

SGLT2 inhibition suppresses glioblastoma cell proliferation via AMPK activation and mTOR-dependent reduction of protein synthesis under glucose starvation

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Abstract

Purpose Glioblastoma (GBM) is a malignant brain tumor classified as WHO grade 4, with a median survival of approximately 15 months. Despite multimodal treatment strategies including surgical resection, chemotherapy, and radiotherapy, recurrence is inevitable. This study tested the hypothesis that cancer-specific metabolic profiles could serve as therapeutic targets for GBM, with the aim of developing effective drug therapies. We investigated sodium-dependent glucose transporter 2 (SGLT2) expression on GBM cells and examined the effect of canagliflozin, a selective SGLT2 inhibitor on glucose uptake. In addition, we evaluated the efficacy of canagliflozin on cell proliferation and survival. Finally, the impact of canagliflozin on subcutaneous tumor growth was evaluated using a xenograft model.

Methods The effects of SGLT2 inhibition in GBM were assessed using cultured human GBM cell lines. SGLT2 expression was evaluated by immunoblot analysis. The effect of canagliflozin on intracellular glucose uptake was analyzed using isotope-labeled glucose, with radioactivity quantified by liquid scintillation counting. In vitro responses to canagliflozin were assessed by cell viability, Ki67 proliferation, and apoptosis assays. Changes in signal transduction were examined by immunoblot analysis, focusing on AMPK activation and mTORC1 inhibition. Global protein synthesis was monitored by the SUnSET (surface sensing of translation) method. In mice xenografted with NGT41 cells, patient-derived GBM cell lines, canagliflozin was administered for 10 days, and in vivo responses were evaluated by measuring tumor volume.

Results SGLT2 expression was detected in GBM cell lines. In NGT41 cells, canagliflozin dose-dependently inhibited intracellular glucose uptake. Canagliflozin also suppressed cell growth, accompanied by a reduction in proliferating cells and an increase in apoptotic cells. Immunoblot analysis demonstrated the activation of AMPK and inhibition of mTORC1 signaling following canagliflozin treatment. Protein synthesis activity during glucose starvation was reduced by canagliflozin. Administration of canagliflozin significantly reduced tumor volume in vivo and decreased the number of Ki67-positive cells in tissue sections.

Conclusions This study demonstrates that the SGLT2 inhibitor canagliflozin induces a glucose starvation in GBM cells, leading to suppression of proliferation. Targeting glucose metabolism via SGLT2 inhibition may represent a promising therapeutic strategy for GBM.

Introduction

Glioblastoma (GBM) is a malignant brain tumor classified as WHO grade 4, with a median survival of approximately 15 months [1]. Despite the availability of multimodal treatments including surgical resection, chemotherapy, and radiotherapy, the overall survival has not improved [2]. Due to the highly invasive nature of GBM, complete removal is nearly impossible, and recurrence is inevitable. Therefore, the development of novel therapeutic strategies is urgently required to improve outcomes.

There has been a growing interest in cancer metabolism because its unique metabolic profile is expected to be an efficient therapeutic target. In pioneering cancer research, Warburg described the characteristic metabolic shift in cancer cells, wherein glucose is highly required and preferentially metabolized to lactate via anaerobic glycolysis [3, 4]. Glucose uptake in cancer cells primarily occurs through glucose transporters (GLUTs), particularly GLUT1, which is frequently overexpressed in various malignancies [5, 6]. Sodium-dependent glucose transporters (SGLTs) carry glucose and other nutrients across the cell membrane to maintain an intracellular sodium gradient [7, 8]. SGLT2 is highly expressed on the brush border membrane of proximal tubular epithelial cells in the kidney. Importantly, SGLT2 expression has also been reported in high-grade astrocytoma [9, 10]. Functionally, SGLT2 inhibition has been shown to block glucose uptake and suppress tumor growth in pancreas and prostate adenocarcinomas and xenograft models [11]. Furthermore, various SGLT2 inhibitors have been reported to suppress the survival and proliferation of cancer cells [12, 13]. These atypical features of SGLT2 inhibitors are thought to reflect a starvation-induced effect in tumor cells, although the underlying molecular mechanisms remain unclear.

AMP-activated protein kinase (AMPK) is a key energy sensor. It is activated in response to increased intracellular AMP/ATP ratio and phosphorylates acetyl-CoA carboxylase (ACC) during energy-depleting stresses such as ischemia and starvation [14, 15]. Activated AMPK suppresses ATP-consuming anabolic pathways, including lipid, glycogen, and protein synthesis [16, 17]. Moreover, AMPK inhibits mammalian target of rapamycin complex 1 (mTORC1) signaling by activating the tuberous sclerosis complex 2 (TSC2) and through direct inhibitory phosphorylation of the Raptor, an mTOR binding partner, thereby limiting cellular proliferation [18, 19]. mTORC1 integrates growth factor signaling and cellular metabolism through substrates such as S6K, underscoring its value as a therapeutic target in cancer [20, 21]. Therefore, activation of AMPK could potentially be a strategic target in cancers. Given the hypoxic and low energy conditions typical of the tumor microenvironment, understanding how cancer cells adapt to metabolic stress is crucial.

We focused on SGLT2, which is expressed in brain tumors, and hypothesized that its inhibition would disrupt the glucose supply to tumor cells, inducing a starvation response. To propose a new treatment for GBM, it is necessary to investigate tumor cell metabolism and elucidate the mechanisms by which tumor cells adapt to starvation. We investigated SGLT2 expression in GBM cell lines and assessed its functional role using the glucose uptake assay under canagliflozin exposure. We further examined the effects of canagliflozin on cell survival and its impact on key metabolic signaling pathways. Additionally, we monitored the capacity for novel protein synthesis. Finally, we evaluated the *in vivo* therapeutic potential of canagliflozin using a GBM xenograft mouse model. Through these studies, we aim to provide preclinical evidence supporting SGLT2 inhibition as a novel therapeutic strategy for GBM.

