboxyethyl)phosphine hydrochloride (TCEP) reducing agent was added at a 50 μM level to both chilled buffers prior to fractionation, which was carried out in a cold room. Fractionation was achieved at a 3 mL/min flow rate while collecting 3 mL fractions. The first 8 fractions were eluted with 1% "Buffer B" (10 mM imidazole) and then stepped up to 5% and 10% "Buffer B" at fractions 9 and 11, respectively. Gradient elution was initiated at fraction 15 at a rate that would have reached 100% "Buffer B" composition over a period of 25 minutes, but was switched back to isocratic elution with 65% "Buffer B" at fraction 30. SDS-10%-PAGE followed by Coomassie Blue staining was used to determine which FPLC fractions contained the highest percentage of $hGAC_{\Delta 1}$.

Glutaminase Assay

$hGAC_{\Delta 1}$ activity was quantified using a modified version of the assay published by Curthoys and Weiss.[11] The standard glutaminase assay mixture contained 20 mM glutamine, 150 mM potassium phosphate, 50 mM Tris acetate (pH 8.6), and 0.2 mM EDTA. The standard glutamate assay mixture contained 80 mM Tris acetate (pH 9.4), 200 mM hydrazine, 0.25 mM ADP, 2 mM NAD+, and 0.2 mg/mL glutamic dehydrogenase. The assay was initiated by adding 2-5 μL of enzyme to 100 μL of glutaminase assay mixture in a test tube at 37oC. The reaction was stopped after ten minutes by the addition of 10 μL of 3 N HCl. Blanks were run for each experiment by adding the HCl to the glutaminase assay mixture before addition of enzyme. The amount of glutamate formed in the initial incubation was determined by adding 1 mL of glutamate assay mixture. After thirty-five minutes at room temperature, the absorbance at 340 nm was measured. Units of activity were then calculated using an NADH extinction coefficient of 6.27 mL μmol-1. Protein was determined using the Bradford dye-binding assay.[12]

Enzyme kinetics experiments were conducted in 96-well clear bottom plates using a Biotek Synergy 4 plate reader to measure path lengths and optical densities. Inhibition studies were carried out with 968 dissolved in dimethylsulfoxide (DMSO). The final DMSO concentrations in the assays ranged between 5 - 10%. Microplate assays were generally initiated by the addition of enzyme, but some assays were initiated by adding glutamine after addition of $hGAC_{\Delta 1}$. The kinetics of 968 inhibition in the presence of 150 mM or 10 mM phosphate was performed by adding 20 μL of either the standard glutaminase assay mixture or an assay mixture containing 10 mM phosphate to the 96-well plate. This was followed by adding 2.5 μL of 968 solutions in DMSO and either 1 μL of a 20-fold dilution of $hGAC_{\Delta 1}$ for wells containing 150 mM phosphate or 2 μL for the wells containing 10 mM phosphate. The reactions were incubated at 37oC for ten minutes and then quenched by the addition of 3 μL of 2 N HCl. The assay was completed by adding 200 μL of the glutamate assay mixture, incubating for thirty-five minutes at room temperature, and recording the absorbance of each well at 340 nm. A similar experiment was performed in the absence of added phosphate by charging a 96-well plate with 35 μL of a glutaminase assay mixture lacking both

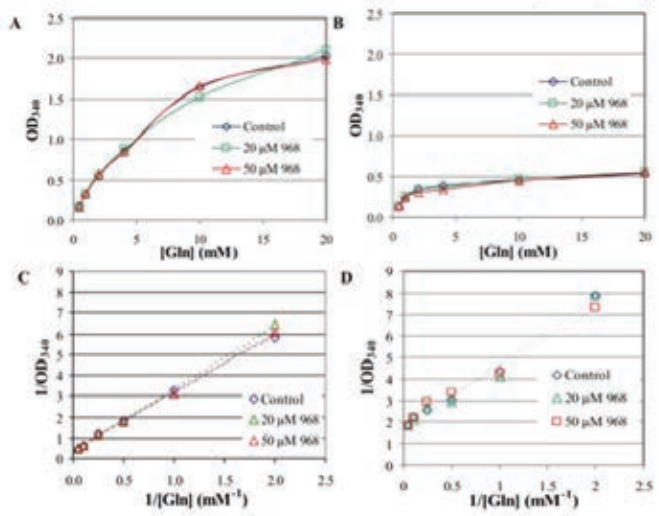

Figure 3: Glutamine saturation profiles for hGACΔ1 determined in the absence and presence of either 20 μM or 50 μM 968. The data are the mean of three measurements for the control and for 20 μM 968 and of two measurements for 50 μM 968. A) Assay at 100 mM phosphate; B) Assay at 10 mM phosphate; C) Lineweaver-Burk plot of 100 mM phosphate data; D) Lineweaver-Burk plot of 10 mM phosphate data.

