BRAIN TUMORS

KHS101 disrupts energy metabolism in human glioblastoma cells and reduces tumor growth in mice

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Pharmacological inhibition of uncontrolled cell growth with small-molecule inhibitors is a potential strategy for treating glioblastoma multiforme (GBM), the most malignant primary brain cancer. We showed that the synthetic small-molecule KHS101 promoted tumor cell death in diverse GBM cell models, independent of their tumor subtype, and without affecting the viability of noncancerous brain cell lines. KHS101 exerted cytotoxic effects by disrupting the mitochondrial chaperonin heat shock protein family D member 1 (HSPD1). In GBM cells, KHS101 promoted aggregation of proteins regulating mitochondrial integrity and energy metabolism. Mitochondrial bioenergetic capacity and glycolytic activity were selectively impaired in KHS101-treated GBM cells. In two intracranial patient-derived xenograft tumor models in mice, systemic administration of KHS101 reduced tumor growth and increased survival without discernible side effects. These findings suggest that targeting of HSPD1-dependent metabolic pathways might be an effective strategy for treating GBM.

INTRODUCTION

Glioblastoma multiforme (GBM) is the most common malignant primary brain tumor in adults and among the most devastating cancers (1). Its overall median time to recurrence after surgery and standard chemoradiotherapy is ~7 months, and the 5-year survival rate remains low (<5%) (2). Preclinical data have shown that small molecules hold therapeutic promise for treating GBM through perturbation of cell death programs (3), epigenetic and transcriptional pathways (4, 5), lethal autophagy (6), and GBM stem cell self-renewal (7). However, GBM biology remains poorly understood, and there is an unmet need for the identification of new targets and the development of alternative therapeutic strategies (2). Although presenting several challenges, phenotypic drug discovery and profiling using small molecules have the advantage of addressing the complexity of diseases, particularly when the molecular target(s) and the underlying mechanisms of action of a small molecule are identified (8, 9).

GBM consists of diverse cell populations that can differ in their tumor-promoting potential (10). Poorly differentiated (stem cell–like) GBM cells can be isolated from patient tumors and expanded for their use in chemical screens and diverse biological investigations using serum-free culture conditions as well as orthotopic xenografts in immunodeficient mice (10–14). Molecular pathways promoting GBM “stemness” have been implicated in tumor development, and phenotypic and molecular similarities between neural stem cells and poorly differentiated GBM cells have been described (10, 15, 16). In this context, the question arises as to whether chemical compounds that induce neural stem cell differentiation may also have a potential for reducing GBM growth.

KHS101 is one such compound that crosses the blood-brain barrier (BBB) and selectively induces neuronal differentiation of hippocampal neural progenitor cells in vitro and in vivo (17). Previous studies have revealed transforming acidic coiled-coil containing protein 3 (TACC3) as a biologically relevant target of KHS101 (17, 18). TACC3 is a known regulator of cell division (19) and an emerging factor in GBM and tumor biology (20–22). The TACC3-targeting and neural differentiation–promoting features of KHS101 prompted us to investigate the compound in human stem cell–like GBM cell models.

As inter- and intratumor heterogeneity is a major impediment to broadly efficacious GBM therapy, we also sought to address whether KHS101 would affect a spectrum of clinically relevant GBM subtypes. To achieve this, we established a panel of different patient-derived primary and recurrent GBM cell models that were characterized through cytogenetic and single-cell gene expression analysis. We observed that KHS101 induces a rapid and selective cytotoxic response in this heterogeneous spectrum of patient-derived GBM cell lines. Accordingly, we sought to identify the mechanisms of action behind the KHS101 anti-GBM activity using gene expression analysis, affinity-based target identification, orthogonal chemical validation, and quantitative proteomics. These investigations were complemented by the analysis of energy metabolism and mitochondrial dynamics, which have been previously implicated in cancer biology and the regulation of cancer stem cell phenotypes (23–25). Furthermore, we investigated the KHS101 anti-GBM activity in established patient-derived tumor xenografts upon systemic administration.
RESULTS

KHS101 selectively induces cytotoxicity in transcriptionally heterogeneous patient-derived GBM cell lines, independent of their molecular subtypes

