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Selective growth inhibition by glycogen synthase kinase-3 inhibitors in tumorigenic HeLa hybrid cells is mediated through NF-κB-dependent GLUT3 expression

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Carcinogenesis and cancer progression, driven by mutations in oncogenes and tumor-suppressor genes, result in biological differences between normal and cancer cells in various cellular processes. Specific genes and signaling molecules involved in such cellular processes may be potential therapeutic targets of agents that specifically interact with the key factors in cancer cells.

Increased glucose uptake is fundamental to many solid tumors and well associated with increases in glycolysis and the overexpression of glucose transporters (GLUTs) such as GLUT1 and GLUT3 at the plasma membrane. Here, we used cell-based screening to identify glycogen synthase kinase-3β (GSK-3β) inhibitors that selectively target GLUT3-expressing tumorigenic HeLa cell hybrids as compared with non-tumorigenic hybrids that express GLUT1 alone. The GSK-3 inhibitors as well as GSK-3β RNAi suppressed GLUT3 expression at the level of transcription, leading to apoptosis. This suppression was associated with NF-κB in a p53-independent manner. Furthermore, GSK-3 inhibitors exhibited a synergistic effect with anticancer agents such as adriamycin and camptothecin in GLUT3-overexpressing colon cancer cells, but little effect in non-producing A431 cells. These results suggest a potential use of GSK-3 inhibitors to selectively kill cancer cells that overexpress GLUT3.

INTRODUCTION

One of the most important considerations in cancer chemotherapy is selective killing of cancer cells without significant toxicity to normal cells. Understanding the physiological differences between normal and cancer cells is essential for the design and development of anticancer drugs with selective anticancer activities. Cancer cells are well known to have accelerated metabolism, higher glucose requirements and increased glucose uptake. Indeed, these characteristics are often associated with increased metastasis and poor survival in cancer patients and have been clinically applied to tumor imaging with positron emission tomography.

Glucose transport is a rate-limiting step for glucose metabolism and is mediated by glucose transporters (GLUTs) in mammalian cells. Increased glucose transport in cancer cells has been associated with increased and deregulated expression of GLUT proteins. There are currently more than 10 members of the GLUT family in mammalian cells. GLUT1, one of the most intensively studied of all membrane transport proteins, is widely expressed in proliferating cells. GLUT3 is the major neuronal glucose transporter predominantly expressed in neuronal cells. In addition, strong expression of both the isoforms is observed in many tumors, such as gliomas, non-small cell lung carcinomas, gastroenterological tumors and ovarian carcinomas. Upregulated expression of the GLUT family is often closely associated with malignancy, although the specific genes and signaling pathways regulating the expression remain undefined.

It has been reported that GLUT1 gene expression is regulated by the hypoxia-induced factor-1 protein. PI3K-AKT signaling also mediates the expression of GLUT1. On the other hand, Kawachi et al. recently showed that the expression of GLUT3 was regulated by NF-κB in a p53-dependent manner in mouse embryonic fibroblasts. Despite that the p53 protein has a critical role in responses to genotoxic stress, p53-independent responses to genotoxic stress have also been reported. Multiple genotoxic stimuli such as anticancer drugs, UV radiation and γ radiation resulted in a suppression of GLUT3 expression and glucose metabolism. These results are consistent with recent findings by us and others, indicating that genotoxic stress controls apoptosis and GLUT3 expression thorough MEK-ERK signaling independently of p53. Our data also suggest that levels of GLUT3 expression affect sensitivity to genotoxic stress in cancer cells. However, the mechanisms underlying cancer cell survival and the expression of GLUTs remain unclear, and little development of chemical compounds or antibodies that specifically target the GLUT family has been reported.