Materials and methods

Reagents

For in vitro experiments, canagliflozin (CANAGLU tablets: Tanabe-Mitsubishi, Japan) was used. Canagliflozin was dissolved in ethanol. A 220 mM stock solution was then prepared and stored at -20°C until use. For in vivo treatment experiments, canagliflozin was diluted in an appropriate solvent and administered to animals.

Cell culture

Cell culture of GBM cell lines was prepared by modification of a previously described method [22]. Briefly, cells were grown in DMEM containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA). Human GBM cell lines (T98G and U87MG) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). NGT41 was a cell line established from disseminated lesions in the spinal cord of a patient with epithelial GBM [23] after obtaining approval from the Institutional Review Board of Niigata University (No. 2016–2583). The NGT191 cell line was also established from a surgical specimen resected from a GBM patient. GBM1 and JHH-DIPG cells were grown in suspension in Neurobasal medium base supplemented with glutaMAX, B27, and N2 (Thermo Fisher Scientific, Rockford, IL, USA).

Glucose uptake assay

To investigate the effect of canagliflozin on glucose uptake into tumors, isotope-labeled glucose was added to the culture medium. The procedure was modified from a protocol we previously described [24]. NGT41 cell line was cultured in DMEM containing 10% fetal bovine serum, then washed and replaced with glucose-free DMEM. Cells were incubated with 2 μ Ci of [^{14} C]-glucose (0.20 mCi/mL) (Perkin-Elmer, USA) for 15 min. Canagliflozin (0, 25, 50, and 100 μ M) was added to the culture 10 min prior to the addition of [^{14} C]-glucose. Uptake was stopped by three washes with ice-cold PBS, and cells were lysed in 200 μ L of 0.1 N NaOH. Samples were then collected, and the radioactivity of [^{14} C]-glucose was measured by a liquid scintillation counter. The ratio of [^{14} C]-glucose to total [^{14} C]-glucose taken up by the cells was calculated. These values were corrected for total protein content, which was quantified by the Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA).

Cell viability assay

The cytotoxic effects of canagliflozin were determined by using the cell proliferation assay reagent WST-1 (Takara Bio, Japan), as described previously [22]. Briefly, glioma cells were seeded at a density of $1-1.5 \times 10^3$ cells/well in 96-well flat-bottomed plates and incubated at 37°C overnight. After, the cells were treated with canagliflozin (at indicated concentrations) for 72 h. Following treatment, WST-1 reagent was added to each well and incubated for 1–2 h at 37°C. Absorbance was measured at 450 nm using a microplate reader. The viability of untreated cells was considered as 100%.

Ki67 proliferation analysis and dead cell assay

To evaluate whether treatments induced cell cycle arrest, GBM cell lines, NGT41, T98G, U87MG, and NGT191 were treated with canagliflozin at indicated concentrations for 72 hours. The proportion of

proliferating cells within the cell population was measured using the proliferation marker Ki67. Additionally, apoptosis assessment was performed. Cells were washed with PBS and trypsinized. Approximately 1×10^5 cells were then fixed. Cell preparations were performed using MUSE Ki67 proliferation and MUSE Annexin V-dead cell assay kits (Millipore, Billerica, MA, USA). Analysis was conducted using the MUSE cell analyzer, following the manufacturer's protocol.

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Immunoblotting

Cells or tumor tissues were sonicated in lysis buffer. Cell lysates were heat denatured as described previously [22]. Protein concentration in the lysates was determined using the Micro BCA Protein assay kit. Equal amounts of protein (10–30 μ g) were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Immunoblotting was performed using anti-phospho-p70S6K (Thr389) [1:500], anti-phospho-S6 (Ser240/244) [1:1000], anti-S6 (1:4000), anti-phospho-acetyl-CoA carboxylase (Ser79) [1:1000], anti-phospho-AMPK α (Thr172) [1:1000], and anti- β -actin [1:4000] polyclonal antibodies (Cell Signaling Technologies, Danvers, MA, USA). Anti-p70S6K antibody and anti-SGLT2 antibody (D6) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Membranes were incubated with the indicated primary antibodies overnight at 4 °C. After the membranes were rinsed with TBST, they were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (1:10000; Santa Cruz, CA, USA). Immunoreactivity was detected by chemiluminescence detection method using the ECL system (Bio-Rad, Hercules, CA, USA). The immunoreactive bands were visualized using GeneGnome (Syngene, Cambridge, UK) and quantified using the Syngene Bio Imaging system (Syngene, Cambridge, UK). β -actin was used as a loading control.

Monitoring of protein synthesis

The SUnSET method was employed for immunoblotting to monitor global protein synthesis during canagliflozin administration [25]. Puromycin, which is structurally similar to aminoacyl-tRNA, is bound to the C-termini of newly synthesized polypeptide chains and forces the stop of elongation. The synthetic peptide chain dissociates from the ribosome when it binds puromycin. Resulting nascent chains were detected with an anti-puromycin antibody. NGT41 was treated with 50 μ M SGLT2 inhibitor canagliflozin (at the indicated times) 30 minutes prior to the addition of 1 μ M puromycin. Finally, cells were lysed in SDS lysis buffer. After centrifugation, the supernatant was collected, and protein concentrations were

determined. 2 μ L of samples were spotted onto a nitrocellulose membrane, and the amount of puromycin uptake was assessed by immunoblotting. The activity was detected by chemiluminescence detection method using the ECL system (Bio-Rad, Hercules, CA, USA). The immunoreactivity was visualized using GeneGnome (Syngene, Cambridge, UK) and quantified using the Syngene Bio Imaging system (Syngene, Cambridge, UK).

