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An Investigation into the Impact of a Glutaminase Inhibitor, Compound 968, on Nrf2 Signaling

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Abstract: Glutaminase is a critical enzyme that catalyzes the process of glutaminolysis for energy synthesis. Meanwhile, glutaminase also contributes to the pathological process of various diseases, such as cancer, neurodegenerative diseases, and inflammation. This leads to the discovery of glutaminase inhibitors for therapeutical uses. However, the mechanisms of the beneficial therapeutical effect of glutaminase inhibitors are still unclear. This pilot study aimed to determine the impact of a well-characterized glutaminase inhibitor, compound 968 (C968), on Nrf2 signaling. We performed molecular docking, luciferase assay, and quantitative PCR to determine the activation of Nrf2 and the expression of several Nrf2-related genes. These experiments found that C968 induced the Nrf2 activation and promoted the expression of Nrf2, heme oxygenase-1 (HO-1), and NAD(P)H Quinone Dehydrogenase-1 (NQO-1). All findings provide evidence that Nrf2 activation could be one of the mechanisms contributing to the therapeutical activity of C968, but more studies are warranted to further confirm this mechanism.

Keywords: glutaminase inhibitor; compound 968; Nrf2; Keap1; neuroinflammation

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1. Introduction

Glutaminase is a mitochondrial enzyme that converts glutamine to glutamate [1]. The expression and function of glutaminase is upregulated, which contributes to tumor growth in several types of cancer [2]. The glutaminase promotes the metabolism of glutamine and glutamate, which provides sufficient energy for tumor growth [3]. In addition, glutamine metabolism also contributes to the tumor metastasis process through interactions with the epithelial–mesenchymal transition (EMT), tumor immunology, and tumor microenvironment [4]. Thus, glutaminase has been developed as an appealing target therapy for various cancers [5]. Numerous glutaminase inhibitors, such as compound 968 (C968, 5-(3-bromo-4-(dimethylamino)phenyl)-2,2-dimethyl-2,3,5,6-tetrahydrobenzo[a] phenanthridin-4(1H)-one, Figure 1), have been developed and characterized in different cancer models, in which telaglenastat (CB-839, 2-(pyridin-2-yl)-N-(5-(4-(6-(2-(3-(trifluoromethoxy)phenyl)acetamido) pyridazin-3-yl)butyl)-1,3,4-thiadiazol-2-yl)acetamide, Figure 1) is an orally bioavailable allosteric glutaminase inhibitor and is the most advanced drug candidate evaluated in clinical trials showing effectiveness in several tumor entities [6].

In the brain, glutaminase is the main glutamate producer enzyme, which is essential for both glutamate and GABAergic transmissions [7]. However, an excessive level of glutamate, produced by glutaminase, leads to neuronal damage and death, which contributes to neuroinflammation brain damage, cardiac arrest, seizures, and trauma [7–9]. Therefore, glutaminase is an important target for pharmacological therapy for a variety of brain

disorders and diseases [7]. However, the mechanisms of glutaminase inhibitors regulation of neuroinflammation are still unclear.

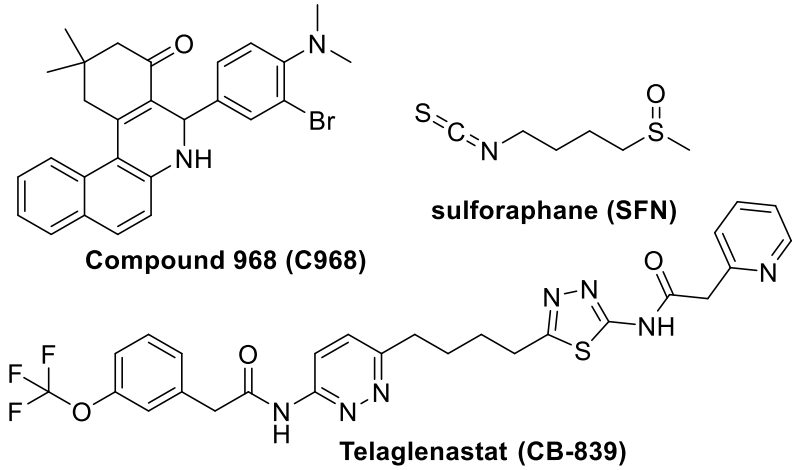

Figure 1. Structures of compound 968 (C968), Sulforaphane (SFN), and telaglenastat (CB-839).

