Glutaminase inhibitor compound 968 inhibits cell proliferation and sensitizes paclitaxel in ovarian cancer

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Abstract: Objective: Our overall goal was to investigate the anti-tumor activity of the glutaminase 1 (GLS1) Inhibitor compound 968 in ovarian cancer cells. The human ovarian cancer cell lines, HEY, SKOV3 and IGROV-1 were used. Cell proliferation was assessed by MTT assay after treatment with compound 968. Cell cycle progression and Annexin V expression were evaluated using Cellometer. Western blotting was performed to determine changes in GLS1, cellular stress and cell cycle checkpoints. Reactive oxygen species (ROS) and glutamate dehydrogenase (GDH) activity were assessed by ELISA assay. Compound 968 significantly inhibited cell proliferation and the expression of GLS1 in a dose-dependent manner in all three ovarian cancer cell lines. Compound 968 induced G1 phase cell cycle arrest and apoptosis. Treatment with compound 968 increased ROS levels and induced the protein expression of calnexin, binding immunoglobulin protein (BiP) and protein kinase RNA-like endoplasmic reticulum kinase (PERK). Deprivation of glutamine increased the sensitivity of cells to paclitaxel, and compound 968 sensitized cells to the anti-proliferative effects of paclitaxel. Compound 968 inhibited cell growth in ovarian cancer cells through induction of G1 phase cell cycle arrest, apoptosis and cellular stress, suggesting that targeting GLS1 provide a novel therapeutic strategy for ovarian cancer.

Keywords: Glutaminase, compound 968, apoptosis, cellular stress, ovarian cancer

Introduction

Ovarian cancer is the deadliest gynecologic malignancy, with an estimated 22,280 cases and 14,240 deaths predicted for 2016 [1]. The majority of ovarian cancers are diagnosed at a late stage, largely due to non-specific symptoms and the lack of a reliable screening test. The mainstay of treatment for ovarian cancer is cytoreductive surgery (to a goal of no gross residual tumor) followed by paclitaxel and platinum based doublet chemotherapy. This treatment strategy provides effective tumor control in more than 80% of patients; however, most patients will develop disease recurrence and drug resistance. Thus, new treatment options for ovarian cancer are urgently needed.

Glutamine is the most abundant nonessential amino acid. It exists in every protein in the human body whose primary function involves energy metabolism, reduction-oxidation homeostasis, macromolecule synthesis and signaling [2, 3]. Although glucose is the major energy source to support cancer cell growth, glutamine contributes to oxidative phosphorylation and glycolysis energy-forming pathways in cancer cells [4]. Glutaminase is an amidohydrolase and rate-limiting enzyme that catalyzes the first step in the glutaminolysis of glutamine to glutamate in reactions that either donate theamide nitrogen to biosynthetic pathways or release it as ammonia. Glutaminase exists as two isoforms, glutaminase 1 (GLS1) and glutaminase 2 (GLS2), and were originally identified in the kidney and liver, respectively [4]. GLS1 is a phosphate-dependent enzyme with two major splice variants: a long form (KGA) and a short form (GAC) [5]. GLS1 is expressed in all mammalian tissue except for liver and is associated
with tumor growth and invasive activities in a number of cancer types [6]. Indeed, most cancer cells cannot survive in vitro in the absence of an exogenous glutamine supply, indicating that cancer cells are glutamine dependent [5, 7]. Inhibition of glutaminolysis or GLS1 activity can induce cell apoptosis and decrease tumor growth in cancer cells and animal models of cancer [5, 6, 8, 9]. Our previous study demonstrated that glutamine restriction results in inhibition of cell growth, induction of apoptosis, G1 phase cell cycle arrest and increased reactive oxygen species (ROS) production through alterations in the mTOR pathway in ovarian cancer cells [10].

Compound 968 is a small molecule that acts as an allosteric regulator of GLS1 and inhibits the activity of KGA and GAC. Several groups have reported anti-tumor activity for compound 968 in lymphoma, breast cancer, glioblastoma and lung cancer in vitro [11-15]. Furthermore, compound 968 has been shown to block oncogenic transformation induced by various Rho GTPases in fibroblasts, without toxic effects on normal cells [11, 16]. Thus, inhibition of GLS1 by compound 968 or other GLS1 inhibitors may provide a new therapeutic strategy for the treatment of different types of cancer, including ovarian cancer. In the current study, we aimed to address the anti-tumorigenic effects and underlying mechanisms of compound 968 in ovarian cancer cells.