Experimental animals

Four-week-old male nude mice (BALB/C-nu/nu, Charles River Laboratories Inc., Yokohama, Japan) were used for in vivo experiments. Mice were housed under aseptic conditions in a plastic cage and provided free access to food and water. Each cage was kept in a colony room (22 ± 1.0 °C) under a 12 h light-dark cycle. All the animal experiments described here were approved by the Animal Committee of Niigata University (No. SA00519) and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals (NIH, USA).

Establishment of the patient-derived xenograft model

For the subcutaneous tumor model, NGT41 cells were suspended in Neurobasal Medium (Life Technologies Corporation, NY, USA) and implanted subcutaneously (1×10^6 cells per place) into nude mice as described previously [23]. When the tumor volume reached 50 mm³, the mice were randomly divided into two groups. Mice were treated daily with solvent (vehicle control) or canagliflozin (30 mg/kg) by oral gavage for 10 days. Tumor size was measured daily with calipers, and tumor volume was calculated using the formula: tumor volume (mm³) = [length (mm) $\times$ width (mm²)] / 2.

Histopathological examination

Pathological examination was performed on mouse subcutaneous tumors formed by xenografts as previously reported [22]. Mice were euthanized at 1 hour following completion of the last canagliflozin treatment. Tumors were resected from mice under deep anesthesia, fixed in 4% paraformaldehyde, and embedded in paraffin. Sections were then cut from the paraffin blocks, and immunohistochemistry was performed. These sections were stained with HE, anti-Ki-67 monoclonal antibody (clone MIB-1; 1:100; Dako, Denmark) and anti-phospho-S6 (Ser240/244) [1:1000] polyclonal antibody (Cell Signaling Technologies, Danvers, MA, USA).

Statistical analysis

Data were expressed as mean $\pm$ SEM and were subjected to parametric analyses. When univariate data with more than two groups showed similar distribution, we used the analysis of variance (ANOVA) to assess the statistical differences among dose and experimental groups, followed by post-hoc test for multiple comparisons. Alternatively, Student's t-test (two-tailed) was used for univariate data analysis of two groups. All statistical analyses were performed using the GraphPad Prism 10 software (GraphPad Software, CA, USA).

Results

SGLT2 is expressed in human GBM cell lines and resected brain tumor specimens, and canagliflozin suppresses intracellular glucose uptake

SGLT2 protein has been reported to be expressed in high-grade glioma cells [10]. To elucidate the functional role of SGLT2 in GBM, we examined its relative expression levels of SGLT2 in human glioma cell lines. Expression of SGLT2 was detected in cultured GBM cell lines (Figure 1A). Furthermore, SGLT2 protein was also expressed in resected tumor specimens obtained from a patient with a meningioma. To determine whether SGLT2 expressed in GBM cell lines contributes to glucose transport into cells, we attempted to inhibit glucose uptake using the SGLT2 inhibitor canagliflozin (Figure 1B). Canagliflozin significantly reduced glucose uptake into NGT41 cells compared to the control cultures (**** $p < 0.0001$ at both 50 μM and 100 μM canagliflozin concentrations). These findings suggest that NGT41 cell proliferation partially utilizes glucose uptake mediated by SGLT2.

To evaluate the efficacy of canagliflozin on cell proliferation, cell viability assays were performed using GBM cell lines (Figure 2A). Canagliflozin significantly reduced NGT41 cell growth, as determined by the WST1 assay (* $p < 0.05$ for 25 μM , ** $p < 0.001$ for 50, 100 μM , and *** $p < 0.001$ for 200 μM canagliflozin vs. control culture). The half-maximal effective dose (ED50) of canagliflozin was estimated to be approximately 50-100 μM across the tested cell lines. Additionally, microscopic observations revealed that canagliflozin affects not only cell survival but also cell morphology (Figure 2B). Similar results were confirmed in experiments adding canagliflozin to representative human GBM cell lines T98G, U87MG, and NGT191 (Supplemental Figure 1A, 1B). Thus, reduced cell survival and morphological changes, such as cell size enlargement, were observed in all GBM cell lines following canagliflozin treatment.

Canagliflozin inhibits proliferation and induces apoptotic cell death in cultured GBM cells

To examine the effect of canagliflozin on proliferative activity, a Ki67 proliferation assay was performed in NGT41 (Figure 3A). A significant reduction in the number of proliferating cells relative to the total cell population was induced by canagliflozin in a dose-dependent manner (* $p = 0.0168$ for 50 μM , *** $p = 0.0003$ for 100 μM canagliflozin vs. control culture. Furthermore, a significant increase in apoptotic cells within the total cell population was observed following canagliflozin treatment (** $p = 0.0018$ vs. control culture: Figure 3B). These results indicate that canagliflozin affects cell proliferation and induces cell death.

Histogram of NGT41 cells after canagliflozin treatment (red: Ki67-positive population, blue: Ki67-negative population). The proportion of proliferating cells is shown as a bar graph. Similar results were obtained from four replicate experiments. One-way ANOVA with Dunnett's multiple comparisons test was applied to statistically analyze: * $p = 0.0168$, *** $p = 0.0003$. (B) Detection of apoptosis in NGT41 treated with canagliflozin. Description of each quadrant (Upper left: dead cells, Upper right: late apoptotic cells or dead cells, lower left: live cells, lower right: early apoptotic cells). Each population was gated and quantified by cell size and cell count, then plotted separately for canagliflozin concentrations

(0 - 100 μ M). Similar results were obtained from four replicate experiments. One-way ANOVA with Dunnett's multiple comparisons test was applied to analyze: $**p = 0.0018$.

