Therapeutic Discovery

Dibenzophenanthridines as Inhibitors of Glutaminase C and Cancer Cell Proliferation

William P. Katt, Sekar Ramachandran, Jon W. Erickson, and Richard A. Cerione

Abstract

One hallmark of cancer cells is their adaptation to rely upon an altered metabolic scheme that includes changes in the glycolytic pathway, known as the Warburg effect, and elevated glutamine metabolism. Glutaminase, a mitochondrial enzyme, plays a key role in the metabolism of glutamine in cancer cells, and its inhibition could significantly impact malignant transformation. The small molecule 968, a dibenzophenanthridine, was recently shown to inhibit recombinantly expressed glutaminase C, to block the proliferation and anchorage-independent colony formation of human cancer cells in culture, and to inhibit tumor formation in mouse xenograft models. Here, we examine the structure-activity relationship that leads to 968-based inhibition of glutaminase and cancer cell proliferation, focusing upon a “hot-spot” ring previously identified as critical to 968 activity. We find that the hot-spot ring must be substituted with a large, nonplanar functionality (e.g., a t-butyl group) to bestow activity to the series, leading us to a model whereby the molecule binds glutaminase at a previously undescribed allosteric site. We conduct docking studies to locate potential 968-binding sites and proceed to test a specific set of docking solutions via site-directed mutagenesis. We verify the results from our initial assay of 968 and its analogues by cellular studies using MDA-MB-231 breast cancer cells.

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Introduction

Cancer cells exhibit altered metabolic activity in which glucose is converted in the presence of oxygen primarily to lactate, that is, the Warburg effect (1). This differs from metabolic activities in healthy cells supplied with oxygen, which instead undergo oxidative phosphorylation (2, 3). These alterations in metabolic activity are key to understanding how cancer cells supply the materials and energy needed to proliferate rapidly and have been reviewed elsewhere (4–8).

One key metabolic enzyme is glutaminase, which catalyzes the hydrolysis of glutamine to glutamate, with glutamate serving as the precursor for α-ketoglutarate, a citric acid cycle intermediate (9, 10). Mammalian cells contain 2 genes that encode glutaminase: the kidney-type (GLS1) and liver-type (GLS2) enzymes (11). Each has been detected in multiple tissue types, with GLS1 being widely distributed throughout the body (12, 13). GLS1 is a phosphate-activated enzyme that exists in humans as 2 major splice variants, a long form (referred to as KGA) and a short form (GAC), which differ only in their C-terminal sequences (14). Both forms of GLS1 are thought to bind to the inner membrane of the mitochondrion in mammalian cells, although at least one report suggests that glutaminase may exist in the intramembrane space, dissociated from the membrane (15–18). The GAC isoform is overexpressed in many human cancers (19).

Recently, we showed that glutaminase activity is elevated in transformed fibroblasts and in human breast cancer cells (20). GAC activation downstream of Rho GTPase signaling requires NF-κB activity, which has been implicated in various human cancers (21–23). Its activation in cancer cells seems to be essential for the elevations in glutamine metabolism that satisfy their biosynthetic and energy requirements (2). The mechanisms by which GAC activity is increased in cancer cells are still not understood. For recombinantly expressed GAC, maximal activity requires inorganic phosphate, which stimulates conversion of enzyme dimers to activated trimers (24–27). While numerous metabolites, including acetyl-CoA, have been shown to stimulate glutaminase activity, the exact mitochondrial localization of GAC seems to vary by tissue, organism, and cell line, making it difficult to hypothesize what might be biologically available to the enzyme for use as an agonist (10, 28, 29).

We have recently reported on 5-(3-bromo-4-(dimethylamino)phenyl)-2,2-dimethyl-2,5,6-tetrahydrobenzo[alphenanthridin-4(1H)-one, designated 968, which inhibits recombinant GAC as well as blocks cancer cell proliferation and the growth of tumors in mouse xenograft models (20). Unlike the GAC inhibitor 6-diazo-5-
oxo-L-norleucine, 968 is neither irreversible nor competitive versus glutamine but rather acts as an allosteric regulator of GAC (30). Initial studies also suggested that the 3-bromo-4-(dimethylamino)phenyl ring of 968 represents a functional hot-spot, with small changes to this ring abolishing activity. This study aims to examine the nature of this critical ring in depth, determine its transferability to other scaffolds, and elaborate upon the requirements for deactivating GAC.