Materials and methods

Cell Culture and reagents

The human ovarian cancer cell lines HEY, SKOV3 and IGROV-1 were used. The HEY and IGROV-1 cell lines were maintained in RPMI-1640 medium supplemented with 5% and 10% fetal bovine serum (FBS), respectively. The SKOV3 cell line was maintained in DMEM/F12 medium supplemented with 10% FBS. To study the effects of compound 968, we used Gibco DMEM/F-12(1:1) medium with L-Glutamine and 15 mM Heps (Catalog Number: 11039), containing 5% HyClone Characterized FBS and supplied with varying concentrations of compound 968. The media was supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were cultured in a humidified 5% CO₂ at 37°C.

Compound 968 was purchased from Calbiochem (Billerica, MA). 3-(4,5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), RNase A, 2', 7'-Dichlorofluorescin diacetate (DCFH-DA) and Paclitaxel were purchased from Sigma-Aldrich (St. Louis, MO). The GDH assay kit was bought from BioVision (Milpitas, CA). The Annexin V FITC kit was purchased from Biolegend (San Diego, CA). The anti-glutaminase (GLS1) antibody was purchased from Abcam (Cambridge, MA), and all the other antibodies were obtained from Cell Signaling (Danvers, MA). Enhanced chemiluminescence (ECL) detection reagents were purchased from GE Health care (Piscataway, NJ). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cell proliferation assay

The ovarian cancer cell lines, HEY, SKOV3 and IGROV-1, were treated with media containing different concentrations of compound 968 (0, 2, 5, 10, 25, 50 uM) for 5 days after seeding cells at 3000 cells/well in 96-well plates in their culture media for 5 h. The media was refreshed at day 3. Cell proliferation was measured by adding 5 ul MTT solution (5 mg/ml) per well for an additional incubation time of 1 h. The MTT reaction was terminated through the replacement of the media by 100 ul DMSO. Viable cell densities were determined by measuring absorbance of metabolic conversion of the colorimetric dye at 570 nm. Each experiment was performed in triplicate and repeated three times to assess for consistency of results.

Cell cycle analysis

The effect of compound 968 on cell cycle progression was assessed using Cellometer (Nexcelom, Lawrence, MA). Cells were plated at a density of 1.5 × 10⁵ cells/well in 6-well plates for 5 h, and then treated with varying concentrations of compound 968 (0, 5, 10, 25 uM) for 48 h. Cells were collected by 0.05% trypsin (Gibco Grand Island, NY), washed with phosphate-buffered saline (PBS) solution, fixed in a 90% methanol solution and then stored at -20°C until cell cycle analysis was performed. On the day of analysis, the cells were washed with PBS and centrifuged, resuspended in 50 ul RNase A solution (250 ug/ml) with 10 mM EDTA, followed by incubation for 30 min at 37°C. After incubation, 50 µl propidium iodide
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(PI) staining solution (2 mg/ml PI, 0.1 mg/ml Azide and 0.05% Triton X-100) was added to each tube and incubated for 10 min in the dark. The cells were assessed by Cellometer. The results were analyzed using FCS4 express software (Molecular Devices, Sunnyvale, CA). Each experiment was repeated at least twice for consistency of response.

Annexin V assay

The effect of compound 968 on cell apoptosis was detected by using the Annexin-V FITC kit. Briefly, $1.75 \times 10^5$ cells/well were seeded into 6-well plates for 5 h, and then the cells were treated with different concentrations of compound 968 (0, 5, 10, 25 uM) for 24 h. The cells were collected by 0.25% trypsin without EDTA. After washing with PBS, cells were resuspended in 100 ul Annexin-V and PI dual-stain solution (0.1 ug Annexin-V FITC and 1 ug PI) for 15 min in the dark and detected by Cellometer. The results were analyzed by FCS4 express software. Each experiment was repeated at least twice for consistency of response.

Reactive oxygen species (ROS) assay

Intracellular reactive oxygen species (ROS) production was detected using DCFH-DA. After treatment of the cells with different concentrations of compound 968 for 24 h, 10 ul of 200 uM DCFH-DA was added into the media and mixed gently. The fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a plate reader (Tecan, Morrisville, NC). Data were normalized based on the viable cell counts measured by the MTT assays. Each experiment was performed in triplicate and repeated three times for consistency of results.