Canagliflozin induces AMPK activation and inhibits mTORC1 signaling in cultured GBM cells

Since canagliflozin inhibited glucose uptake and suppressed viability of GBM cells, we examined its effects on intracellular signaling pathways. Activation of AMPK was assessed by western blot analysis using an anti-phospho AMPK (Thr172) antibody against NGT41 cell lysates (Figure 4A). Phosphorylation of AMPK was increased by canagliflozin treatment ($*p = 0.0232$ for 50 μ M, $****p < 0.0001$ for 100 μ M canagliflozin vs. control culture). Consistent with this, ACC phosphorylation was increased following canagliflozin exposure ($**p = 0.0028$ vs. control culture). Furthermore, we examined the effect of canagliflozin on mTORC1 signaling. The phosphorylation status of mTORC1 was evaluated by western blot analysis using anti-phospho p70S6K (Thr389) antibody (Figure 4B). Canagliflozin reduced the phosphorylation of p70S6K in dose dependent manner, with maximal inhibition observed at 100 μ M ($*p = 0.0299$ for 25 μ M, $***p = 0.0005$ for 50 μ M, and $****p < 0.0001$ for 100 μ M canagliflozin vs. control culture). Furthermore, canagliflozin suppressed the phosphorylation of S6, a downstream substrate of p70S6K ($*p = 0.0185$ for 50 μ M, and $***p < 0.0002$ for 100 μ M canagliflozin vs. control culture), indicating that canagliflozin inhibits mTORC1 activity and its downstream signal cascade. Similar trends were confirmed in experiments where canagliflozin was added to human GBM cell lines T98G, U87MG, and NGT191. AMPK activation and mTORC1 signaling inhibition by canagliflozin were shown by immunoblotting (Supplemental Figures 2 and 3). These results indicate that canagliflozin activates AMPK and ACC signaling in GBM cell lines.

Global protein synthesis in GBM cells is suppressed by canagliflozin.

To verify the effect of canagliflozin on translation, we evaluated its activity on protein synthesis activity using the SUNSET method (Figure 4C). Nascent peptide synthesis was significantly reduced 2 hours after treatment with 50 μ M canagliflozin in NGT41 ($****p < 0.0001$ canagliflozin vs. control culture), and similarly reduced in T98G, U87MG, and NGT191 cells (Supplemental Figure 4), although

the effect was modest in T98G cells. These findings suggest that canagliflozin inhibits novel protein synthesis in GBM cells.

Canagliflozin reduces tumor growth in subcutaneous human GBM xenografts without significant body weight loss or toxicity

We established a tumor model by subcutaneously transplanting NGT41 cells into BALB/C nude mice. The anti-proliferating activity of canagliflozin was verified in vivo following previously reported methods. After tumor establishment, mice were orally administered vehicle or canagliflozin (30 mg/kg) daily for 10 days. Tumor growth was markedly suppressed in the canagliflozin-treatment group compared with the vehicle group, and tumor volume did not increase during the treatment period (Figure 5B). No apparent adverse events, such as weight loss, were observed with canagliflozin treated mice.

Immunohistochemical analysis of tumor sections revealed decreased staining for Ki67 (clone MIB1), and phospho S6 in the canagliflozin treated group (Figure 5C). Immunoblot analysis of protein extracts from resected tumors at the end of treatment showed significantly decreased phosphorylation levels of p70S6K and S6 (Figure 5D).

Discussion

Glucose metabolism is essential processes for all the cells to produce ATP, the cellular energy currency. In many tumor cells, including GBM, metabolic reprogramming called Warburg shift has been observed. In these cells, glucose is mainly metabolized by aerobic glycolysis instead of regular oxidative phosphorylation. Although this process is thought to be beneficial to tumor growth, it is less efficient to produce ATP. To produce enough ATP, tumor cells require much glucose. In addition to glucose transporter (Glut) family molecules, GBM is reported to express SGLT2, that is expressed almost only in kidney among normal tissues.

For some SGLT2 inhibitors, biological activities beyond glucose uptake have been reported. As a direct effect on myocardial cells, SGLT2 inhibitors applied to SGLT2-low expressing myocardial cells and vascular endothelial cells demonstrated anti-inflammatory and oxidative stress-suppressing effects [26]. Empagliflozin has shown to prevent myocardial infarction, independent of SGLT2 and improve cardiac function and reduce oxidative stress in heart failure model using SGLT2 knockout mice [27]. Furthermore, as a regulatory effect on autophagy, empagliflozin ameliorates acute kidney injury in a mouse nephrectomy model by improving glomerular hyperfiltration, resolving lysosomal dysfunction, and correcting impaired autophagy [28]. Canagliflozin also inhibits mitochondrial complex-1 activity and exerts antiproliferative effects on lung cancer cells [29]. To isolate these direct effects of SGLT2 inhibitors, siRNA to knock down SGLT2 expression was attempted in U251 GBM cells, demonstrating decreased cell viability, AMPK activation, and mTOR suppression [30]. This research suggests an antitumor effect based on energy supply blockage mediated by SGLT2, supporting our findings that AMPK-dependent signaling under a low-glucose condition induced by canagliflozin contributes to reduced survival and proliferation in GBM cells. AMPK is activated in response to low energy status and phosphorylates Raptor and TSC2. This results in the inhibition of mTORC1 [18, 19, 31–33]. Therefore, the activation of AMPK and inhibition of mTORC1 resulting from glucose uptake suppression can be considered a pharmacological effect of canagliflozin.

The role of AMPK in tumor suppression remains controversial. In clinical samples, the U87MG human malignant glioma cell line, and a mouse astrocytoma model, a correlation between AMPK activity and tumor cell proliferation was confirmed. In this context, AMPK is known to increase Rb phosphorylation, release E2F transcription factor, and promote cell cycle progression [34]. Furthermore, AMPK activation increases glioma cell survival by inducing autophagy and suppressing apoptosis via caspase-3 and p53 [35]. Therefore, AMPK possesses a dual role, acting tumor-promotively under specific conditions [36]. We investigated the effect of AMPK activation on glioma cell proliferation. AMPK activation, mediated by canagliflozin-induced glucose uptake, significantly reduced the number of Ki67-positive cells undergoing

proliferation. Furthermore, AMPK activation promoted apoptosis. In a mouse xenograft model using NGT41 cells, canagliflozin treatment significantly reduced tumor volume. In the subcutaneous tumor, mTOR signaling was suppressed, and a decrease in Ki67-positive cells was observed. However, the contribution of AMPK to cell cycle progression or arrest, and its role in maintaining cancer metabolism, remain poorly understood. Interestingly, AMPK knockdown or overexpression causes similar effects in cells: the cell cycle halts, leading to aneuploidy [37]. This indicates that appropriate switching of AMPK signaling is essential for the progression of mitosis. The development of rhythmic AMPK regulation synchronized with the cell cycle may be required in cancer treatment.