Nuclear factor erythroid 2–related factor 2 (Nrf2) is a key regulator of the antioxidant and anti-inflammatory responses [10]. Activation of Nrf2 signaling is the first line of homeostatic response against oxidative stress, proteostasis, and inflammation [11–13]. Nrf2 activators have shown beneficial effects in most chronic diseases, such as neurodegenerative, vascular, and metabolic diseases [14]. Kelch-like ECH-associated protein 1 (Keap1), as the negative regulator of Nrf2 signaling, facilitates the ubiquitination and degradation of Nrf2 by binding to Nrf2. When reactive oxidative species (ROS) increase, oxidated Keap1 promotes the dissociation and translocation of Nrf2 to the nucleus so that the transcription of a series of antioxidant genes, including heme oxygenase-1 (HO-1), and NAD(P)H Quinone Dehydrogenase-1 (NQO-1), can be induced [15]. Several Nrf2 activators, such as sulforaphane (SFN, 1-Isothiocyanato-4-(methylsulfinyl) butane, Figure 1), have been developed for treating a variety of chronic diseases, such as multiple sclerosis [14]. SFN was initially isolated from *Brassicaceae* plants [16]. The SFN interacts with Keap1 and promotes the disassociation of Keap1/Nrf2 [17]. The protective effects of SFN on neurological disorders has been tested in preclinical models and clinical trials [14,18].

Previous studies have revealed some synergistic effects between Nrf2 signaling pathway and glutaminase activity [15]. However, no direct effect of glutaminase inhibitors on the Nrf2 signaling pathway has been previously reported. This study aimed to determine the potential impact of a glutaminase inhibitor, C968, on the Nrf2 signaling pathway. The findings from this study shed light on the mechanisms underlying the anticancer efficacy of glutaminase inhibitors.

2. Materials and Methods

C968 was synthesized in house following the procedures previously described in [19].

We first carefully examined the interaction of C968 with Keap1 in the Nrf2 binding pocket through molecular modeling studies. The molecular docking experiment on C968 binding to Keap1 was performed with Autodock Vina [20]. Crystal structure of Keap1 with cpd16, a reported inhibitor of Keap1-Nrf2 interaction (PDB ID: 4IQK), was retrieved from RCSB Protein Databank [21]. The protein was prepared by employing AutoDock Tools (version 1.5.6, Scripps Research Institute, La Jolla, CA, USA) software. The original compound cpd16 bound to the protein was deleted first, together with all water molecules. Then all the missing hydrogens and Kollman charges were added to this modified protein structure. The resulting format file was saved in a pdbqt file for further study. The number of grid points were set to 40 × 40 × 40, and the grid spacing was 0.375 Å. With the energy range set to be 4, eight docking runs were performed using a Lamarckian genetic algorithm and flexibility on all active torsions were allowed on C968.

We next evaluated the impact of C968 on Nrf2 activation using BV2 microglial cells stably expressing antioxidant element (ARE) luciferase reporters, which was generously provided by Dr. Valeri Mossine from the University of Missouri. BV2 microglial cells were seeded in a 96-well plate and cultured overnight in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS, CellGro brand, VWR, Radnor, PA, USA) and 1% Pen/Strep. The next day, cells were serum-starved in serum-free DMEM for 1 h, followed by treatment with a series concentration (started from 10 uM) of sulforaphane (SFN, purchased from Fisher Scientific, Hampton, NH, USA), C968, or telaglenastat (MedChemEpress, Monmouth Junction, NJ, USA) for 3 h. The cell culture medium was removed, and then 60 µL of lab-made luciferase lysis buffer was added to each well. The green fluorescence (GFP) was measured in the mini-plate reader (Biotek, Winooski, VT, USA). Then 30 µL of cell lysates were transferred to a solid-white plate. The luminescence was read immediately after adding 30 µL of luciferase substrates (lab made).

