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ORIGINAL ARTICLE

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 *GULT3*-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.[1–5] Indeed, these characteristics are often associated with increased metastasis and poor survival in cancer patients[6–9] and have been clinically applied to tumor imaging with positron emission tomography.[10,11]

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.[12] There are currently more than 10 members of the GLUT family in mammalian cells.[13] GLUT1, one of the most intensively studied of all membrane transport proteins, is widely expressed in proliferating cells.[13,14] GLUT3 is the major neuronal glucose transporter predominantly expressed in neuronal cells.[13,15] 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.[16–19] Upregulated expression of the GLUT family is often closely associated with malignancy,[12,20] 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.[21–23] PI3K-AKT signaling also mediates the expression of GLUT1.[24] On the other hand, Kawauchi *et al.*[25] 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.[26–28] Multiple genotoxic stimuli such as anticancer drugs, UV radiation and γ radiation resulted in a suppression of *GLUT3* expression and glucose metabolism.[29] 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.[30,31] Our data also suggest that levels of *GLUT3* expression affect sensitivity to genotoxic stress in cancer cells.[31] 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.[32–34] CGL4, a tumorigenic hybrid, expressed both GLUT1 and GLUT3, whereas CGL1, the tumor-suppressed hybrid, expressed GLUT1 alone.[34]

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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-3β (GSK-3β) 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-κB signaling in a p53-independent manner, leading to apoptotic cell death. Furthermore, GSK-3β inhibition induced a synergistic cytotoxic effect in *GULT3*-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 mM.

We compared the cytotoxicity between CGL4 and CGL1 cells of each drug at various concentrations by using a cell counting kit-8 viability assay (CCK-8). The results were assigned as $S_{CGL1/CGL4}$; the log ratio of the normalized cell number in CGL1 divided by the normalized cell number in CGL4 (Figure 1a). A positive $S_{CGL1/CGL4}$ score indicates that the drug was selectively lethal or inhibited the growth of CGL4 cells. In contrast, a negative $S_{CGL1/CGL4}$ 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 $S_{CGL1/CGL4}$ scores (Figures 1b and c). Unexpectedly, these inhibitors showed low $S_{CGL1/HeLa-S3}$ 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 μM, 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-3α/β Y276/Y216 in three cell lines (Figure 1e). However, a marked reduction in the phosphorylation of GSK-3α/β Y276/Y216 was observed in the cell lines after treatment for 24 h with 10 μM GSK-3 IX, which induced substantial activation of caspase-3 only in CGL4 cells (Figure 1e). To confirm these findings, *GSK-3β*-specific siRNA was transfected into these cell lines (Figure 1f). A correlation between the reduction in GSK-3β 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-3β 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 μM) or kenpaullone (20 μM). 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-3α/β by 10 μM 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-3β* siRNA, which mediated the downregulation of GSK-3β, resulted in an apparent decrease in the expression of *GLUT3* but not *GLUT1* in CGL4 cells (Figure 2c). Knockdown of GSK-3β by siRNA was further confirmed in HeLa cell hybrids at the protein level, which resulted in efficient reduction of GSK-3β protein in CGL4 as well as HeLa and CGL1 cells, accompanied by a decrease in glucose consumption only in CGL4 cells (Figure 2d). 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 (ETOP) and CPT (Figure 2a, Watanabe *et al.*[31]), although there was no difference in toxicity between 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). The changes in *GLUT3* expression were not significantly affected by p53-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-κB activity in CGL4 cells