The findings of the present study highlight SGLT2 as a promising metabolic target in GBM. The high glucose dependence of GBM suggests that blocking glucose uptake via SGLT2 could impair tumor growth. Although the role of AMPK in cancer remains to be fully understood, our study supports the hypothesis that SGLT2 inhibitors act as indirect AMPK activators and have antitumor potential.

Conclusions

This study demonstrates that canagliflozin induces metabolic stress and suppresses GBM growth through modulation of the AMPK/mTOR axis. The unique biological effects of SGLT2 inhibitors may contribute to the development of novel GBM therapies. Future studies should explore integrating SGLT2 inhibitors with standard chemotherapy to enhance treatment outcomes.

Declarations

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Contributions

Conception and design of the work: T.E., N.T., and M.N.; acquisition and analysis of data: T.E. and N.T.; interpretation of data: T.E., N.T., M.Ok., J.W., R.O., Y.T., M.Oi., and M.N.; original draft: T.E., N.T., and M.N.; critical review of manuscript and editing: N.T., M.Ok., J.W., R.O., Y.T., M.Oi., and M.N.; approved the final version to be published: all authors; agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: all authors

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Figures

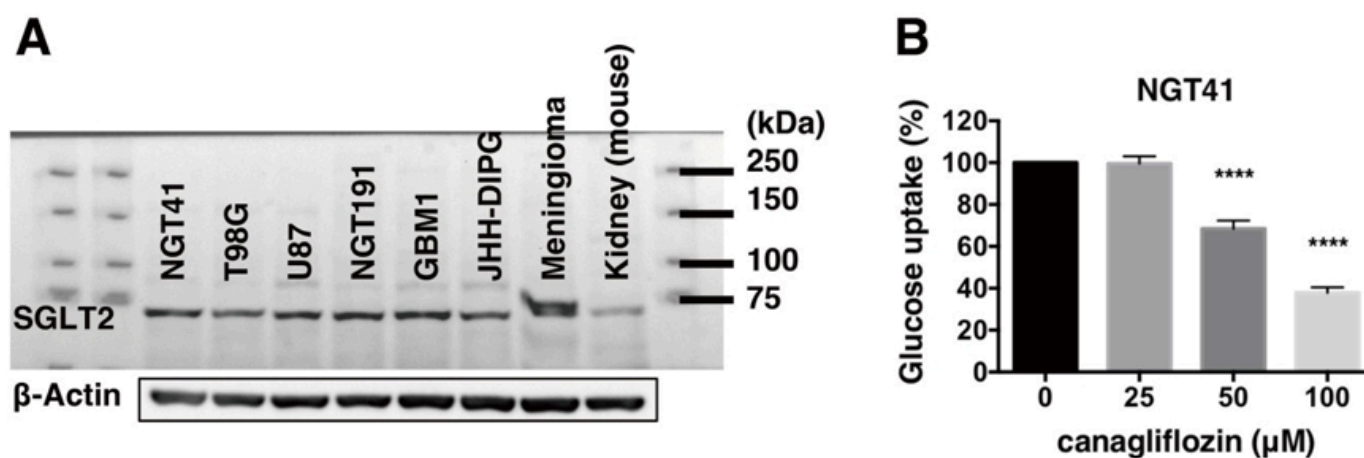


Figure 1

Expression and functional role of SGLT2 in GBM cell lines

(A) Western blot analysis showing SGLT2 protein expression in cultured GBM cell lines. Mouse kidney, the renal cortex was used as a control sample for SGLT2 expression. “Meningioma” denotes the surgical specimen removed from a meningioma patient. β -actin was used as internal control levels in all cases. (B) Significant reduction of glucose uptake by the SGLT2 inhibitor canagliflozin in NGT41 cells. The bar graph represents glucose uptake at each canagliflozin concentration (0, 25, 50 and 100 μ M) in NGT41. Data are shown as mean $\pm$ SEM from three replicates. One-way ANOVA with Dunnett’s multiple comparisons test was applied to statistically analyze: **** $p < 0.0001$.

Canagliflozin reduces the survival of cultured GBM cell lines.