Materials and Methods

BPTES was a gift from Dr. Scott Ulrich (Ithaca College, Ithaca, NY). Other inhibitors were purchased from ChemBridge or Specs. RPMI-1640 and FBS were obtained from Invitrogen. Site-directed mutagenesis was conducted with the QuikChange Mutagenesis Kit (Stratagene) and primers were obtained from Integrated DNA Technologies. All other reagents were from Fisher Scientific or Sigma-Aldrich. Cell line MDA-MB-231 was purchased from American Type Culture Collection (ATCC) and cultured at 37°C, in 5% CO2, using RPMI-1640 media supplemented with 10% FBS. Conditions different than those recommended by ATCC were maintained to allow optimal growth of multiple cell lines in the same incubator unit. Cell lines are consistently treated with RPMI-1640 when possible to minimize possible conditional differences between cell lines for multline experiments used in other studies. The cells were used within 4 months of resuscitation from ATCC stocks authenticated by ATCC via short tandem repeat (STR) analysis.

Recombinant GAC

GAC was expressed in Escherichia coli and purified as previously described (20). Mouse GAC (residues 128–603) was cloned into the pET28a vector from Novagen, expressed as a His6-tagged protein in E. coli, and purified by ion exchange and size-exclusion chromatography. Mutagenesis was conducted on mouse GAC (residues 72–603), cloned into the pET28a vector, referred to as Δ72 GAC.

Recombinant protein assays

Inhibitors were solvated in dimethyl sulfoxide (DMSO). Assay vessels were charged with 1 μL of inhibitor and/or DMSO. Fifty-five microliters of an aqueous solution containing 48 mmol/L Tris-acetate (pH 8.6), 21 mmol/L glutamine, and 50 nmol/L recombinant GAC was added. Fifteen microliters of water or 1 mol/L potassium phosphate, pH 8.2, were immediately added to the reaction mixture. The mixture was incubated 10 minutes at 37°C, and then 10 μL of ice-cold 2.4 mol/L hydrochloric acid was added. A second vessel (218 μL) contained 114 mmol/L Tris-HCl (pH 9.4), 0.35 mmol/L ADP, 1.7 mmol/L β-NAD, and 1.3 units of glutamate dehydrogenase. A third vessel contained an identical solution except that it lacked NAD+. Twenty microliters of the initial reaction mixture was added to the second and third vessels, which were then incubated at room temperature for 45 minutes, and then the absorbance at 340 nm was measured for each mixture. The third reaction was treated as a baseline control. Experiments were carried out in duplicate.

Cell assays

Cells that were 70% to 80% confluent were trypsinized and dispensed into 12-well culture plates (1.6 × 10^4 cells per well). Each well was brought to 1 mL of media. Cells were allowed to adhere to the wells for 24 hours and then counted ( assay day 0). Then, and every 48 hours thereafter, media were exchanged for media containing either 10 μmol/L of an inhibitor diluted from a 3 mmol/L DMSO stock, or an equivalent amount of DMSO (0.33% DMSO by volume). Cells were counted every 48 hours for 6 days by removing the media, rinsing the cells with room temperature PBS, incubating at 37°C for 5 minutes in 0.5 mL trypsin-EDTA solution, followed by light agitation to dissociate the cells from the plate, and the addition of RPMI-1640 complete media (0.5 mL) to quench trypsin activity. Cells were then counted on a hemocytometer (3 measurements were averaged per sample). All experiments were carried out in triplicate.

Docking

Docking studies were conducted with Autodock 4.2 in Cygwin 1.5.25. Autodock input files were prepared with MGLTools 1.5.2. Molecules were drawn in ChemBioOffice 2010, and energy minimized using the MMFF94 force field in ChemDraw 3D. Docking was carried out with a genetic algorithm. The docking procedure is detailed in Supplementary Methods. Visualization was carried out with PyMOL 0.99, and graphics were prepared in that software.