Glutamate dehydrogenase (GDH) activity assay

Intracellular GDH activity was measured by using the GDH assay kit. HEY cells were treated with compound 968 for 24 h. Cell lysates were prepared in cold GDH lysis buffer. Ten to 20 ul of cell lysates were transferred into a new 96-well plate, then GDH assay buffer was added until the total volume was 50 ul. Next, 100 ul of reaction mix (82 ul GDH assay buffer, 8 ul GDH developer and 10 ul glutamate) was added to each well. The concentration of GDH was measured at wavelength of 450 nm in a plate reader after incubating for 3 min at 37°C. The results were normalized on the basis of the total protein concentration of each sample. The experiment was performed in triplicate and repeated twice to assess for consistency of results.

Western blot analysis

Total protein was extracted from ovarian cancer cells using RIPA buffer (Boston Bioproducts, Ashland, MA), and the protein was quantified with the BCA assay kit (Thermo Scientific, Rockford, IL). Protein samples with equal loading (30 ug) were separated by 10-12% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk and then incubated with a 1:1000 dilution of primary antibodies for overnight at 4°C. The membranes were washed and incubated with a secondary peroxidase-conjugated antibody for 1 h at room temperature. The membranes were developed using enhanced ECL at Alpha Innotech Imaging System (Protein Simple, Santa Clara, CA). After developing, the membranes were re-probed using an antibody against α-tubulin to confirm equal loading. The bands’ intensity were measured and normalized to α-tubulin. Each experiment was repeated at least twice for consistency of results.

Statistical analysis

Data are expressed as mean ± standard error. Data were compared using the two-tailed Student’s t test with $P<0.05$ considered statistically significant. Analysis of synergy was calculated using CalcuSyn software (Biosoft, Cambridge, UK). Combination index (CI)<1 indicates synergism, CI=1 indicates additive effects and CI>1 indicates antagonism.

Result

Compound 968 inhibited ovarian cancer cells proliferation

To investigate the inhibitory effects of compound 968 on cell growth in ovarian cancer cell lines, we treated three ovarian cancer cell lines (HEY, SKOV3, IGROV-1) with compound 968 for 5 days. Our results indicate that compound 968 inhibited proliferation in all three cell lines in a dose-dependent manner.
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We tested the effect of compound 968 on the expression of GLS1 after 24 h of treatment. The GLS1 antibody recognizes two isoenzymes of glutaminase: glutaminase 1 (GLS1) and glutaminase (GAC). We found decreased expression of GLS1 after treatment with compound 968 in all three cell lines (Figure 1B). Given that GDH converts glutamate to α-ketoglutarate in glutamine catabolism, we next examined if GDH activity was affected after treatment with compound 968 in HEY cells. Treatment with compound 968 significantly decreased the activity of GDH in the HEY cell line after 24 h treatment. These data suggest that compound 968 has the ability to inhibit cell proliferation and reduce glutamine metabolism in ovarian cancer cells (Figure 1C).

Compound 968 induced cell cycle G1 arrest

Studies indicate that glutamine is necessary for cell cycle progression [17]. We examined the effect of compound 968 on cell cycle progression in all three ovarian cancer cell lines and found that treatment with compound 968 for 48 h resulted in G1 phase cell cycle arrest. In addition to an increased proportion of cells in G1 phase, we noted a concomitant decreased proportion of cells in S phase (Figure 2A). Western blotting showed compound 968 treatment caused reduced protein expression of CDK4 and cyclin D1 with increased expression of p21 and p27 (Figure 2B). These results are consistent with the effects of glutamine metabolism on cell cycle changes.

Compound 968 induced cell stress

Given that glutamine metabolism is involved in modulating reduction-oxidation homeostasis
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A  

968  C  5  10  25 (µM)

Hey

SKOV3

IGROV-1
Figure 2. Compound 968 caused cell cycle G1 phase arrest. The HEY, SKOV3 and IGROV-1 ovarian cancer cell lines were treated with compound 968 at different concentrations for 48 h. Cell cycle analysis was performed using Cellometer. Treatment with compound 968 caused G1 phase cell cycle arrest in all three ovarian cancer cell lines (A). Cells were treated with various concentrations of compound 968 for 24 h, Western blotting showed compound 968 decreased cyclin D1 and CDK4 expression and increased p21 and p27 expression in all three cell lines (B). Data are shown as mean ± SEM of two experiments (*P<0.05).
and oxidative stress [4, 10], we tested the effects of compound 968 on the production of intracellular reactive oxygen species (ROS) and protein expression of endoplasmic reticulum stress. We treated all three ovarian cancer cell lines with compound 968 for 24 h. We found that treatment with compound 968 significantly increased ROS production in a dose dependent manner (Figure 3A). Western blotting showed the protein expression of protein kinase-like endoplasmic reticulum kinase (PERK), calnexin and binding immunoglobulin protein (BiP) increased after treatment with compound 968 (Figure 3B), suggesting that compound 968 induces significant cellular stress in ovarian cancer cells.