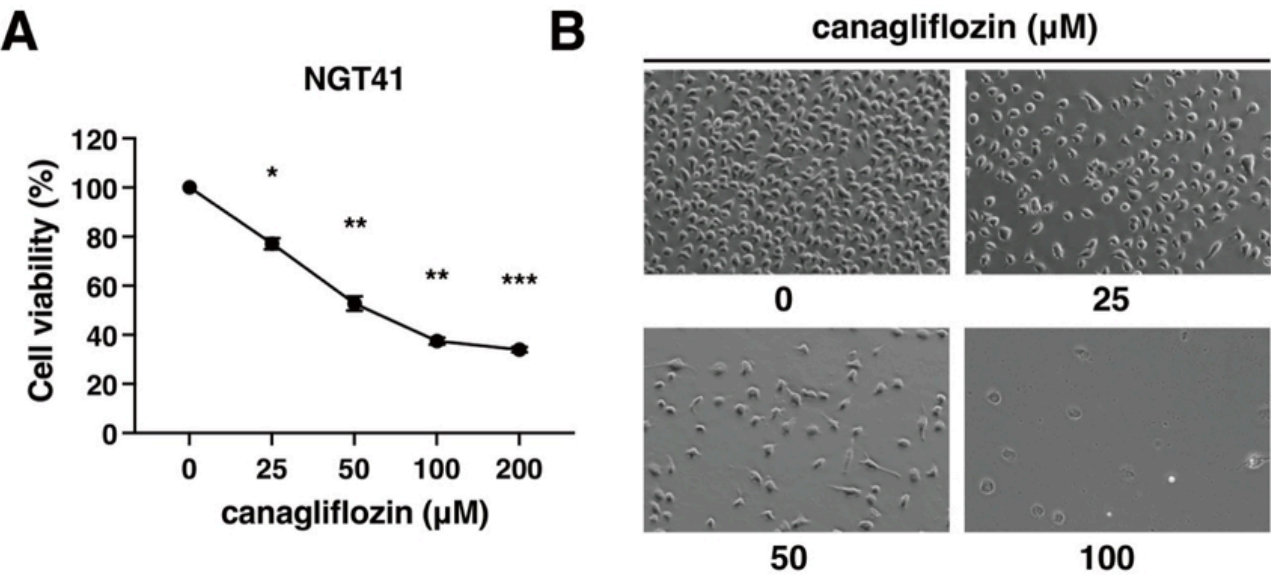


Figure 2

Inhibitory effect of canagliflozin on the proliferation of cultured GBM cell lines

(A) Dose responses of canagliflozin on cell viability in human GBM cells NGT41. Cell viability was assessed using the WST-1 assay after NGT41 cells were treated with indicated concentrations (0 - 200 μ M) of canagliflozin for 72 hours. The viability of untreated cells (vehicle control: 0 μ M canagliflozin) was considered 100 %. Data are presented as mean $\pm$ SEM. Similar results were obtained from triplicate experiments. One-way ANOVA with Dunnett’s multiple comparisons test was applied to statistically analyze: **** $p < 0.0001$. (B) Microscopic image of NGT41 cells 72 hours after canagliflozin treatment at indicated concentrations.

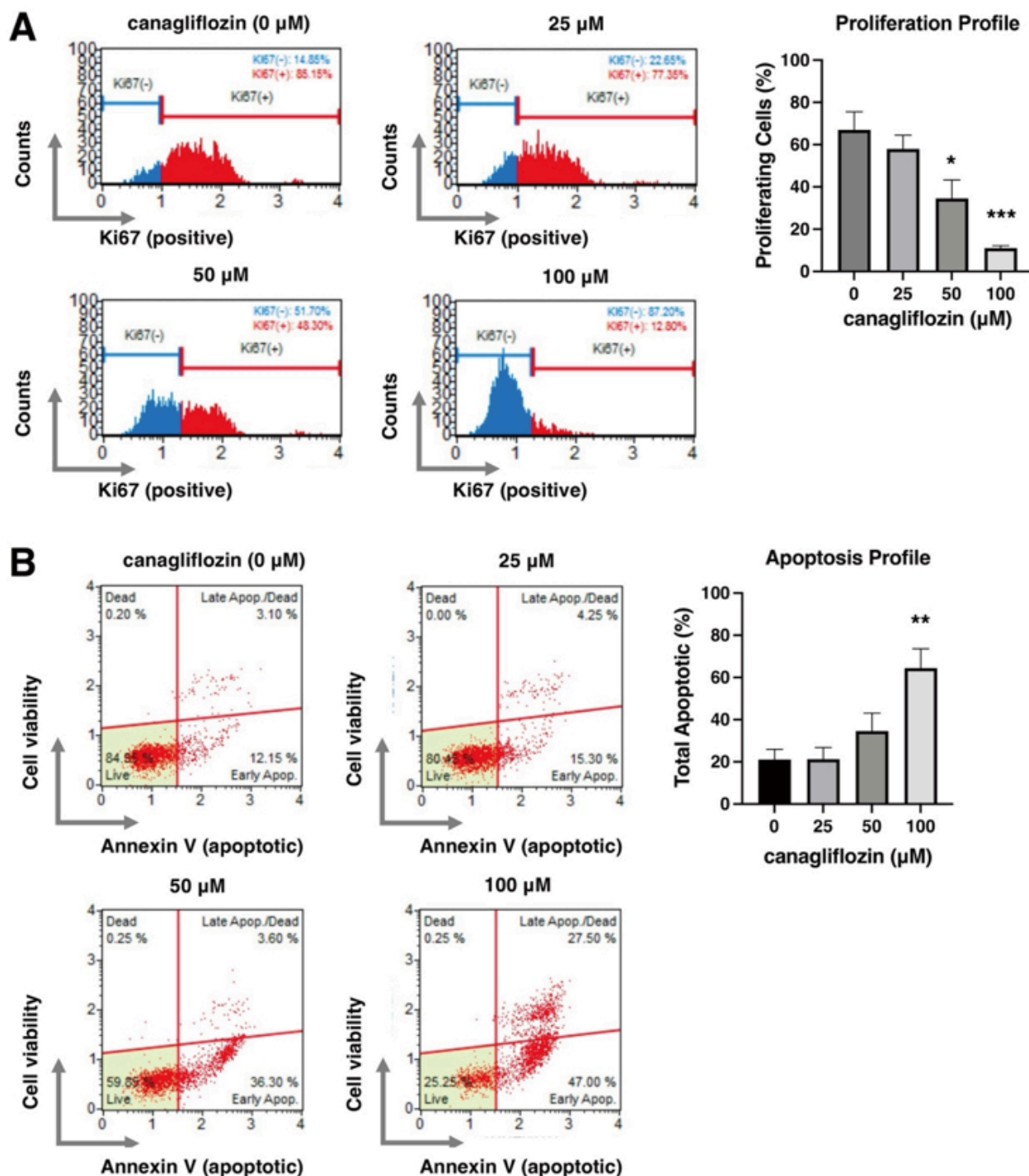


Figure 3

Cell death induced by canagliflozin in cultured NGT41 cells: Ki67 proliferation assay and dead cell analysis

Histogram of NGT41 cells after canagliflozin treatment (red: Ki67-positive population, blue: Ki67-negative population). The proportion of proliferating cells is shown as a bar graph. Similar results were obtained from four replicate experiments. One-way ANOVA with Dunnett's multiple comparisons test was applied to statistically analyze: * $p = 0.0168$, *** $p = 0.0003$. (B) Detection of apoptosis in NGT41

treated with canagliflozin. Description of each quadrant (Upper left: dead cells, Upper right: late apoptotic cells or dead cells, lower left: live cells, lower right: early apoptotic cells). Each population was gated and quantified by cell size and cell count, then plotted separately for canagliflozin concentrations (0 - 100 μ M). Similar results were obtained from four replicate experiments. One-way ANOVA with Dunnett's multiple comparisons test was applied to analyze: $**p = 0.0018$.