Kawauchi *et al.*[25] showed the importance of the transcriptional activity of NF-κB to *Glut3* expression in mouse embryonic fibroblasts.[25] A previous study linked the inhibition of GSK-3 to the negative regulation of NF-κB activity.[36] We investigated the relationship between NF-κB activity and *GLUT3* expression in CGL4 cells. We found the expression to be significantly inhibited by siRNA for *p65*, a component of NF-κB (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-κB activity. p65-dependent transcriptional activity was suppressed by both GSK-3 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-κB.
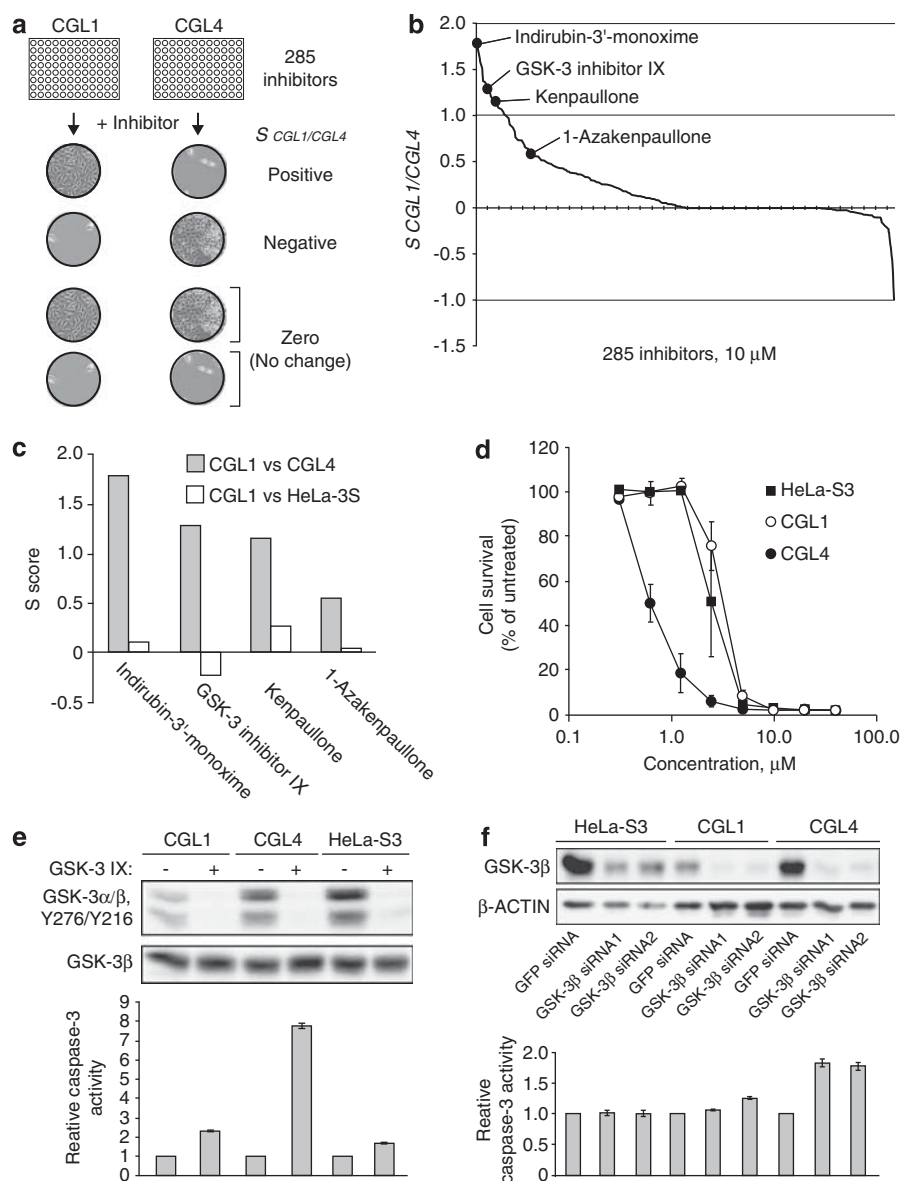
Figure 1. A screen to discover agents that inhibit the growth of CGL4 cells. (**a**) A scheme of the drug screen. CGL1 or CGL4 cells grown in 96-well plates were exposed to a chemical library of 285 compounds for 72 h. The logarithm of the normalized cell number in CGL1 versus CGL4 provides a summary statistic ($S_{CGL1/CGL4}$) for each compound. (**b**) Results of the screening. $S_{CGL1/CGL4}$ is plotted for all compounds. (**c**) Part of the results, $S_{CGL1/CGL4}$ (gray bar) and $S_{HeLa-S3/CGL4}$ (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) Phospho-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-3 β* siRNA2. (Lower panels) Apoptotic detection by caspase-3 activity using a fluorescence probe.