We have previously demonstrated tumor-associated expression of GLUT1 or GLUT3 in human cell hybrids derived from cervical carcinoma HeLa cells and normal fibroblasts. CGL4, a tumorigenic hybrid, expressed both GLUT1 and GLUT3, whereas CGL1, the tumor-suppressed hybrid, expressed GLUT1 alone.
This tumor-associated GLUT3 expression is regulated at the level of transcription at least.\(^{34}\) Based on this background, we used a screening method to identify drugs that predominantly kill a tumorigenic HeLa cell hybrid as a model of GLUT3-overexpressing cancer cells. By screening a library of inhibitors, we identified several glycogen synthase kinase-3-\(^{\beta}\) (GSK-3-\(\beta\)) inhibitors as potential lead compounds. These inhibitors suppressed GLUT3 expression at the transcriptional level in HeLa cells and human cell hybrids. We also demonstrated that this suppression occurred through NF-\(\kappa\)B signaling in a p53-independent manner, leading to apoptotic cell death. Furthermore, GSK-3-\(\beta\) inhibition induced a synergistic cytotoxic effect in GLUT3-overexpressing colon cancer cells (Caco-2) when combined with DNA-damaging agents such as adriamycin (ADR) and camptothecin (CPT), but had little effect in non-producing carcinoma (A431).

**RESULTS**

Chemical screening for inhibitors that predominantly inhibit the growth of cancer cells

Previously, we have reported that a tumorigenic HeLa cell hybrid CGL4 expressed GLUT3, which was undetectable in non-tumorigenic CGL1 cells.\(^{34}\) The increased glucose uptake by the tumorigenic cells was shown to be well associated with the level of GLUT3 expression.\(^{34}\) We have hypothesized that this tumor-associated GLUT3 expression may be regulated by a putative tumor-suppressor gene on chromosome 11, whose deletion or inactivation leads to the tumorigenesis of the HeLa cell hybrids.\(^{35}\) To understand the physiological and molecular mechanism(s) underlying the putative tumor-suppressor function, we screened for inhibitors that selectively kill tumorigenic CGL4 cells in a library of 285 chemicals prepared by the Screening Committee of Anticancer Drugs (SCADS, http://gantoku-shien.jfcr.or.jp/). The compounds were mainly commercially available antitumor drugs and kinase inhibitors, dissolved in DMSO at 10 \(\mu\)M.

We compared the cytotoxicity between CGL4 and CGL1 cells of each drug at various concentrations by using a cell counting kit-8 viability assay (CKC-8). The results were assigned as \(S_{\text{CGL4/CGL1}}\); the log ratio of the normalized cell number in CGL1 divided by the normalized cell number in CGL4 (Figure 1A). A positive \(S_{\text{CGL4/CGL1}}\) score indicates that the drug was selectively lethal or inhibited the growth of CGL4 cells. In contrast, a negative \(S_{\text{CGL4/CGL1}}\) score indicates that the drug selectively killed CGL1 cells. A score of zero means similar effects on both the hybrids.

Due to this assay, we identified a number of GSK-3 inhibitors with high \(S_{\text{CGL4/CGL1}}\) scores (Figures 1b and c). Unexpectedly, these inhibitors showed low \(S_{\text{CGL1/HeLa-S3}}\) scores (Figure 1c), suggesting their toxicity to be greater in CGL4 cells than HeLa-S3 cells, which showed a lower level of GLUT3 expression (Supplementary Figure S1). Consistent with the results of primary screening (Figures 1c and b), treatment with the GSK-3 inhibitors reduced the viability of CGL4 cells in a dose-dependent manner (Figure 1d and Supplementary Figure S2). Growth was inhibited at a half-maximal inhibitory concentration (IC\(_{50}\)) of 0.66 \(\mu\)M, fivefold lower than the concentration in non-tumorigenic CGL1 cells (Supplementary Table S1). A non-GSK-3 inhibitor, vinblastine, inhibited the growth of both hybrid cells similarly (Supplementary Figure S3 and Supplementary Table S1).