**Compound 968 induced cell apoptosis**

The effect of compound 968 on apoptosis was evaluated in all three ovarian cancer cells. We treated the cell lines with varying concentrations of compound 968 for 60 h, and found significantly increased expression of Annexin V with increasing concentrations of compound 968 (Figure 4A). We performed western blotting and found decreased expression of MCL-1, a member of the Bcl-2 family, after 24 h treatment with compound 968 (Figure 4B). These results demonstrate that treatment with compound 968 results in increased apoptosis in ovarian cancer cell lines.

**Compound 968 sensitized paclitaxel in ovarian cancer cells**

Inhibition of glutaminolysis has been previously shown to sensitize paclitaxel in paclitaxel-resistant breast cancer cells [18]. We assessed the effect of glutamine and compound 968 on sensitivity to paclitaxel in ovarian cancer cell lines. As expected, increasing glutamine concentrations resulted in increased cell proliferation and decreased cell sensitivity to low dose paclitaxel in all three ovarian cancer cell lines (Figure 5A). MTT assays demonstrated that the combination of 5 uM of compound 968 with paclitaxel at varying concentrations resulted in synergistic inhibitory effects in all three ovarian cancer cell lines after 72 h treatment (Figure 5B, CI<1). These results suggest that compound 968 treatment increased the sensitivity of the ovarian cancer cells to paclitaxel.

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**Figure 3.** Treatment of Compound 968 caused cellular stress. Compound 968 induced intracellular reactive oxygen species (ROS) production in a dose dependent manner after 24 h treatment in all three ovarian cancer cell lines (A). HEY, SKOV3 and IGROV-1 cells were treated with compound 968 for 24 h. Western blotting found that compound 968 induced the expression of PERK, calnexin and BiP (B). Data are shown as mean + SEM of two experiments. (*P<0.05).
Figure 4. Treatment of Compound 968 induced significant cell apoptosis. Cells were treated with compound 968 for 60 h. Apoptosis was detected using Annexin-V FITC assay. Compound 968 increased Annexin V expression in all three cell lines (A). Western blotting indicated treatment with compound 968 for 24 h decreased the expression of the anti-apoptotic protein, MCL-1 (B). Data are shown as mean ± SEM of two experiments (*P<0.05).
Figure 5. Compound 968 sensitized paclitaxel in ovarian cancer cells. The cells were treated with different concentrations of paclitaxel (0, 0.1, 1.0, 10, 50 nM) in media with various concentrations of glutamine for 72 h. MTT assays showed that deprivation of glutamine or low concentrations of glutamine increased sensitivity to paclitaxel (A). The cells were treated with 5 compound 968 in combination with different concentrations of paclitaxel for 48 h. Compound 968 synergized with the anti-proliferative effects of paclitaxel in the ovarian cancer cell lines (B, CI<1). Data are shown as mean ± SEM of three experiments (*P<0.05).
Discussion

We found that compound 968 inhibits cell proliferation, induces G1 phase cell cycle arrest, and initiates apoptotic cell death in the HEY, SKOV3 and IGROV-1 ovarian cancer cell lines in a dose dependent manner. Increased ROS production was also seen with increasing doses of compound 968. Moreover, low concentrations of glutamine resulted in increased sensitivity of ovarian cancer cells to paclitaxel, while compound 968 treatment resulted in significant synergy when combined with paclitaxel to inhibit ovarian cancer cell growth. These results support a role for targeting GLS1 or inhibiting glutamine metabolism as a valuable strategy for the treatment of ovarian cancer [6, 10, 13].

Glutamine is a major source of carbon and energy to promote cell growth and viability in cancer. The activity of glutaminase (GLS) and glutamine levels correlate with cancer cell proliferation in vitro [19]. High level of GLS1 protein expression is associated with worse overall survival in patients with ovarian cancer and silencing of GLS1 via siRNA in glutamine dependent SKOV3 cell-bearing mice results in a significant reduction in tumor weight and tumor volume when compared to controls [20]. The depletion of glutamine results in inhibition of cell proliferation, induction of G1 phase cell cycle arrest, and apoptosis through mTOR/S6 pathway in ovarian cancer cells. GLS knockdown results in reduced mitochondrial membrane potential in glioma cells, suggesting that mitochondrial dysfunction plays a role in inducing apoptosis with inactivity of GLS [21]. Recent studies indicate that the response to glutamine deprivation in cancer cells is dependent on their different genetic and epigenetic background [9, 13].