Results

Structure–activity relationship of GAC inhibitors

We set out to identify modifications to the dibenzophenanthonidine scaffold of 968 that lead to optimal inhibitory activity, with the hope of obtaining chemical tools useful for studying glutaminase activity in cancer model systems, as well as possibly shedding some insight into the mechanism by which glutaminase becomes activated. Initial characterizations of the effects of 968 on glutaminase activity and oncogenic transformation (20) suggested that bromine or a similar soft, electronegative group was required at the position 3 of the phenyl hot-spot ring (H-ring), with an alkyl-substituted hydrogen bond acceptor group being required at the position 4. To expand this structure–activity relationship (SAR), we concentrated upon substituents with subtly different size, shape, or electronic properties than those already identified. We began by screening compounds 1 through 19 (representative compounds are shown in Table 1; all compounds are shown in Supplementary Table S1) against the recombinant GAC enzyme, using a variant of the 2-step assay developed by Kenny and colleagues (9) and Curthoys and Lowry (31). Several compounds have negative inhibition...
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<th>Structure</th>
<th>Percentage inhibition</th>
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values; these generally reflect small variations in readings at the high- absorbance (low-inhibition) range of the assay. Some values may suggest allosteric activation and will be pursued in future work.

While most compounds fit the previous SAR, compound 17 stood out. Although 17 was less potent than 968, it was surprisingly active considering that it lacked the bromine atom on the H-ring. The primary difference between 17 and the less active 14 is steric bulk. Like 968, 17 exhibited a dose-dependent effect (Fig. 1). To see whether bulkiness could compensate for the loss of the bromine atom, compounds 20 through 26 were tested. These experiments showed that an isopropyl (22) or tert-butyl (23) group at the position 4 of the H-ring restored the activity lost upon removal of the bromine atom. The methylthiol group of 25 provided significant activity as

<table>
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<td>96 ± 1</td>
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NOTE: GAC in Tris-acetate buffer (pH 8.6) was exposed to glutamine (21 mmol/L) and inhibitor at 10, 25, and 50 μmol/L concentrations, and then inorganic phosphate (136 mmol/L) was added. The solution was incubated for 10 minutes, at which time glutamate was measured and percentage inhibition values were determined.
well. The nitrile group of 26 showed no activity, suggesting that not only is steric bulk important but it must be oriented toward the para-position of the H-ring. The H-ring of 22 was better than that of 14, but less effective than the H-rings in either 5 or 968, suggesting that it is most effective to hold this steric bulk nonplanar to the ring system. In 14, the optimal dimethylamine group geometry would be heavily weighted by delocalization of the nitrogen lone pair into the benzene ring, positioning the group coplanar to the ring. In 968, the bromine atom enters into steric clash with the dimethylamine, pushing the methyl groups out of the plane of the ring, causing it to mimic the orientation of the functional groups of 22 or 23. Such orientations are observed upon energy minimization of 968 with the MMFF94 force field (Fig. 2A). The ketone on the main scaffold forces the H-ring into an orientation nearly perpendicular to the scaffold. Thus, the para-substituent of the H-ring optimally occupies much of the plane of the main scaffold.

**Examining other scaffolds**

We previously showed that the 968 H-ring, when attached to a resin, was capable of affinity precipitating...
GAC from transformed fibroblast lysates (20). As binding to GAC could be achieved without the benzophenanthridine scaffold, we were interested in examining the transferability of the H-ring to other scaffolds, hoping to identify GAC inhibitors that exhibited improved binding affinity and aqueous solubility. We examined compounds 27 through 55 (Table 1; Supplementary Table S1), which contained similar or identical derivatives of the H-ring and were selected to represent a diverse region of chemical space. Many of these scaffolds showed very low potency when assayed for their ability to inhibit GAC. This suggests a key difference between a molecule exhibiting binding affinity, enabling it to bind GAC in cell lysates, and inhibitory potency, which may require additional contacts. Most surprising were compounds 27 and 44, which possessed small alterations to the 968 scaffold. While 44 was approximately 50% more potent than homolog 17, 27 was significantly less potent than its homolog, 968 (Fig. 1). This was surprising given that 35 and 40, which contain radically different side chains than 968, exhibited excellent inhibitory potency. Because 35 and 40 each had potentially reactive centers, we tested to see whether their inhibitory activity changed as a function of their incubation time with the enzyme. In each case, inhibitory potency was noticeably reduced when the compound was preincubated with GAC and glutamine in the assay buffer, suggesting that the compounds might be unstable in water (Supplementary Fig. S3).