We next determined whether GSK-3 inhibitors suppress the phosphorylation of GSK-3 in these HeLa cell hybrids. Western blot analysis showed the phosphorylation of GSK-3-\(\beta\)/Y276/Y216 in three cell lines (Figure 1e). However, a marked reduction in the phosphorylation of GSK-3-\(\beta\)/Y276/Y216 was observed in the cell lines after treatment for 24 h with 10 \(\mu\)M GSK-3 IX, which induced substantial activation of caspase-3 only in CGL4 cells (Figure 1e). To confirm these findings, GSK-3-\(\beta\)-specific siRNA was transfected into these cell lines (Figure 1f). A correlation between the reduction in GSK-3-\(\beta\) protein and activation of caspase-3 was observed in CGL4 cells but not in non-tumorigenic CGL1 cells and parental HeLa cells. These results suggest a key role of GSK-3-\(\beta\) in the proliferation and sustained survival of GLUT3-expressing cancer cell lines.

GSK-3 inhibitors and GSK-3 downregulation suppress GLUT3 gene expression

As GLUT3 is overexpressed in tumorigenic CGL4 cells that suffered selective cytotoxicity from GSK-3 inhibitors, we examined if inhibition of GSK-3 affects the expression of GLUT3 and consumption of glucose in CGL4 cells. GSK-3 IX and kenpaullone significantly inhibited GLUT3 gene expression in a dose-dependent manner (Figure 2a). The accumulation of GLUT3 mRNA was further quantified by the RT–PCR method and shown to be reduced 40–50% by treatment with GSK-3 IX (10 \(\mu\)M) or kenpaullone (20 \(\mu\)M). In contrast, the expression of GLUT1 was unaffected by these inhibitors (Figure 2a), suggesting a link between GSK-3 and the GLUT3 expression during growth suppression in cancer cells. Whereas a reduction in the phosphorylation of GSK-3-\(\beta\)/Y by 10 \(\mu\)M of GSK-3 IX was similarly observed in all three cell lines tested, glucose consumption was markedly impaired only in the tumorigenic CGL4 cells (Figure 2b).

As expected, transfection of GSK-3-\(\beta\)-specific siRNA, which mediated the downregulation of GSK-3-\(\beta\), resulted in an apparent decrease in the expression of GLUT3 but not GLUT1 in CGL4 cells (Figure 2c). Knockdown of GSK-3-\(\beta\) by siRNA was further confirmed in HeLa cell hybrids at the protein level, which resulted in efficient reduction of GSK-3-\(\beta\) protein in CGL4 as well as HeLa and CGL1 cells, accompanied by a decrease in glucose consumption only in CGL4 cells (Figure 2a). Thus, GSK-3 may control cell growth through GLUT3-dependent glucose metabolism in tumorigenic CGL4 cells.

Suppression of GLUT3 expression by GSK-3 inhibitors is independent of p53

Recently, we have reported the suppressive effects on GLUT3 expression of DNA-damaging agents, such as ADR, etoposide (ETO) and CPT (Figure 2a, Watanabe et al.\(^{31}\)) in tumorigenic HeLa cell hybrids. As these effects were no different in tumorigenic and non-tumorigenic HeLa cell hybrids. As these effects were independent of p53, we determined whether or not p53 influences the suppressive effect of GSK-3 inhibitors on GLUT3 expression in CGL4 cells. The p53 protein was fully depleted by adding siRNA in CGL4 cells treated with either GSK-3 IX or ADR (Supplementary Figure S4a). A change in GLUT3 expression were not significantly affected by p53-specific siRNA (Supplementary Figure S4b). As a control experiment, the expression of GLUT3 was suppressed by ADR in the p53-depleted CGL4 cells (Supplementary Figure S4b).