The mechanisms of action of compound 968's inhibition on cancer cell proliferation are less well characterized. Treatment of MDA-MB-231 cells with compound 968 resulted in downregulation of 11 anti-apoptotic genes, upregulation of several critical tumor suppressor genes (including VHL, MGMT and FHIT), and alternations of the expression for genes related to cell cycle progression, suggesting that inhibition of cell growth by compound 968 was related to induction of apoptosis and cell cycle arrest in breast cancer cells. These changes in gene expression were accompanied by an increase in histone H4K16ac and a decrease in H3K4me3 suggesting that altered regulation of cell cycle progression through histone modifications may also play a role [13, 22, 23]. In this study, we evaluated the impact of compound 968 on cell cycle arrest and apoptosis in three ovarian cancer cell lines. Our results show that compound 968 inhibited cell growth through induction of G1 phase cell cycle arrest and augmented Annexin-V expression, along with decreased expression of CDK4, cyclin D1 and MCL-1, and increased p21 and p27 expression in all three cell lines. These results are consistent with experiments showing deprivation of glutamine causes apoptosis and cell cycle arrest in some types of cancer [10, 24-26].

Deprivation of glutamine in cell culture can increase ROS production and induce endoplasmic reticulum stress [10]. Glutamine metabolism protects against oxidative stress by increasing reduced glutathione (GSH) levels, an antioxidant, and providing a source of nicotinamide adenine dinucleotide phosphate (NADPH) [27]. Depletion of GLS1 by shRNA exhibited lower levels of both GSH and oxidized glutathione (GSSG) [21], indicating that GLS1 activity was involved in the process of oxidative stress. Treatment with BPTES, an allosteric inhibitor of glutaminase, in lung cancer cells resulted in increased ROS levels through inhibition of glucose flow into the Kreb's cycle and reduced oxidative phosphorylation in the mitochondria [28]. In agreement with our previous study of glutamine metabolism in ovarian cancer, we found that compound 968 at varying concentrations led to decreased GLS1 expression and increased ROS production in a dose dependent manner with accompanied increased expression of ER stress markers including Calnexin, BIP and PERK. These changes ultimately culminated in cell death and decreased proliferation in all three ovarian cancer cell lines evaluated. Recent studies have implicated that histone H4K16AC acetylation and histone acetyltransferase, Mof, are associated with oxidative stress induced by hydrogen peroxide and that H4K16AC regulates the expression of the ROS-generating enzyme, NADPH oxidase 4 (Nox4) [29, 30]. Given that treatment with compound 968 increased H4K16AC level in breast cancer cells, we postulate that epigenetic regulation was involved in the cellular stress process.
induced by compound 968. The complexity of cellular oxidative stress and endoplasmic reticulum stress and the exact mechanisms by which compound 968 induces both stresses provide opportunities for further investigation. Hence, in combination with the above results, we believe compound 968 inhibits cell proliferation through multiple pathways including ROS formation, cell cycle arrest and apoptosis.

Paclitaxel is a standard chemotherapeutic agent for the treatment of ovarian cancer. It has been reported that paclitaxel can increase glutamine uptake and GLS1 expression in breast cancer cells. High expression of GLS1 and upregulation of glutamine metabolism are associated with paclitaxel resistant breast cancer cells and knockdown of GLS1 by siRNA results in sensitization of paclitaxel-resistant breast cancer cells to paclitaxel [18]. Glutamine deprivation creates synthetic lethality to paclitaxel in breast and prostate cell lines bearing K-Ras-mutations [26]. Targeting GLS1 by CB-839, a novel selective glutaminase inhibitor, significantly inhibited tumor cell growth in vitro and in vivo [25, 31, 32]. CB-839 in combination with paclitaxel largely increased the sensitivity to paclitaxel in xenograft models of triple-negative breast cancer [31]. After treating the ovarian cancer cell lines with different concentrations of glutamine in combination with paclitaxel, we observed that low concentrations or depletion of glutamine considerably increased the sensitivity of the cells to low doses of paclitaxel in all three cell lines. We also found that the combination of compound 968 and paclitaxel significantly and synergistically increased the sensitivity of the ovarian cancer cell lines to paclitaxel. Together, our results indicate that glutaminase appears to be a promising therapeutic target for ovarian cancer and worthy of further investigation.

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Disclosure of conflict of interest

None.

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