Docking studies

Given the unique nature of the SAR and the inefficacy of alternate scaffolds, we wanted to determine the possible location of the 968-binding site on GAC. Docking studies were conducted using the human GLS1 crystal structure 3CZD, which was the only X-ray crystal structure available at the time. Two potential binding pockets presented themselves: the glutamine-binding site and a pocket formed at the interface of 2 monomers, between their collective N- and C-termini (Fig. 2B and C). This pocket seemed promising, as previous studies showed that 968 is not competitive with glutamine (20). Autodock was used to carry out a docking operation over the entire surface of the GAC dimer. A majority of docked structures were found in the binding pocket formed at the interface of the 2 monomers. We have recently obtained the X-ray structure of the full-length human GAC protein (manuscript in preparation), in which much of the N-terminus is visible, and conducted docking operations on this structure, resulting in similar poses for 968 at the same binding pocket (Fig. 2C).

Much of the affinity of 968 for GAC seems to be due to hydrophobic interactions and shape complementarity, which likely explains why we were not able to substitute new scaffolds easily. We have identified only a single potential electronic interaction between the protein (i.e., the δ N-H of arginine 539) and the 968 bromine atom, supporting the SAR conclusion that the bromine atom is optional but may provide some contribution to binding affinity. This could explain why the smaller chloride atom does not help binding affinity; however, the distance between the bromine and hydrogen atom centers (~5 Å), as well as recent experiments (see below), suggest that such an interaction is unlikely. The dimethylamine of the H-ring projects directly out of the site, and effectively makes no protein contacts, but it might interact with flexible protein regions not visible in current crystal structures.

Binding site analysis

To support the docking studies, we conducted a mutational analysis of GAC, examining residues that exhibit potentially important contacts with 968. We began by assessing the potency of 968 against full-length wild-type GAC (Δ72 GAC), to assess the importance of the N- and C-termini to the inhibitory capability of 968. 968 exhibits an IC50 of 19 μmol/L against full-length GAC, which is slightly weaker than its inhibitory potency against the shorter GAC construct (IC50 ~10 μmol/L), suggesting that the N- and C-termini do not make a major contribution to 968-binding affinity.

We then focused upon 2 key residues: Arg 539, in the event that it formed a weak hydrogen bond with the bromine atom of 968, and Phe 532, which could potentially undergo a π-stacking interaction with 968 (Fig. 2D). Each residue was mutated to leucine to eliminate the hydrogen bonding or π-stacking potential, respectively, while minimally altering the overall size of the residue. Assays were conducted with protein concentrations normalized such that each protein (Δ72 GAC, and the R539L and F532L mutants) exhibited similar phosphate-stimulated enzymatic activity (at equimolar levels, R539L is 50% as active as wild-type, whereas F532L is 33% as active). The potency of 968 was decreased for each mutant, such that detectable inhibition was not observed at concentrations up to 50 μmol/L. When similar experiments were carried out using compound 23 (IC50 vs. wild-type GAC of 12 μmol/L), IC50 values of 40 and 36 μmol/L were obtained for the R539L and F532L GAC mutants, respectively. While a loss of inhibition was expected for the F532L mutant, the inability of 23 to inhibit the R539L mutant ruled out the possibility of a hydrogen bond between 968 and Arg 539, as compound 23 lacks an electronegative substituent near the arginine residue. What may be more likely is the differences observed between compounds containing bromine versus a chloride atom at the H-ring reflect a desolvation effect.

Effects of GAC inhibitors on cancer cell proliferation

While recombinant GAC is activated via the addition of inorganic phosphate, this is unlikely responsible for stimulating its enzymatic activity in cells, given the levels of phosphate required for activation (>50 mmol/L). Similarly, GAC is likely bound to a membrane in cells, which could affect changes to the protein structure, and possibly render our conclusions from the docking and mutational...
analyses valid for studies only on the recombinant enzyme. We thus examined whether the compounds exhibited the same relative effectiveness in cells as when assaying recombinant GAC. MDA-MB-231 human breast cancer cells were subjected to each compound at a concentration of 10 μmol/L, and the proliferation of these cells was monitored over 6 days.