Downregulation of GSK-3 inhibits NF-\(\kappa\)B activity in CGL4 cells

Kawachi et al.\(^{25}\) showed the importance of the transcriptional activity of NF-\(\kappa\)B to GLUT3 expression in mouse embryonic fibroblasts.\(^{25}\) A previous study linked the inhibition of GSK-3 to the negative regulation of NF-\(\kappa\)B activity.\(^{36}\) We investigated the relationship between NF-\(\kappa\)B activity and GLUT3 expression in CGL4 cells. We found the expression to be significantly inhibited by siRNA for p65, a component of NF-\(\kappa\)B (Figures 3a and b). In contrast, knockdown of p65 did not affect the expression of GLUT1. We next examined whether the effect of GSK-3 inhibition involves suppression of NF-\(\kappa\)B activity. p65-dependent transcriptional activity was suppressed by both GSK-3-\(\beta\) siRNA (Figure 3c) and GSK-3 IX (Figure 3d) in CGL4 cells. Thus, it is suggested that GSK-3 inhibition contributes to the suppression of GLUT3 expression through negative regulation of the transcriptional activity of NF-\(\kappa\)B.
Constitutive expression of GLUT3 renders cells more resistant to GSK-3-mediated death

As a signaling pathway in GLUT3 expression through GSK-3 would be important for survival of tumorigenic CGL4 cells, we next assessed the role of GLUT3 in cell death induced by GSK-3 inhibitors. In previously constructed CGL4/gt3 cells in which GLUT3 was stably overexpressed using a viral promoter,34 GLUT3 mRNA was shown to be about fourfold higher than in the parental CGL4 cells (Figure 4a). After a remarkable reduction in the phosphorylation of GSK-3α/β by 5 μM GSK-3 IX (Figure 4b), higher levels of GLUT3 expression and glucose consumption remained observable in the CGL4/gt3 cells (Figures 4b and c).

We examined the effect of GSK-3 inhibitor on growth in vitro using various amounts of GSK-3 IX for 72 h. As shown in Figure 4c, a dose-dependent cytotoxic effect of GSK-3 IX was observed. The IC50 value of GSK-3 IX for CGL4 cells was 0.68 ± 0.05 μM, whereas CGL4/gt3 cells were more resistant, with an IC50 of about 7.03 ± 0.07 μM (Figure 4d). These results suggest that the increased GLUT3 expression is rather resistant to GSK-3 inhibitors acting on the growth of parental CGL4 in which endogenous GLUT3 expression was suppressed. In fact, GLUT3 promoter activity was markedly suppressed by treatment with GSK-3 IX and GSK-3β siRNA in CGL4 cells (Supplementary Figure S5).

Figure 1. A screen to discover agents that inhibit the growth of CGL4 cells. (a) A scheme of the drug screen. CGL1 or CGL4 cells grown in 96-well plates were exposed to a chemical library of 285 compounds for 72 h. The logarithm of the normalized cell number in CGL1 versus CGL4 provides a summary statistic (SCL1/CGL4) for each compound. (b) Results of the screening. SCL1/CGL4 is plotted for all compounds. (c) Part of the results, SCL1/CGL4 (gray bar) and SHeLa-S3/CGL4 (white bar) are plotted for four compounds. (d) The viability of HeLa-S3, CGL1 or CGL4 cells cultured with GSK-3 IX for 72 h. Data are expressed as the mean ± s.d. (n = 3). (e) upper panels) Phosho-GSK-3 detection by western blot analysis of protein extract from each cell line after 8 h treatment with 0.1% DMSO (−) or 10 μM GSK-3 IX (+). (Lower panels) Apoptotic detection by caspase-3 activity using a fluorescence probe. (f) upper panels) GSK-3 detection by western blot analysis of protein extract from each cell line after 24 h treatment with 50 nM GFP siRNA, 50 nM GSK-3β siRNA1 or 50 nM GSK-3β siRNA2. (Lower panels) Apoptotic detection by caspase-3 activity using a fluorescence probe.
Synergistic cytotoxicity induced by GSK3 inhibitors and DNA-damaging agents in GLUT3-upregulated tumor cells