GBM is characterized by intra- and intertumor heterogeneity that may hinder therapeutic success (26–28). To represent this molecular heterogeneity, we established six patient-derived tumor cell models from primary GBM (GBM1, GBM4, and GBM13), recurrent GBM (GBM20), and rare GBM subtypes such as gliosarcoma (GBM11) and recurrent giant cell GBM (GBM14) (table S1). We hypothesized that single-cell quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis using a selection of 85 classifier genes from the published proneural, neural, classical, and mesenchymal GBM molecular subtypes (28), and an additional selection of genes playing roles in GBM stemness and proliferation, and the cell cycle, could indicate the molecular subtype heterogeneity within our GBM cell lines. Transcriptome profiling was carried out (after microfluidic chip–based capture of individual cells) by single-cell qRT-PCR of ~45 randomly selected cells from each tumor model and from the noncancerous adult brain neural progenitor (NP) cell line 1 (derivation is described in (14)). On the basis of the expression of the aforementioned classifier genes, principal components analysis (PCA) indicated transcriptional heterogeneity between the different GBM (and NP1) cell lines and between individual cells within each model (Fig. 1A and file S1). Using computational analysis by cell cycle normalization, data discretization, and supervised classification, we stratified our cell lines based on their single-cell transcriptional phenotypes (file S1). Our analysis showed that the GBM cell lines used here have either single (mesenchymal or proneural) or double (classical/proneural or mesenchymal/proneural) GBM subtype signatures (Fig. 1B and file S1).

To evaluate the role of KHS101 in tumor cell proliferation, we treated three GBM cell lines harboring different molecular signatures with 7.5 μM KHS101 for 120 hours and assessed cell growth by real-time imaging. Independent of classical, proneural, and mesenchymal molecular subtypes, KHS101 markedly attenuated tumor cell growth as compared to the cells treated with the vehicle [dimethyl sulfoxide (DMSO)]. KHS101 did not affect cell proliferation in the noncancerous NP1 cell line (Fig. 1C). We then evaluated KHS101 in all six GBM cell lines and showed that KSH101 exhibited dose-dependent cytotoxic properties in all patient-derived GBM cell models and also in the U251 and U87 GBM cell lines (Fig. 1D and fig. S1A). Neither bone morphogenetic protein 4 (BMP4)–induced differentiation of GBM cells (29) nor reduced oxygen tension (5% O2) (30) interfered with KHS101-induced cytotoxicity, and NP cells were refractory to KHS101 treatment under both 21% and 5% O2 conditions (fig. S1B).

KHS101 promotes autophagy and apoptosis in GBM cells

To examine the distinct cellular phenotypes of KHS101-treated GBM and NP cells, we carried out electron microscopy (EM) and immunocytochemistry-based imaging 12 hours after KHS101 (7.5 μM) addition to the GBM cell cultures. KHS101-treated GBM1 cells displayed a pronounced development of intracellular vacuoles compared to NP1 cells and to GBM cells treated with the vehicle (Fig. 2Aa, top). Concomitantly, microtubule-associated proteins 1A/1B light chain 3B (LC3B)–positive autophagosomal compartments increased in GBM1 and all other tested GBM cell models compared to the NP1 line (Fig. 2, A and B). The KHS101-induced macroautophagy phenotype, measured as LC3B-stained cytoplasmic area, was concentration-dependent in three different GBM cell cultures tested (GBM1, GBM11, and GBM20) and was not detected in NP1 cells after a 12-hour treatment period (Fig. 2C). The effect of KHS101 on GBM autophagic flux was further confirmed by cellular accumulation of the cationic amphiphilic tracer dye CYTO-ID in the GBM1 cell line (Fig. 2D). Consistently, EM imaging indicated the degradation of electron-dense cytoplasmic cellular content (fig. S1D). The KHS101-induced autophagic phenotype was accompanied by a proapoptotic cell fate shown by a marked increase in caspase 3/7 activation (luminescent assay) in the GBM1, GBM11, and GBM20 cell lines after a 48-hour treatment period (compared with the NP1 model; Fig. 2, E and F). A significant accumulation of annexin V–positive apoptotic cells was also observed in GBM1 cells 48 hours after KHS101 treatment (fig. S1E). However, chemical inhibition of late-stage autophagy using bafilomycin A1 did not prevent this KHS101-induced apoptotic cell death (fig. S1E), suggesting that the latter is not autophagy-dependent.