Furthermore, we investigated the expression of Nrf2 and downstream genes, heme oxygenase-1 (HO-1) and NAD(P)H Quinone Dehydrogenase-1 (NQO-1), in cells treated with or without C968. BV2 cells were seeded in a 6-well plate and cultured overnight. The next day, cells were serum-starved (in DMEM without FBS and P/S) for 1 h, followed by treatment with/without 3.3 µM of SFN, or 10 µM C968 or telaglenastat for 3 h. The total RNA was extracted using the Qiagen RNeasy mini kit (Qiagen, Germantown, MD, USA) following the protocol. The RNA was quantified using the NanoDrop (Thermo Scientific, Waltham, MA, USA), and 1 ug of total RNA was used for the cDNA synthesis using the iScript™ Reverse Transcription Supermix (BioRad, Hercules, CA, USA). The expression of target genes was measured using iQ™ SYBR® Green Supermix on the BioRad c1000Touch Thermal Cycler/CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA, USA). The program used was 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, with a final extension at 72 °C for 2 min and a melt curve analysis. The primers used were Nrf2-F—5′ TCT ATG TGC CTC CAA AGG 3′; Nrf2-R—5′ CTC AGC ATG ATG GAC TTG GA 3′0; HO-1-F—5′ CGC CTT CCT GCT CAA CAT T 3′; HO-1-R—5′ TGT GTT CCT CTG TCA GCA TCA C 3′; NQO-1-F—5′ TTC TCT GGC CGA TTC AGA G 3′; NQO-1-R—5′ GGC TGC TTG GAG CAA AAT AG 3′; glyceraldehyde phosphate dehydrogenase (GAPDH)-F—5′ TCC TGC ACC ACC AAC TGC TTA G 3′; GAPDH-R—5′ GAT GAC CTT GCC CAC AGC CTT G 3′. Cycle thresholds (CT) of each target were normalized to the CT of GAPDH in the same qPCR run. The resulting normalized CTs were transformed by 1/2ˆCT to generate a relative quantity for each target in each sample. The treatment effects were analyzed by one-way ANOVA and Dunnett multiple comparison tests using GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA, USA). A p value < 0.05 was considered to indicate statistical significance.

3. Results and Discussion

There are several mechanisms for a compound to active Nrf2 signaling pathway, with the most commonly pursued one being the modulation of Keap1–Nrf2 interaction [22]. Most of the well-known Nrf2 activators are irreversible inhibitors of Keap1 with high electrophilic motifs that can covalently bind to the cysteine residue of Keap1 to enhance the disassociation of Nrf2 [23]. More recently, developing small molecules capable of competing with Nrf2 for the binding pocket in Keap1 have emerged as an attractive approach to directly interfere with the Keap1–Nrf2 interaction and activating Nrf2 signaling [24]. C968, with no electrophilic feature in its structure, will not bind to Keap1 covalently. Therefore, its activity in activating Nrf2 signaling probably comes from its binding to Keap1 in the interface of the Keap1–Nrf2 interaction, thus preventing Nrf2 from binding to Keap1. The results from our docking experiments confirmed this hypothesis. The analysis revealed that C968 binds to Keap1 in the same binding pocket as the reported Keap1–Nrf2 interaction inhibitor cpd16 [24] with a high binding affinity (Binding affinity = −9.4 kcal/mol) (Figure 2).

This interaction of C968 with Keap1 excludes Nrf2 from binding to Keap1, which results in the disassociation of Nrf2 and activation of Nrf2 signaling pathway.

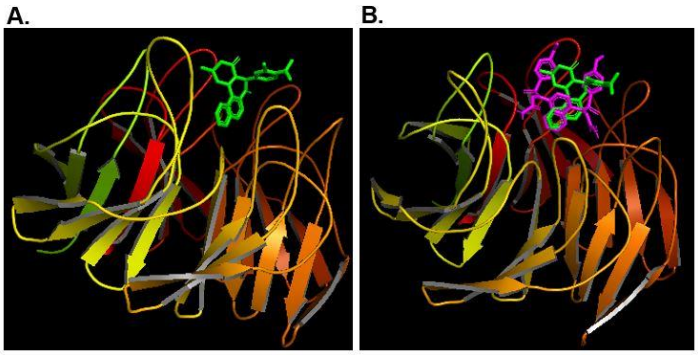

Figure 2. Docking images of compound 968 (C968) with Keap1. (**A**) Docking image of C968 binding to Keap1. (**B**) Overlap of C968 docking image with X-ray structure of cpd16 with Keap 1(PDB ID: 4IQK) with Keap1 (Purple is cpd16 and green is C968).