As the present results indicate selective cytotoxicity of GSK-3 inhibitors in GLUT3-expressing tumor cells (Figure 1c), upregulation of GLUT3 expression may be directly linked to the GSK-3 inhibitor-induced cell death. To test this possibility, Caco-2 cells, which are p53-null colonic carcinoma cells, were tested for combined sensitivity to GSK-3 IX (0–20 μM) and ETOP (1.5625–200 μM). As a control experiment, a skin cancer cell line, A431, in which GLUT3 mRNA was nearly undetectable (Figure 5a, Supplementary Table S2), was similarly examined. The expression of GLUT1 was also determined in both cell lines (Figure 5a, Supplementary Table S2).

After 72 h of each treatment, cell viability was measured by a CCK-8 assay. A significant increase in sensitivity to GSK-3 IX was evident in Caco-2 cells when compared with A431 cells (Figure 5b, Supplementary Table S3). In contrast, Caco-2 cells were more resistant to ETOP than the A431 cell line (Figure 5b, Supplementary Table S3). Then, we tested the combined effects of either 0.5–1.0 μM ETOP and 0.15625–20 μM GSK-3 IX or 1.5625–200 μM ETOP and 0.5–1.0 μM GSK-3 IX. We found that ETOP-mediated cell death at the subtoxic doses (6.2–50 μM) was markedly enhanced by GSK-3 IX (0.25–4 μM) in Caco-2 cells (Figure 5c). A similar effect of GSK-3 IX (0.25–4 μM) was obtained with another DNA-damaging agent, CPT (Supplementary Figure S6). In A431 cells, however, these synergistic effects were not seen (Figure 5d, Supplementary Table S3 and Supplementary Figure S6), suggesting that a GSK-3 inhibitor combined with a DNA-damaging agent would be effective against GLUT3-overexpressing tumor cells.

DISCUSSION

The targeting of glucose metabolism may have therapeutic potential against tumor cells. In this study, we employed a screening strategy using a pair of HeLa-derived cell hybrids to search for small molecules that selectively kill tumorigenic cells overexpressing GLUT3. We identified several commercially available GSK-3 inhibitors with selective killing activity, demonstrating a novel role for GSK-3 in the control of GLUT3 expression in tumor cell growth. Although GSK-3 is known to be
essential in glycan synthesis, it has also been shown to be a crucial regulator of cell structure, metabolism and survival.49,50 GSK-3 also functions as a pro-survival factor in pancreatic cancer,41 as well as a modulator for apoptosis in colon cancer cells.42 and for Alzheimer’s disease.43 Our findings indicate an additional role of GSK-3 in survival in GLT3-expressing cancer cells. It should be noted that this inhibition by GSK-3 occurs independent of p53.

The inhibition of GSK-3 has been shown to result in the reduction of intracellular glucose levels in glioma cells, although the molecular mechanism for this reduction remains unknown.44 We observed that GSK-3 inhibitors reduced GLUT3 expression and glucose consumption in tumorogenic CGL4 cells. Thus, GSK-3-dependent changes in the intracellular glucose concentration in glioma cells might be partly associated with GLUT3 expression.

We also demonstrated that the GSK-3-dependent downregulation of GLUT3 expression is associated with NF-kB impaired by both GSK-3 inhibitors and siRNA (Figure 3). These observations are consistent with the finding that GLUT3 gene expression in mouse embryonic fibroblasts is dependent on NF-kB.25 Although the mechanism underlying the inactivation of NF-kB followed by GSK-3 inhibition remains unclear, it should be noted that GSK-3 has an important role in the constitutive NF-kB signaling in pancreatic cancer by regulating IKK activity.45 Thus, cascades involved in NF-kB signaling through IkB46 and β-catenin47,48 may be partly linked to the GSK-3-dependent GLUT3 expression and cell growth. However, the direct link between GLUT3 expression and cell growth remains unclear. More studies including in vivo models are needed to clarify this subject.