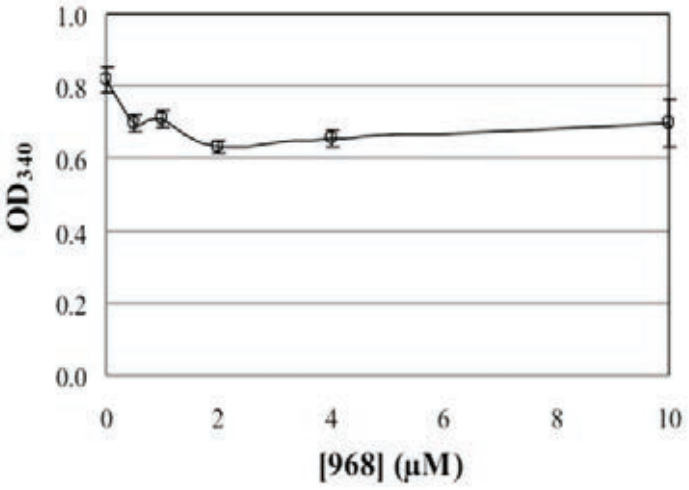

Figure 4: Effect of 0.5-10 μM 968 on hGACΔ1 activity in the absence of added phosphate. The data represent the mean of four measurements ± the standard deviation.

phosphate and glutamine. This was followed by the sequential addition of 5 μL of a 2-fold dilution of $hGAC_{\Delta 1}$, 4 μL of 968 solutions in DMSO, and 16 μL of 25 mM glutamine.

To replicate the previous experimental protocol, 75 μL of a solution containing 64 mM Tris acetate, 0.27 mM EDTA, and 4.4 μg of $hGAC_{\Delta 1}$ were added to each well of a 96-well plate.[9] Subsequently, 5 μL of various dilutions of 968 in DMSO were added and the samples were incubated at 35oC for 30 minutes. Then, 35 μL of 60 mM glutamine were added and the wells were incubated for another hour at 35oC. Glutamate formation was determined by transferring 10 μL of each sample to 200 μL of standard glutamate assay mixture and incubating for forty-five minutes at room temperature.

Glutamine saturation profiles were obtained by adding 10 μL of glutaminase assay mixture containing either 250 mM or 25 mM phosphate to the wells of a 96-well plate, followed by adding 10 μL of glutamine solutions ranging from 1.25 to 50 mM. To each well, 2.5 μL of either DMSO or 0.2 mM or 0.5 mM 968 solutions in DMSO were added. The reactions were initiated by the addition of 2.5 μL of a 5-fold dilution of $hGAC_{\Delta 1}$ and incubated for ten minutes at 37oC. The assays were completed by adding 200 μL of standard glutamate assay mixture, incubating for thirty-five minutes at room temperature, and then reading the absorbance at 340 nm.

The phosphate activation profiles were conducted by charging a 96-well plate with 20 μL of glutaminase assay mixtures in which the phosphate concentration ranged from 6.25 mM to 187.5 mM, followed by 2.5 μL of DMSO or either 0.2 mM or 0.5 mM 968 in DMSO. The reactions were initiated with 2.5 μL of a 5-fold dilution of $hGAC_{\Delta 1}$ and incubated for ten minutes at 37oC. The assays were completed as for the glutamine saturation profiles.

Mitochondria were isolated by differential centrifugation of rat brain that was homogenized in isotonic sucrose.[13] The purified mitochondria were diluted to a protein concentration of either 5.2 or 2.4 mg/mL. Samples containing 100 μL of the mitochondrial preparations were incubated for thirty minutes at room temperature with 2.5 μL of either DMSO or 968 solutions ranging in concentration from 0.22 to 10.9 mM. The wells of a 96-well plate were charged with 20 μL of glutaminase assay mixtures containing 150 mM, 10 mM, or 0 mM potassium phosphate followed by 5 μL of the 968/mitochondria mixtures (2.4 mg/mL levels for the wells containing phosphate and 5.2 mg/mL mitochondrial solution for the wells lacking phosphate). The plate was incubated at 40oC for ten minutes and the assay completed using 200 μL of glutamate assay mixture for each well as for the glutamine saturation profiles experiments.