Sulforaphane is a well-characterized Nrf2 activator and has shown a beneficial impact on reducing pain and improving opioid therapy [25]. As expected, sulforaphane strongly promotes Nrf2 activation in BV2 microglial cells (Figure 3A). Treatment with C968 dose-dependently activated Nrf2 signaling (Figure 3B) and exhibited similar potency as that of SFN (C978, 0.83 ± 0.13 nM; SFN, 0.52 ± 0.16 nM). However, another glutaminase inhibitor, telaglenastat, showed no impact on Nrf2 activation (Figure 3B). This finding demonstrates that the interaction between C968 and Keap1, the negative regulator of Nrf2 signaling, causes the disassociation of Keap1 and Nrf2, leading to the activation of this signaling pathway.

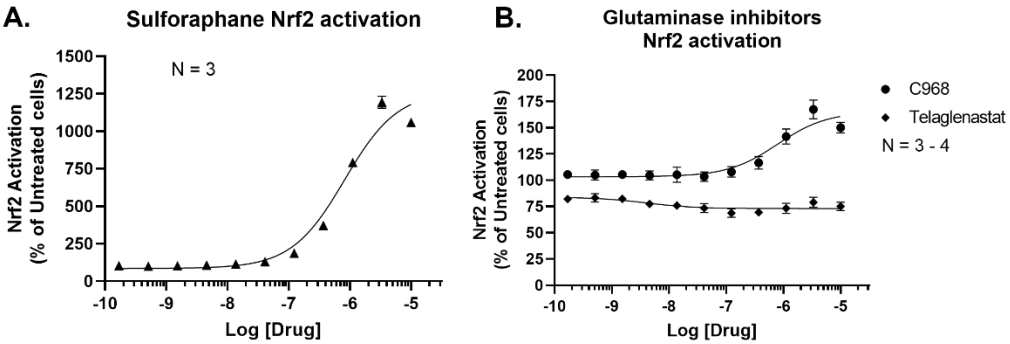

Figure 3. Compound 968 (C968) activates Nrf2 signaling in a dose-dependent matter. BV2 microglial cells stably expressing antioxidant element (ARE) reporter were treated with a series concentration (started from 10 μM) of (**A**) sulforaphane, (**B**) C968 or telaglenastat. Cells were lysed, and the green fluorescence (GFP) and luciferase activity were measured in the mini-plate reader. The luminescence data were normalized by the GFP data, and the normalized data from non-treated cells were considered as 100%. Data from at least 3 independent experiments are presented as mean ± SEM.

To further confirm the impact of C968 on the Nrf2 signaling pathway, we performed quantitative PCR to measure the expression of Nrf2, HO-1, and NQO-1. We chose 3.3 μM sulforaphane based on the dose-dependent curve of Nrf2 activation (Figure 3A). Treatment with sulforaphane promoted the expression of HO-1 and NQO-1 but not Nrf2 (Figure 4). This could be explained by sulforaphane disrupting the association of Nrf2 and Keap1 instead of changing the expression of Nrf2. We also found that treatment with C968 not only increased the expression of HO-1 and NQO-1 but also promoted Nrf2 expression (Figure 4). However, it is unclear whether C968 activates Nrf2 via Keap1-dependent and/or

-independent mechanisms [26]. Similar to the activity on Nrf2 activation, telaglenastat had a very limited effect on the expression of evaluated genes (Figure 4).