The observed dependence on GSK-3 provides a potential therapeutic target in tumor cells defined by overexpression of the GLUT3 gene. In lung, rectal, ovarian, laryngeal and breast carcinomas, higher levels of GLUT1 or GLUT3 were significant markers of decreased survival of patients.49-54 A close association between 18F-FDG uptake and increased glucose metabolism (GLUT1 and GLUT3) is observed in pulmonary pleomorphic carcinoma,55 which warrants a search for new therapeutic approaches using GSK-3 inhibitors. As GSK-3 has many key roles in regulating a diverse range of cellular functions, including glycan metabolism, transcription, translation, cell cycle and apoptosis,39 it has potential applications as a drug target for diabetes and neurodegenerative disorders, including Alzheimer’s disease.56

The present findings should contribute to the discovery of GSK-3 inhibitors as antitumor agents for GLUT3-expressing cells. The side effects of GSK-3 inhibitors should also be considered. For example, as GSK-3 normally has suppressive roles in Wnt, signaling via hedgehog and Notch might be mimicked by GSK-3 inhibition, potentially increasing the risk of oncogenesis.57 The cytotoxicity of GSK-3 inhibitors may be another issue and GSK-3 inhibition induces apoptosis through a TRAIL-mediated mechanism.44 However, our screenings help to focus on the clinical advantages of GSK-3 inhibitors against GLUT3-expressing tumor cells and the findings shown here may have some benefits for cancer therapy and a clinical safety.

Results of synergistic killing by GSK-3 inhibitors and DNA-damaging chemotherapeutic agents for GLUT3-expressing Caco-2 cells but not GLUT3-depleted cancer cell lines lead to another implication for future studies (Figures 5c and d, Supplementary Figure 6 and Supplementary Table S3). ETOP or CPT, a standard

Figure 3. GLUT3 suppression caused by GSK-3 inhibition is accompanied by downregulation of NF-kB activity. (a) Expression of GLUT3, GLUT1 and p65 in CGL4 cells transfected with control- or p65 siRNA was determined by RT-PCR (upper panel) and quantitative real-time PCR (lower panel). (b) p65 detection by western blot analysis of protein extract from cells treated for 48 h with either 50 nM GFP siRNA, 50 or 100 nM p65 siRNA. (c, d) NF-kB luciferase reporter activities in GSK-3β siRNA- (c) or GSK-3 IX- (d) transfected CGL4 cells. *P<0.01 for the indicated comparison (t-test).

Figure 4. Correlation between GLUT3 gene expression and cytotoxicity of GSK-3-inhibition. (a) GLUT3 expression in CGL4 or CGL4/gt3 cells treated with DMSO (−) or 5 μM GSK-3 IX (+) determined by RT-PCR (upper panel) and quantitative real-time PCR (lower panel). (b) Phospho-GSK-3 detection by western blot analysis of protein extract from each cell line after 24 h treatment with 0.1% DMSO (−) or 5 μM GSK-3 IX (+). (c) Glucose consumption in CGL4 or CGL4/gt3 cells transfected with control (−) or GSK-3 IX (+). Values were calculated from a standard curve. (d) Survival of CGL4 or CGL4/gt3 cells treated with various concentrations of GSK-3 IX. Cells were treated with GSK-3 IX or DMSO for 72 h and viable cell numbers were counted as described in ‘Materials and methods’. Cell survival relative to the DMSO control (100% value) is plotted. *P<0.01 for the indicated comparison (t-test).
DNA-damaging agent, is often used in patients with malignant tumors. Because a GSK-3 inhibitor and DNA-damaging agent would be theoretically applicable only in GLUT3-upregulated cancer cells, lower doses of these agents could be provided to the proper cancer targets.