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 IC_{50} value of GSK-3 IX for CGL4 cells was 0.68 ± 0.05 μM, whereas CGL4/gt3 cells were more resistant, with an IC_{50} 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).
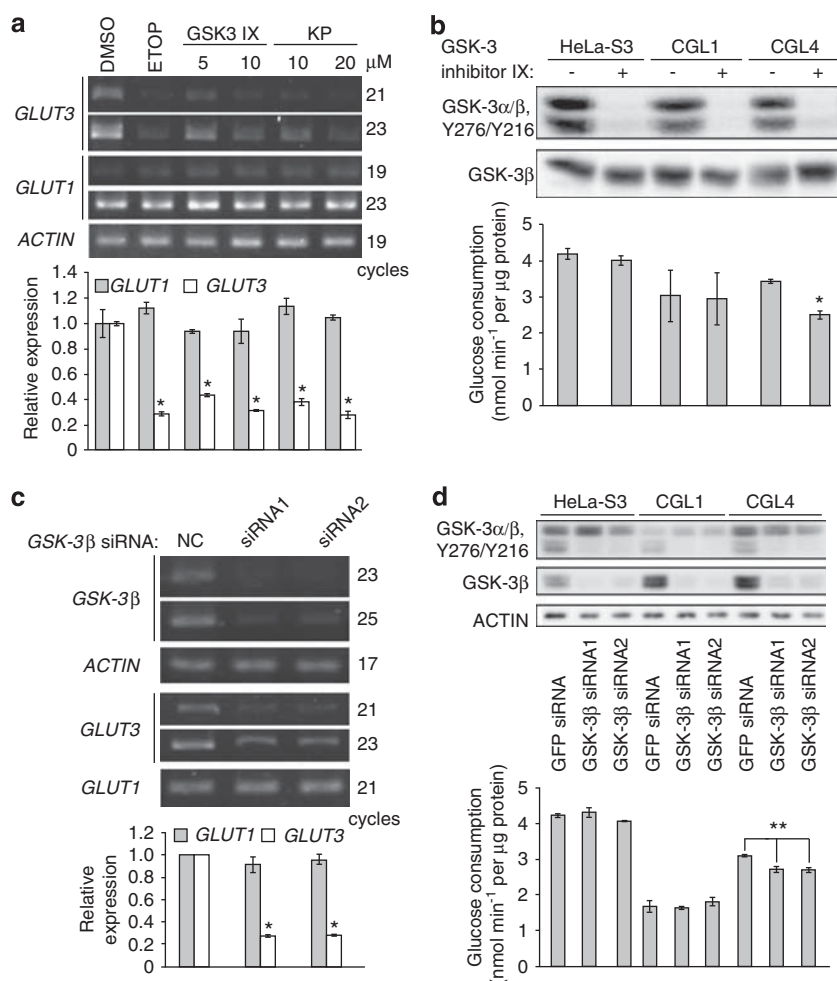
Figure 2. GSK-3 inhibition results in downregulation of *GLUT3* expression and glucose consumption. (**a**) Changes in *GLUT3* or *GLUT1* expression in CGL4 cells treated with DMSO, the GSK-3 inhibitor IX or kenpaullone determined by RT–PCR (upper panel) and quantitative real-time PCR (lower panel). 50 μM ETOP was used as a positive control for suppression of *GLUT3* expression. (**b**) Phosho-GSK-3 detection by western blot analysis of protein extract after 24 h treatment with 0.1% DMSO (−) or 10 μM GSK-3 IX (+) (upper panels). Glucose consumption was also assessed (lower panel). (**c**) *GLUT3* expression in CGL4 cells transfected with control- or *GSK-3β* siRNA was determined by RT–PCR (upper panel) and quantitative real-time PCR (lower panel). (**d**) Knockdown of GSK-3 was detected by western blot analysis of protein extract from each cell line after 24 h treatment with 50 nM GFP siRNA or 50 nM *GSK-3β* siRNA (upper panels). Glucose consumption in these cells was assessed (lower panel). *$P < 0.01$ or **$P < 0.05$ for the indicated comparison (*t*-test).