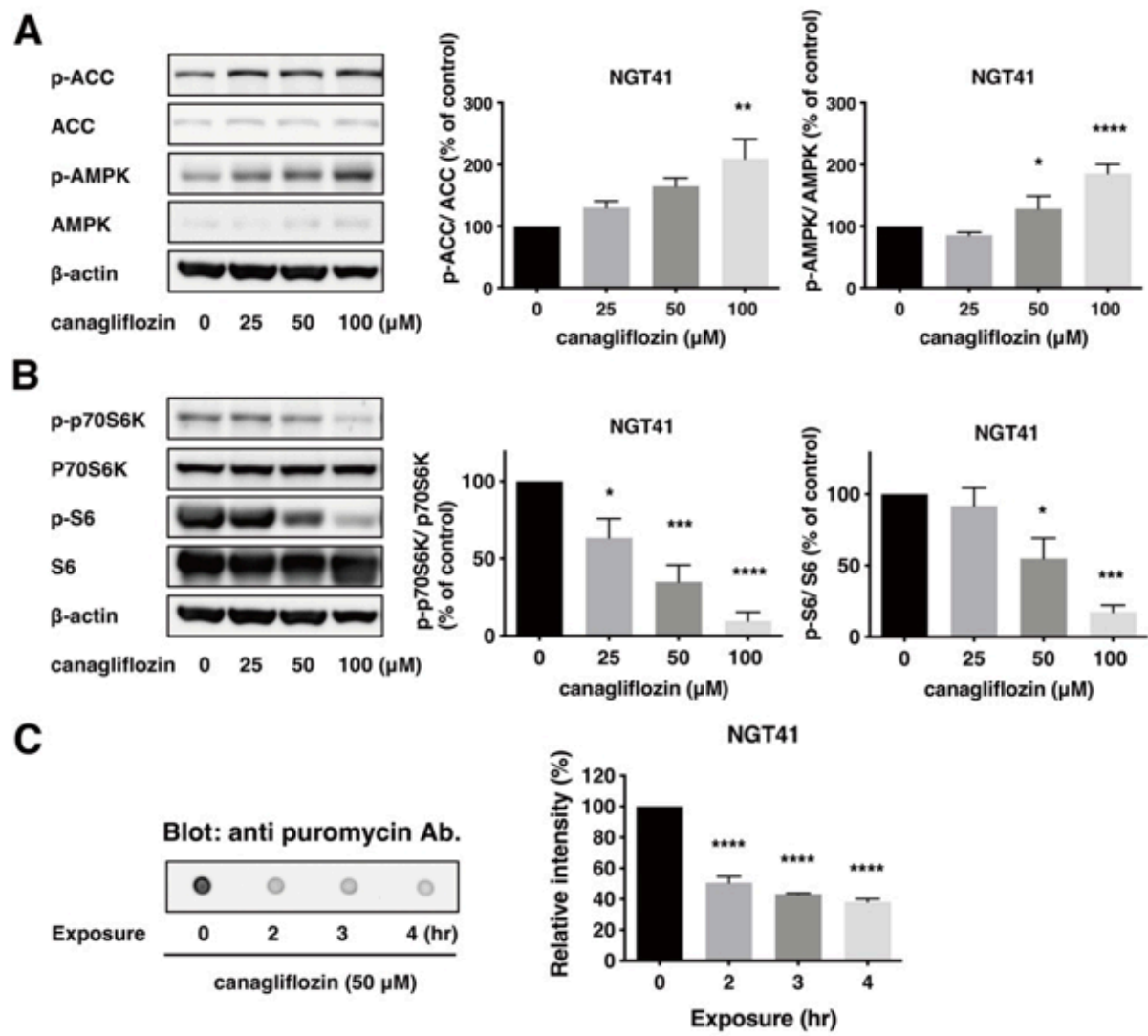


Figure 4

Effects of canagliflozin on AMPK activation and mTOR signaling in GBM cell lines: impact on global protein synthesis activity

(A) Dose response of canagliflozin on the activation of ACC and AMPK in NGT41, analyzed by Western blotting. Bar graphs represent phospho/total ACC ratio (left panel) or phospho/total AMPK ratio (right panel), indicated by corresponding band intensities. β -actin was used as a loading control. Cells were treated with the indicated concentrations (0 - 100 μ M) of canagliflozin for 72 hours. Similar results were obtained from four replicate experiments. One-way ANOVA values for Dunnett's multiple comparisons of

canagliflozin treatment: $**p = 0.0028$ (p-ACC/ACC ratio), $*p = 0.0232$, $****p < 0.0001$ (p-AMPK/AMPK ratio). (B) Dose response of canagliflozin on mTOR signaling in NGT41. The phosphorylation levels of p70S6K (Thr389) and S6 (Ser240/244) were analyzed by Western blotting. The level of phosphorylated and total p70S6K (left panel) or phosphorylated and total S6 (right panel) was indicated the corresponding bands. β -actin was used as a loading control. Similar results were obtained from four replicate experiments. One-way ANOVA values for Dunnett's multiple comparisons of canagliflozin treatment: $*p = 0.0299$, $***p = 0.0005$, $****p < 0.0001$ (p-p70S6K/P70S6K ratio), $*p = 0.0185$, $***p = 0.0002$ (p-S6/S6 ratio). (C) Immunoblotting using anti-puromycin antibody against synthesized polypeptide chains. NGT41 cells were treated with canagliflozin (50 μ M) for the indicated times, followed by puromycin administration and sampling. The bar graph represents intensity of spot, data are presented as mean $\pm$ SEM. Similar results were obtained from triplicate experiments. One-way ANOVA with Dunnett's multiple comparisons test was applied: $****p < 0.0001$.