Understanding the molecular mechanism by which GSK-3 contributes to the proliferation of GLUT3-expressing tumor cells awaits future experiments. It is interesting that overexpression of GLUT3 using a viral vector rendered CGL4 cells more resistant to GSK-3 inhibition in glioma cells.44 Tumors with a constitutively active AKT associated with loss of phosphatase and tensin homolog deleted from chromosome 10 (PTEN) gene may be sensitive to GSK-3 inhibitors. In addition, p27Kip1 is a critical effector in GSK-3-induced suppression of GLUT3 expression and cell growth.38 Thus, the expression levels of GLUT3 as well as GLUT1 may provide useful pharmacodynamic information for clinical trials in malignant tumors. More studies will be needed to clarify how GSK-3 inhibition is clinically applicable to the selective killing of GLUT3-expressing tumor cells.

**MATERIALS AND METHODS**

Inhibitor, antibodies, reagents and RNA interference

The library (http://gamoku-shien.jfcr.or.jp/) used was kindly provided by the SCADS supported by a Grant-in-Aid for Scientific Research on the Priority Area ‘Cancer’ from the Ministry of Education, Culture, Sports, Science and Technology, Japan. The mouse monoclonal antibody to β-actin was purchased from Sigma (St Louis, MO, USA), while those to human phospho-GSK-3 and GSK-3β were from BD Biosciences (Bedford, MA, USA). The rabbit monoclonal antibody to human p65 was purchased from Epitomics (Burlingame, CA, USA). The enhanced chemiluminescence (ECL) kit was obtained from GE Healthcare (Chalfont St Giles, UK). The lipofectamine 2000 reagent and siRNAs to GSK-3β (Supplementary Table S4) and p65 (RELA: HSS184266) were purchased from Invitrogen (Carlsbad, CA, USA). ADR, CPT, ETOP, GSK-3 inhibitor IX and kenpaullone were obtained from Calbiochem (La Jolla, CA, USA).

**Figure 5.** Synergic effect of GSK-3 inhibitors on the cytotoxic action of DNA-damaging agents in GLUT3-unregulated cancer cells. (a) RT–PCR (upper panel) was performed to examine GLUT1 and GLUT3 expression in CGL1, CGL4, Caco-2 or A431 cells. (b) The viability of Caco-2 or A431 cells treated with ETOP (left) or GSK-3 IX (right) for 72 h. Data are expressed as the mean ± s.d. (n = 3). (c, d) Survival of Caco-2 (c) or A431 (d) cells treated with various concentrations of GSK-3 IX with or without ETOP. Cell survival relative to the DMSO control (100% value) is plotted.
Screening
Inhibitor screening was conducted using a subset of the SCADS library containing 285 compounds in three 96-well microplates. HeLa-S3, CGL1 and CGL4 cells were seeded in duplicate 96-well plates on day 0, and each inhibitor was added at 10 μM on day 1. Cell viability was determined on day 4 by the water-soluble tetrazolium salt (WST-8) assay using cell counting kit-8 (Dojindo, Kumamoto, Japan) as described previously.9 The cell viability from duplicate plates was averaged, and a 5-score was calculated using the following formula: S CGL1 or HeLa-S3/CGL4 = log10 (% viability of CGL1 or HeLa-S3/ % viability of CGL4).

Cell culture and transfection
HeLa-S3 was obtained from the American Type Culture Collection (Manassas, VA, USA). CGL1, CGL4 and CGL4/gt3 (GLUT3-overexpressing) cells were previously established as tumorigenic derivatives of a hybrid, ESH5, obtained from HeLa D98/AH2 cells and normal human fibroblasts.32-35.60 HeLa-S3, CGL1, CGL4 and CGL4/gt3 cells were cultured in DMEM (Invitrogen) containing 5% fetal bovine serum (MBL, Nagoya, Japan), supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) under humidified 5% CO2/95% air at 37 °C. This was performed as reported.31

Semi-quantitative RT–PCR
Semi-quantitative RT–PCR was performed as described previously.31 Briefly, total RNA for preparing RT–PCR templates was extracted using TRIzol (Invitrogen). The cDNA was synthesized from 1 μg of total RNA and then subjected to PCR. Primer sequences are described in Supplementary Table S4. RT–PCR results are representative of at least three independent experiments.