Results

Human GAC (hGAC) is encoded by exons 1-15 of the *GLS1* gene that is located on chromosome 2.10 The enzyme used in this study, $hGAC_{\Delta 1}$, is a truncated version of hGAC that lacks the sequence encoded by the first exon, which contains the mitochondrial targeting signal. This modification prevents aggregation without affecting the glutaminase activity.[10] The recombinant $hGAC_{\Delta 1}$ enzyme includes an N-terminal His_6-sequence to facilitate its purification by nickel-affinity chromatography. Attempts to purify the $hGAC_{\Delta 1}$ by gravity elution with buffers containing imidazole proved to be surprisingly non-reproducible. However, gradient elution using an FPLC proved more effective. The enzyme required approximately 200 mM imidazole to elute from the nickel-affinity column. Analysis of the eluted fractions by SDS-PAGE demonstrated that considerable purification of $hGAC_{\Delta 1}$ was accomplished (Fig. 1). The bands corresponding to $hGAC_{\Delta 1}$ migrated slightly below the 55-kDa protein standard. The identification of this band as $hGAC_{\Delta 1}$ was confirmed by a Western blot using a rabbit polyclonal antibody to glutaminase (Aviva Systems Biology) that recognizes both the KGA and GAC isoforms. Protein concentrations for fractions 20 and 21 were determined to be 0.81 mg/mL and 0.69 mg/mL, respectively. The calculated specific activities for the two fractions were 102 units/mg and 95 units/mg, respectively. Based upon this analysis, fractions 20 and 21 were combined and used for the kinetic

analyses.

In the absence of phosphate, the purified glutaminase exists as a dimer that has little activity.[14] The addition of phosphate promotes the formation of a tetramer that is highly active. The phosphate activation profile of $hGAC_{\Delta 1}$ was determined in the presence of 20 and 50 µM 968 and compared to a control containing the same level of DMSO (Fig. 2). The control assay exhibited an activation profile that is characteristic of the enzyme purified from kidney mitochondria when assayed in the absence of DMSO.[14] The activation profiles for the control and the 20 µM 968 samples were identical. However, the profile obtained with 50 µM 968 exhibited a very slight inhibition at all concentrations of phosphate. Thus, 968 may be a weak inhibitor of the $hGAC_{\Delta 1}$ enzyme.

The potential inhibitory effect of 968 on $hGAC_{\Delta 1}$ activity was also characterized by analysis of its effect on activity with increasing concentrations of glutamine. The glutamine saturation profiles were determined with 10 mM and 100 mM phosphate and a five-fold dilution of the purified enzyme (Fig. 3A and B). The addition of either 20 µM or 50 µM 968 produced no inhibition at either concentration of phosphate. Michaelis constants for glutamine activation under high and low phosphate conditions were determined using Lineweaver-Burk double reciprocal plots (Fig. 3C and D) and Hanes-Woolf plots of glutamine concentration divided by activity vs. glutamine concentration (data not shown). This analysis indicated that the K_m for glutamine was decreased when the enzyme was assayed with the lower concentration of phosphate (Table I). However, the addition of either concentration of 968 had no effect on the apparent binding of glutamine to $hGAC_{\Delta 1}$.

As a follow up to the apparent lack of inhibition by 968, an assay was run in the absence of added phosphate (Fig. 4). In this experiment the $hGAC_{\Delta 1}$ was pre-diluted only by a factor of two, the final DMSO concentration was 7%, and the assay was initiated by the addition of glutamine. A modest inhibition by 968 was detectable without added phosphate. This analysis is not consistent with the previous report that 10 µM 968 was sufficient to produce a potent inhibition of GAC.[9] Therefore, the glutaminase assay was repeated exactly as described in the previous publication. In this assay, undiluted $hGAC_{\Delta 1}$ was pre-incubated for thirty minutes at 35oC with various concentrations of 968 in a phosphate-free buffer before initiating the assay through the addition of glutamine. This experiment produced greater variability, but showed no clear evidence of inhibition of $hGAC_{\Delta 1}$ by 968 (Fig 5).

The possibility that 968 inhibits glutamine transport into the mitochondria rather than glutaminase itself was examined by conducting an assay using whole mitochondria isolated from rat brain in place of the $hGAC_{\Delta 1}$ construct. In this analysis, the mitochondria were incubated with various concentrations of 968 for thirty minutes at room temperature before initiating the assay. A 30 % inhibition of the mitochondrial glutaminase activity was observed when assayed with 150 mM phosphate, but no inhibition was apparent when the assays were performed with 10 mM phosphate or with no added phosphate (Fig. 6).