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[37] having greater expression of *GLUT3* (Figure 5a, Supplementary Table S2), 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.[9,20,38] In this study, we employed a screening strategy using a pair of HeLa-derived cell hybrids[35] 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
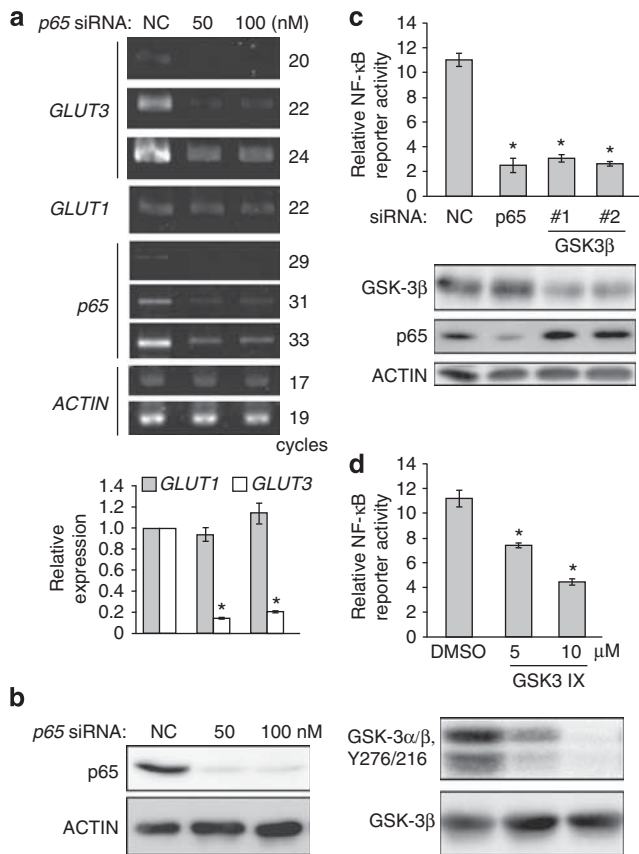
Figure 3. *GLUT3* suppression caused by GSK-3 inhibition is accompanied by downregulation of NF-κB activity. (**a**) Expression of *GLUT3*, *GLUT1* and *p65* in CGL4 cells transfected with control- or *p65* siRNA was determined by RT–PCR (upper panel) and quantitative real-time PCR (lower panel). (**b**) p65 detection by western blot analysis of protein extract from cells treated for 48 h with either 50 nm GFP siRNA, 50 nm or 100 nm *p65* siRNA. (**c**, **d**) NF-κB 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).

essential in glycogen synthesis, it has also been shown to be a crucial regulator of cell structure, metabolism and survival.[39,40] 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 GLUT3-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 tumorigenic CGL4 cells. Thus, GSK3-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-κB 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-κB.[25] Although the mechanism underlying the inactivation of NF-κB followed by GSK-3 inhibition remains unclear, it should be noted that GSK-3 has an important role in the constitutive NF-κB signaling in pancreatic cancer by regulating IKK activity.[45] Thus, cascades involved in NF-κB signaling through IκB[46] and β-catenin[47,48] may be partly linked to the GSK-3-dependent *GLUT3* expression and cell growth. However, the direct link between *GLUT3* expression
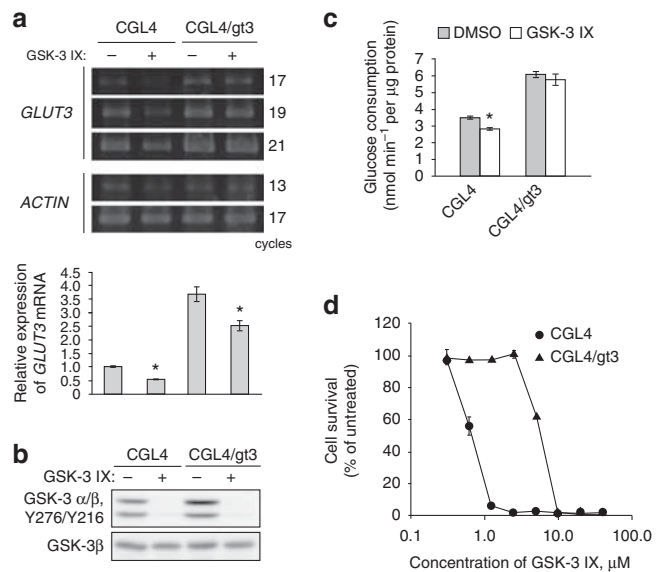
and cell growth remains unclear. More studies including *in vivo* models are needed to clarify this subject.