Quantitative real-time PCR analysis
This was performed using TaqMan Universal Master Mix II (Applied Biosystems, Foster City, CA, USA) under the following conditions: 15 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, using a 7500 Real-time PCR system (Applied Biosystems). The predesigned primer and probe sets for human GLUT1, GLUT3 and β-actin are commercially available (Applied Biosystems; GLUT1: Hs00892681_m1, GLUT3: Hs00359840_m1, β-actin: Hs99999903_m1). Threshold-cycle (Ct) values were automatically calculated for each replicate and used to determine the relative expression of the gene of interest relative to reference genes for both treated and untreated samples by the 2−ΔΔCt method.

Plasmids
Human GLUT3 promoter–reporter constructs (GLUT3-P-S1-4) were generated by subcloning of the upstream 5′ region of the human GLUT3 gene into pGL4.79 (Promega, Madison, CA, USA) upstream of the renilla luciferase gene. pGL4.13 (Promega) was used as the control plasmid. The NF-κB reporter plasmid (p55IgK-Luc) was kindly provided by Dr K Kawasaki (Douishisha Women’s College).

Transcriptional reporter assays
For the GLUT promoter-luciferase assay, CGL4 cells were cotransfected with either GLUT promoter-Luc or vector plasmids (pGL4.79) or pGL4.13 as an internal control for the transfection rate. For the NF-κB reporter assay, CGL4 cells were cotransfected with either p55IgK-Luc or vector plasmids (pGL4.79) or pGL4.13 as an internal control for the transfection rate. A dual-luciferase assay kit (Promega) was used according to the manufacturer’s instructions. The activity levels were expressed relative to the vector control.

Immunoblotting
Cells were cultured in DMEM plus 5% (v/v) fetal bovine serum (MBL) overnight. After transfection or the addition of appropriate inhibitors, the cells were cultured for another 24 h. They were then harvested and lysed in lysis buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1.0 mM dithiothreitol, 20 mM glycerophosphate, 2 mM Na3VO4, 1% NP40 and 1 mM phenylmethylsulfonyl fluoride). Whole-cell lysate was electrophoresed on a 10% SDS–PAGE gel, transferred to PVDF membranes and immunoblotted with the antibody. β-Actin was used as a loading control.

Cell viability analysis
Cell viability was determined with cell counting kit-8 (CCK-8, Dojindo) according to the manufacturer’s protocol. Briefly, 2.5 × 104 cells were plated onto 96-well plates and treated with the appropriate inhibitor as indicated in figure legends. After incubation at 37 °C in 5% CO2/95% air for 72 h, cell viability was calculated relative to the DMSO control.

Measurements of caspase-3 activity
Caspase-3 activity was measured by a fluorometric assay using AC-DEVD-AMC peptide (Sigma) as a fluorogenic substrate for Caspase-3. Cells were harvested and lysed in lysis buffer (20 mM HEPES (pH 7.5), 0.1% TritonX-100 and 5 mM dithiothreitol). Whole-cell lysate was then incubated with the caspase-3 fluorogenic substrate AC-DEVD-AMC at 37 °C for 1 h. The cleavage of the peptide was quantified in a spectrofluorometer with an excitation wavelength of 360 nm and emission wavelength of 460 nm.

Measurements of glucose consumption
Cells were seeded in 12-well dishes and the medium was changed after an overnight culture. Cells (about 90% confluent) were incubated for 24 h and then the culture medium was collected for measurements of glucose concentrations using a Glucose (GO) assay kit (Sigma). Glucose consumption was calculated from a standard curve.

Statistical analysis
The statistical significance of differences in data was determined using the unpaired Student’s t-test. A P-value <0.01 or <0.05 was considered statistically significant.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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