The results obtained for several randomly selected inhibitors are shown in Fig. 3. The percentage inhibition observed in MDA-MB-231 cells exposed to 10 μmol/L 968 for 6 days was most similar to that obtained when incubating recombinant GAC with 25 μmol/L 968. We suspect that this reflects the differences in the ability of 968 to bind to the enzyme in cells and block its activation via posttranslational modification(s) (20) versus its ability to bind to the recombinant enzyme and antagonize the phosphate-stimulated activity. Figure 4 shows the inhibition profiles for several of the least and most potent compounds examined, which indicate that for compounds active in cells, relative potency largely follows the SAR obtained from the recombinant enzyme.

Discussion

Our study has led us to an unexpected SAR, where the H-ring needs to only hold a group of sufficient steric bulk para to the 968 core system. The bromine atom of 968 seems to be primarily responsible for imposing a nonplanar orientation on the nearby dimethylamine, which should otherwise organize nearly parallel to the ring to maximize resonance energy gains. Mutagenesis studies verify that the bromine atom is not involved in any electronic interaction with the enzyme.

Because the H-ring attached to a bead was originally used to snare GAC from transformed cell lysates, we assumed that this ring would be transferable to alternate scaffolds (20). However, this was not the case. Fewer than 10 molecules were found to exhibit a high potency against recombinant GAC, and of these, only 40 was effective in cells. Clearly, the H-rings of the 968 class of compounds are not transferable to other scaffolds, and 968 must make additional contacts of functional significance with the enzyme.

These observations led to our docking studies, in which a deep pocket was located within the GAC dimer, composed of equal surface areas from 2 protein monomers. Our docking experiments are consistent with this pocket serving as a potential 968-binding site and were further supported by mutational analysis, where altering either of 2 local residues predicted to make key contacts resulted in a loss of inhibitory ability. However, docking analyses also suggest that the dimethylamine group of the H-ring does not contact the protein. We had originally thought that this might be due to protein flexibility not visible in
the crystal structure, until we made a serendipitous discovery that shed additional light on the inhibitory actions of 968.

In our recombinant protein assay, inorganic phosphate is required to activate GAC and is typically the final reagent added to the reaction. However, we noticed that if GAC was incubated with phosphate before its exposure either to 968 or various 968 analogues, significantly less inhibition of the enzyme occurred, relative to when the enzyme was exposed to 968 before adding phosphate or glutamine (Supplementary Figs. S1 and S2). These findings suggest that 968 has little ability to bind to the GAC molecules already activated by inorganic phosphate or via the posttranslational modifications that drive enzyme activation in cancer cells (20).

Prior kinetic analysis indicated that 968 is not a glutamine antagonist, nor does it compete with the binding of either glutamine or inorganic phosphate (20). While we do not yet know the exact mode by which 968 exerts an allosteric inhibition of GAC, collectively our data from static light scattering and small-angle X-ray scattering analyses suggest that 968 does not alter the dimer-to-tetramer transition, which is essential for enzyme activation (manuscript in preparation). Whereas inorganic phosphate, and presumably posttranslational modifications, drives GAC to an activated, tetrameric species, 968-Inhibited enzyme occurs, drives GAC to an activated, tetrameric species, 968 seems to stabilize the enzyme either as an inactive dimer and/or inactive tetramer (see Fig. 5). Structural studies conducted with bacterial mGlu have suggested that glutamine binding causes the movement of residues 26 to 29, which cap the glutamine-binding site temporarily (32). 968 may cause a similar effect when added to GAC, effectively shutting off the glutamine-binding site, perhaps by holding open the protein N- and C-termini. Although such a model remains to be proven, it would help to explain the SAR, in which a suitably large functional moiety on the H-ring might wedge open the N- and C-termini of GAC. This would also be consistent with our docking model, which places the H-ring of 968 at the outer edge of the pocket formed near the N- and C-termini of the enzyme. An interesting consequence of such a function would be that the H-ring is primarily responsible for inhibition rather than for binding affinity, which also helps to explain why we were unable to obtain potency by transferring the ring to other scaffolds. Because 968 shows little ability to inhibit the phosphate-activated enzyme, this suggests that it probably prevents GAC from undergoing the posttranslational modifications in cancer cells that are necessary for its activation, which would be consistent with our findings that 968 has a persistent effect upon mitochondrial GAC activity when cells are treated with the drug before harvesting the mitochondria (20).