The observed dependence on GSK-3 provides a potential therapeutic target in tumor cells defined by overexpression of the *GLUT3* gene. In lung, colorectal, 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 ^{18}F-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 glycogen 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 finding 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
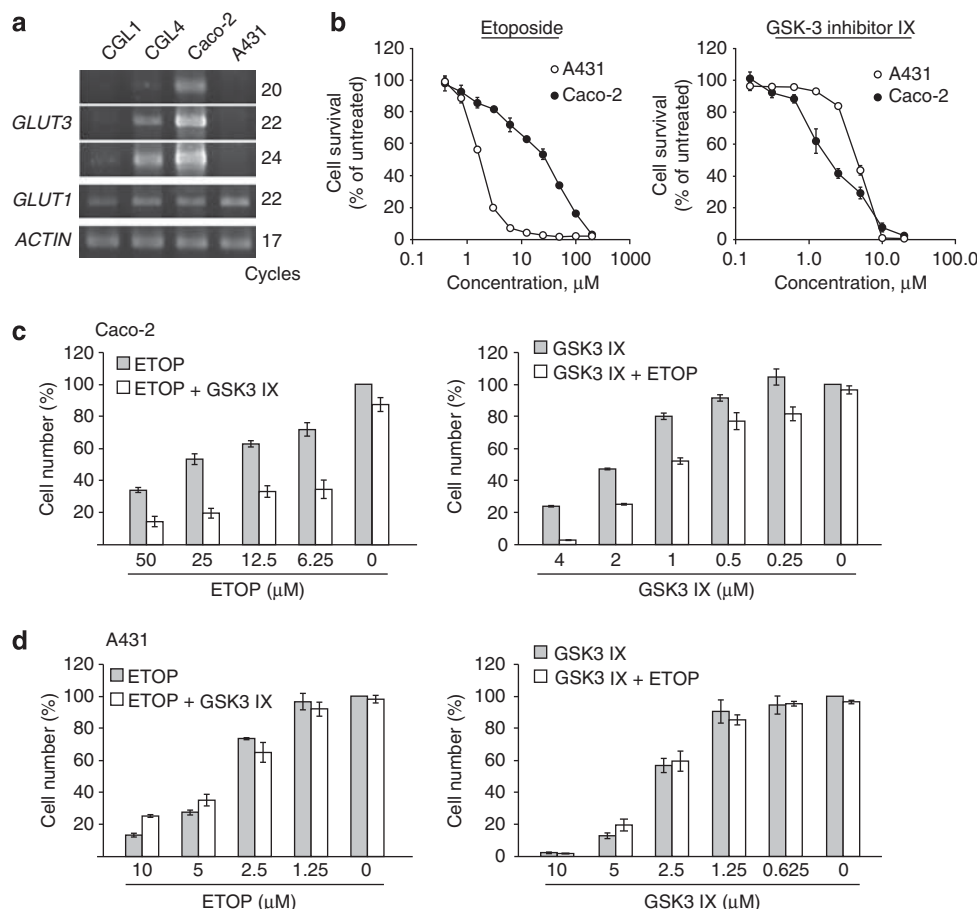
Figure 5. Synergic effect of GSK-3 inhibitors on the cytotoxic action of DNA-damaging agents in *GLUT3*-unregulated cancer cells. (**a**) RT–PCR (upper panel) was performed to examine *GLUT1* and *GLUT3* expression in CGL1, CGL4, Caco-2 or A431 cells. (**b**) The viability of Caco-2 or A431 cells treated 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.

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-mediated cytotoxicity (Figures 4a–d). These results suggest that some GSK-3-dependent signals have a crucial role as a mediator of GSK-3-induced cytotoxicity. It was previously been reported that deregulation of the PI-3K (phosphatidylinositol 3-kinase) pathway and activation of AKT may be important for 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, p27[Kip1] is a critical downstream mediator of the cell cycle arrest associated with GSK-3 inhibition in MLL-transformed cells.[58] The involvement of these effectors in GSK-3-induced suppression of GLUT3 expression and apoptosis needs to be clarified.

In conclusion, chemical screening with a pair of HeLa cell hybrids identified GSK-3 inhibitors as useful for the selective killing of GLUT3-expressing tumor cells. This study showed that (i) the induction of pro-apoptotic effects by the drugs is mediated through the activation of caspase-3; (ii) inhibition of *GLUT3* expression is associated with inhibition of NF-κB activity; and

(iii) synergistic cytotoxicity between GSK-3 inhibitors and DNA-damaging agents can be expected.

The results suggest for the first time that GSK-3 is a molecular target for GLUT3-expressing tumor therapy. As possible target cells are limited in this study, it would be examined in more cell types. During the preparation of this manuscript, chemotherapeutic agents that attack GLUT1 in renal tumor cells were identified.[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

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.[59] The cell viability from duplicate plates was averaged, and a *S*-score was calculated using the following formula: S CGL1 or HeLa-S3/CGL4 = $\log_{10}$ (% 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% CO_2/95% air at 37 °C, as described previously.[34] Transfection 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 TRlzol (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 (C_t) 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 $2^{-\Delta\Delta Ct}$ 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 (Doushisha 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 incubated 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 Na_3VO_4, 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, 2.5×10^3 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% CO_2/95% air for 72 h, cell viability was calculated relative to the DMSO control.

Measurement 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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Supplementary Information accompanies the paper on the Oncogenesis website (http://www.nature.com/oncsis)