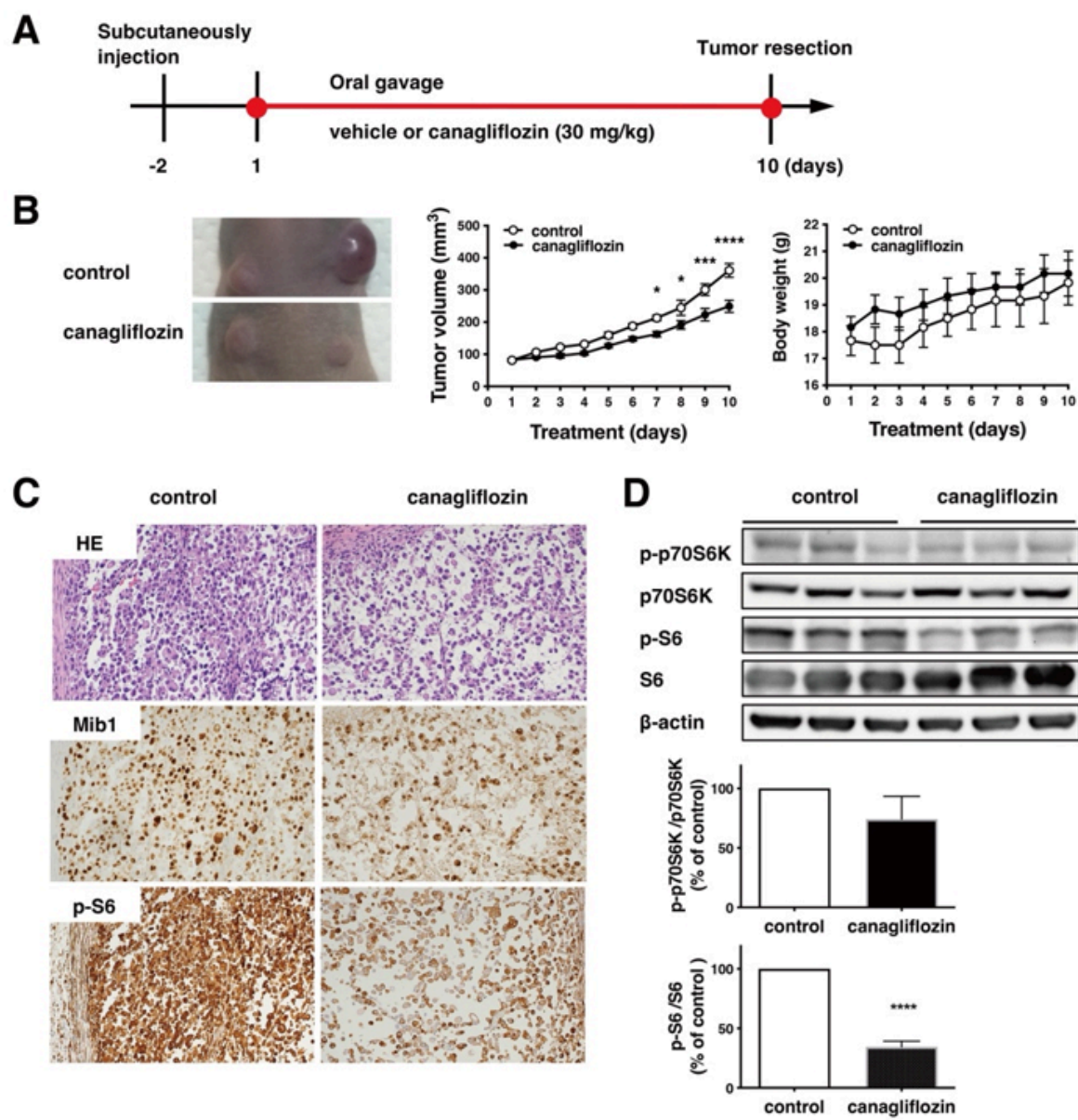


Figure 5

Canagliflozin suppresses tumor growth of NGT41 xenograft in BALB/C nude mice

Schematic representation of the in vivo experimental design. (B) The curves represent changes in tumor volume (left panel) and mouse body weight (right panel) during canagliflozin administration. Data are shown as mean $\pm$ SEM (n = 6 each). Statistical analysis of tumor growth rates was performed using two-way ANOVA with Bonferroni's multiple comparisons test: * p = 0.0361 (day 7), * p = 0.0214 (day 8), *** p = 0.0001 (day 9), **** p < 0.0001 (day 10). No severe adverse effect, including death, were observed between groups. (C) Representative macro and micro image of tumor sections. Immunohistochemical staining of canagliflozin-treated tumors for Ki67 (clone MIB1) and phospho S6. (D) Immunoblot analysis of protein extracts from resected tumors (upper). Phosphorylation levels of p70S6K (Thr389) and S6 (Ser240/244) were analyzed. Bar graphs show the levels of phosphorylated and total p70S6K (upper) and phosphorylated S6 and total S6 (lower), quantified after standardizing the ratio of phospho/total P70S6K. β -actin was used as a loading control. Data are shown as mean $\pm$ SEM (n = 3 each). Unpaired t-test values for comparisons between vehicle and canagliflozin group: *** p = 0.0002.

Supplementary Files

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