Discussion

The principle goal of this study was to characterize the kinetics of 968 inhibition of a human GAC construct with the hope that this data might be used to direct future modifications to the chemical structure of 968. Although assays conducted in the absence of phosphate suggested that 968 produced a slight inhibition of $hGAC_{\Delta 1}$ (Fig. 4), the glutamine saturation profiles showed that 968 in fact had almost no effect on the activity of this glutaminase construct (Fig. 3). Furthermore, phosphate activation profiles gave no indication that 968 had any greater effect on $hGAC_{\Delta 1}$ at low phosphate

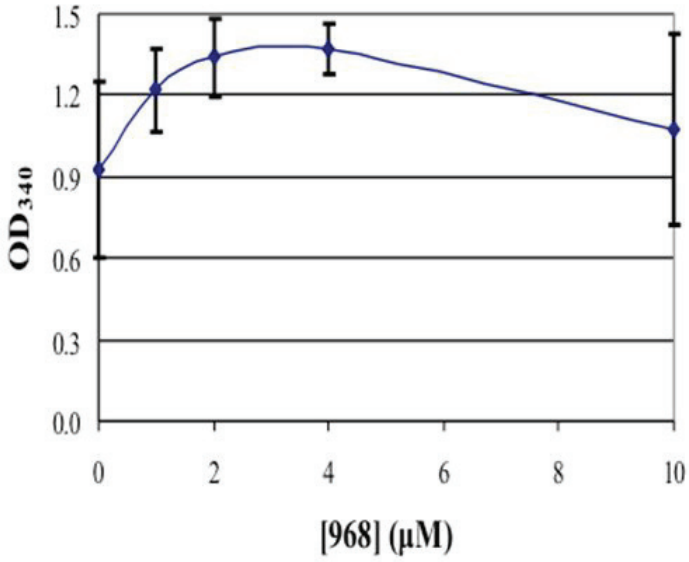

Figure 5: Effect of pre-incubating $hGAC_{\Delta 1}$ and the inhibitor 968 in the absence of added phosphate. The data are the mean of three measurements ± the standard deviation.

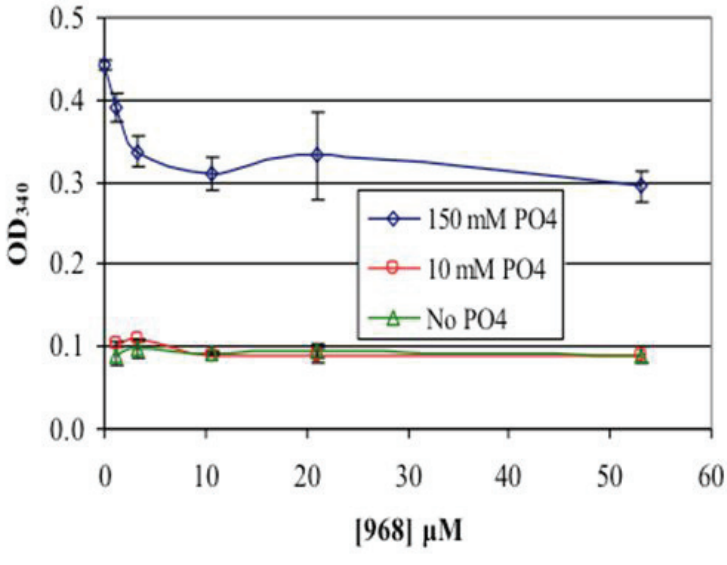

Figure 6: 968 inhibition of glutaminase activity in whole rat brain mitochondria at various phosphate levels. Freshly isolated rat brain mitochondria were pre-incubated with increasing concentrations of 968 and with three different concentrations of phosphate. The 150 mM phosphate data represent the mean of 6 measurements obtained from two experiments, while the 10 mM phosphate and no phosphate data are the mean of two measurements and three measurements, respectively, from a single experiment.

Table 1: Effect of 968 on the Michaelis Constants for Glutamine

	[PO4-3]	[968]	Slope	Intercept	R^2	K_m
Lineweaver-Burk:	100 mM	0 μM	2.74	0.419	0.9986	6.6 mM
		20 μM	3.01	0.313	0.9973	9.6 mM
		50 μM	2.84	0.357	0.9988	8.0 mM
Hanes-Woolf		0 μM	0.344	2.94	0.9914	8.6 mM
		20 μM	0.329	3.03	0.9943	9.2 mM
		50 μM	0.354	2.86	0.9889	8.1 mM
	[PO4-3]	**[968]**	**Slope**	**Intercept**	**R^2**	**K_m**
Lineweaver-Burk	10 mM	0 μM	3.01	1.71	0.9908	1.8 mM
		20 μM	2.97	1.66	0.9782	1.8 mM
		50 μM	2.64	1.93	0.9830	1.4 mM
Hanes-Woolf		0 μM	1.76	3.09	0.9954	1.8 mM
		20 μM	1.70	3.09	0.9967	1.8 mM
		50 μM	1.68	3.60	0.9909	2.1 mM

Linear transformations of the hyperbolic data from Fig. 3 were used to determine the Km values from the glutamine saturation profiles conducted at either 100 mM or at 10 mM phosphate in the absence or presence of either 20 μM or 50 μM 968.

concentrations than at the standard phosphate level used in glutaminase assays (Fig. 2).