The action of 968 seems to resemble that of BPTES, another allosteric inhibitor of glutaminase (33, 34). However, while 968 is unable to inhibit the phosphate-activated enzyme, BPTES is able to do so, suggesting alternate modes of inhibition for each molecule. BPTES binds between the helical interfaces where 2 GAC dimers come together to form a tetramer, near the glutamine-binding site, and opposite the proposed 968-binding site. We have observed an additive effect when MDA-MB-231 cells are dosed with 968 and BPTES simultaneously. When 968 and BPTES were added to cells at concentrations equal to their IC50 values (4.2 and 3 μmol/L, respectively), an 83% inhibition of cell proliferation was observed, compared with the maximum expected 50% inhibition for 2 competitive drugs at IC50 concentrations, further suggesting that 968 and BPTES act via different mechanisms.

It is worthwhile to consider the relationship between the inhibitory actions of 968 activity against recombinant GAC, versus its effects on the growth of MDA-MB-231 cells. The latter case involves GAC activation as an outcome of signals downstream from Rho GTPases and NF-κB (20), whereas the former represents assays of the recombinant enzyme activated by inorganic phosphate. These distinctions likely account for the results obtained with compounds 4 and 5, which exhibit similar inhibitory activities to 968 when assaying recombinant GAC, but are noticeably less effective at inhibiting cancer cell proliferation (Table 1 and Fig. 3). It is difficult to know whether a particular compound will function in both assays, although for those compounds that do, we see a similar SAR in the 2 assay systems (Fig. 4). Indeed, the number of compounds active in both systems suggests that 968, and its derivatives, offers realistic possibilities for therapeutic intervention.
Our docking model helps explain the low activities of other molecules that otherwise seem to fit the SAR as well. Compound 16 has an H-ring substituent, which is large enough to fit the SAR, and would not present planar to the ring. However, the model shows that very few hydrogen-bonding opportunities are available in the cavity, and thus a charged group, such as the organic acid of 16 would compromise binding. Quinoline 27 is less potent than the related naphthyl compound 968. This is most likely due to the quinoline nitrogen atom lying near the backbone carbonyl of Pro498, creating an electronic repulsion. We have not considered the docking of the diverse alternate scaffolds examined, but the model seems to hold for all of the close 968 derivatives, which we have investigated.

In summary, we have further elucidated the SAR surrounding the H-ring of the GAC inhibitor 968, following the examination of 26 dibenzophenanthridines. The bromine atom of 968, previously thought critical to binding, is not essential and may play a primarily supportive role in providing ideal shape to otherwise unsuitable para-substituents, such as the dimethylamine on the H-ring. We have found that the H-ring is not easily transferable to other molecular structures. We have located a potential binding pocket on an X-ray crystal structure of GAC, which could accommodate 968, given previous competition studies. Finally, we have proposed a model by which both 968 and inorganic phosphate may act to differentially regulate GAC activity. With further study and supporting evidence, this model may yield additional strategies for blocking the activation of GAC in cancer cells, a step that is essential for the metabolic changes necessary to sustain malignant transformation.

Disclosure of Potential Conflicts of Interest
W. P. Katt is a consultant to Zuma Biosciences. No potential conflicts of interest were disclosed by other authors.

Authors’ Contributions
Conception and design: W. P. Katt, J. W. Erickson, R. A. Cerione
Development of methodology: W. P. Katt, J. W. Erickson
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W. P. Katt, S. Ramachandran
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W. P. Katt, J. W. Erickson
Writing, review, and/or revision of the manuscript: W. P. Katt, S. Ramachandran, J. W. Erickson, R. A. Cerione
Study supervision: R. A. Cerione

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