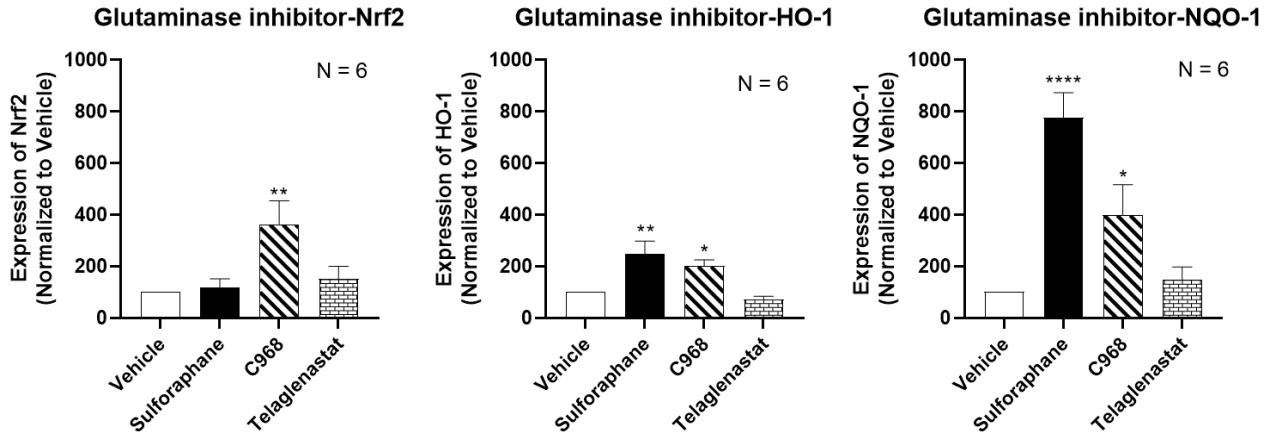

Figure 4. Compound 968 promotes the expression of Nrf2, HO-1, and NQO-1. BV2 cells were treated with sulforaphane, C968, and telaglenastat for 3 h. The total RNA was extracted for reverse transcription, and the expression of Nrf2, HO-1, and NQO-1 was measured by quantitative PCR using GAPDH as loading control. The normalized data from non-treated cells were considered as 100%. Data from 4 independent experiments are presented as mean ± SEM. *, **, and ****, $p < 0.05$, 0.01, and 0.0001, respectively, versus vehicle group by one-way ANOVA with Dunnett post-hoc test.

In summary, through binding to Keap1 at the interface between Keap1 and Nrf2, C968 exhibits the capability of interfering with Keap1–Nrf2 interaction to activate Nrf2 signaling, which could be one of the mechanisms of its beneficial therapeutical effects in addition to its glutaminase inhibitory activity. Our study revealed that C968 not only activated Nrf2 but also promoted the expression levels of Nrf2, HO-1 and NQO-1 (Figure 5). However, more studies are needed to reveal a better understanding of how C968 regulates the Nrf2 signaling pathway and how this mechanism is used as a potential therapeutical approach for neurological disorders.

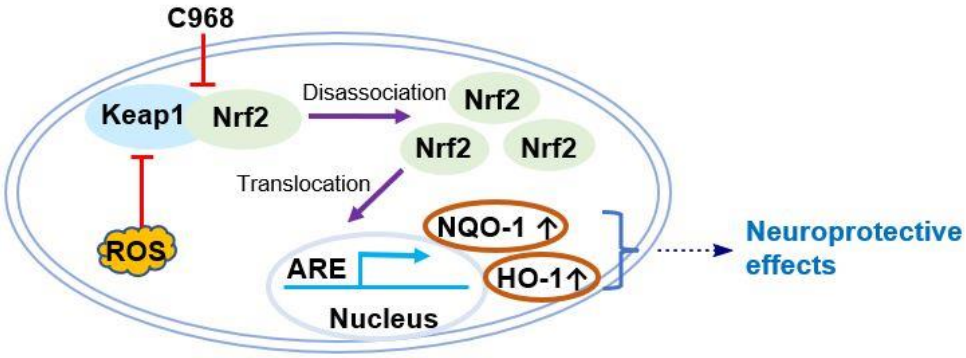

Figure 5. Compound 968 (C968) activates Nrf2 signaling.

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