The first disclosure of compound 968 was by Iconix Pharmaceuticals in a patent application filed in 2001 claiming its use as a modulator of Rho C activity.[15] The association of 968 with glutaminase inhibition first appeared in a publication focusing primarily on 968's ability to block transformation of cells by the oncogenic guanine nucleotide exchange factor Dbl.[9] Glutaminase was identified as the target of 968 by affinity purification experiments using 968 conjugated to biotin. Pre-incubation of GAC with 10 μM 968 produced greater than 80% inhibition of the glutaminase activity. Glutamine saturation profiles, presented as supplementary material, supported the authors' contention that 968 is not a competitive inhibitor with respect to either glutamine or phosphate ion, but rather functions as an allosteric inhibitor.

It is difficult to reconcile the discrepancies between the results of the current study and those from the original 968 disclosure. Even attempts to replicate the published protocol afforded no indication of $hGAC_{\Delta 1}$ inhibition by 968 (Fig. 5). One identifiable difference between the studies is the fact that the original report used the mouse ortholog of human GAC in assaying glutaminase inhibition. However, the inhibition of oncogenic activity in the earlier study was demonstrated using human cells. It should also be noted that the His_6-tag used to isolate cloned mouse GAC in the original study was cleaved prior to use in kinetic experiments, while the tag was left in place in the $hGAC_{\Delta 1}$ used in the present study.

Some understanding regarding the lack of agreement between the original 968 publication and the current study may be gleaned from the revelation that 968 is apparently ineffective at inhibiting glutaminase that has been activated by phosphate.[16] Although efforts were made in the present study to assess inhibition by 968 in the absence of added phosphate, the $hGAC_{\Delta 1}$ used in all experiments had been isolated using a buffer containing 10 mM phosphate. In this connection, it is interesting to consider the hypothesis that accumulation of phosphate in mitochondria is what triggers increased GAC-based glutaminase activity in cancerous cells.[17] Should this in fact be the case, the use of 968 in treating cancers characterized by high GAC activity might be futile.

A somewhat different account of the role of 968 in glutaminase inhibition can be found in a recent patent application filed by Cornell University.[18] It is stated in this application that 968 does not directly inhibit glutaminase catalytic activity. The patent presents data indicating that GAC isolated from cancer cells is more active in the absence of phosphate than GAC isolated from non-transformed cells. The patent also suggests that only in cancer cells is mouse GAC phosphorylated at Serine 103 (corresponding to Serine 95 in human GAC). The authors suggest that 968 may block this phosphorylation and thus prevent the phosphate-independent activation of GAC that is unique to cancer cells. The glutaminase construct used in this study, $hGAC_{\Delta 1}$, lacks the first 123 amino acids from the N-terminus. Thus, it lacks the predicted site of phosphorylation and its activation is highly dependent upon phosphate. However, this serine residue is conserved in the rat brain glutaminase. Thus, it is tempting to ascribe the limited inhibition of glutaminase activity from the rat brain assay of the current study to a portion of the glutaminase that is not phosphorylated (Fig. 6), although the question would remain as to why inhibition was only evident under high phosphate concentrations.

Another consideration with respect to the failure of this study to show GAC inhibition by 968 is that the inhibitory properties of 968 have been observed to diminish with time.[16] This is a surprising observation, since 968 might be expected to be quite thermally stable and show little propensity to react with water

or with oxygen. Precautions were taken to confirm the identity of the 968 sample used in the present study through electrospray time-of-flight mass spectroscopy. However, we could not reproduce the inhibition of GAC using the exact conditions described previously and could only show partial inhibition of the crude rat brain glutaminase. Thus, we cannot exclude the possibility that our sample of 968had undergone some non-detected change that resulted in loss of activity. In conclusion, 968 may yet prove to be a promising material from which therapeutic cancer treatments may be developed. However, it appears that studies directed towards improvements of the efficacy of 968 will not be able to rely on assays predicated on the direct inhibition of glutaminase activity.

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