KHS101 selectively disrupts metabolic pathways in GBM cells

TACC3 is a known target of KHS101 in rodent neural progenitor cells (17). KHS101 has been shown to cause cellular destabilization of TACC3, hence reducing endogenous TACC3 protein levels over time (18). Western blot analysis showed that KHS101 did not reduce TACC3 expression by >20% after a 12-hour treatment period in GBM1 cells; however, TACC3 appeared reduced by >40% from 18 hours onward (fig. S2A). Accordingly, KHS101-mediated TACC3 reduction was not linked to the rapid increase in autophagy marker positivity (from <10 to >80% CYTO-ID–positive cells) measured over a treatment period of 12 hours in GBM1 cells treated with 7.5 μM KHS101 (Fig. 3A). Consistently, the appearance of GBM cell vacuoles was observed ~4 hours after KHS101 addition by time-lapse microscopy (movie S1). The KHS101-induced increased autophagy was not seen in TACC3 knockdown cells (fig. S2, B and C), hence excluding TACC3 down-regulation as a critical player in mediating KHS101 cytotoxicity in the GBM context. Microarray transcriptome profiling (ArrayExpress, accession E-MTAB-5713) and gene enrichment analysis of KHS101-treated GBM1 cells (using the hypergeometric distribution) indicated that, in addition to differentially regulated cell cycle pathways (Fig. 3B, left), genes associated with oxidative phosphorylation (OXPHOS) and the tricarboxylic acid (TCA) cycle were significantly modulated by KHS101 treatment (P < 4 × 10–5; Fig. 3B and fig. S3A). The KHS101 effect on metabolism-related gene expression was observed using a selection of 25 differentially expressed (>2-fold) marker mRNAs indicating alterations in glycolytic [hexokinase 2 (HK2) (31, 32)], oxidative [heme oxygenase 1 (HOX1) (33)], and proapoptotic [harakiri, BCL2-interacting protein (HRK) (34)] pathways as well as down-regulation of known GBM stemness markers [nitric oxide synthase 2 (NOS2) (15), inhibitor of DNA binding 1, helix-loop-helix protein (ID1) (35), and oligodendrocyte transcription factor 2 (OLIG2) (36)] (Fig. 3B, right). This KHS101-induced gene signature was confirmed by qRT-PCR in both primary (GBM1) and recurrent (GBM20) tumor models but was observed neither in KHS101-treated NP1 cells nor in untreated TACC3 knockdown GBM1 cells (Fig. 3C).

To investigate whether the observed changes in metabolic gene expression were linked to a direct effect of KHS101 on GBM cell metabolism and mitochondrial function, metabolic phenotyping was carried out using five different GBM cell models, the NP1 and NP2 lines, and normal (nontransformed) human astrocytes (NHAs) using protocols described in (37). Extracellular flux analysis was carried...
out upon the addition of vehicle or KHS101, and a metabolic phenotype was obtained by plotting the basal oxygen consumption rates (OCRs; indicative of OXPHOS/mitochondrial respiration) of the different cell models as a function of their basal extracellular acidification rates (ECARs; an approximation of glycolytic activity). In contrast to the NP1, NP2, and NHA cell lines, the GBM cell lines exhibited elevated OCR and/or ECAR corresponding to “aerobic” (GBM20), “aerobic/energetic” (GBM1 and GBM13), and “energetic” (GBM11 and GBM14) baseline phenotypes (Fig. 3D). Upon acute KHS101 treatment, the noncancerous control cells remained within a “modest” energetic phenotype window and exhibited a moderate increase in glycolytic activity (≤2-fold; P = 0.05). In contrast, KHS101 induced a significant hypoxic shift (P < 0.0001) and a switch in metabolic baseline phenotype across all tested GBM cell models, independent of their molecular subtypes (Fig. 3D).

**KHS101 affects glycolysis and the TCA cycle in GBM cells**

Next, we examined intracellular fractional enrichment of glucose-derived carbon through glycolysis and the TCA cycle in the GBM1 and NP1 cell models using stable isotope substrate labeling with U-13C glucose (Fig. S4) (38). Four hours after the addition of KHS101 (7.5 μM), 13C label enrichment was not significantly different for glucose (Fig. 4A) or fructose 6 phosphate (Fig. 4B). However, we found a selective impairment of glycolysis in GBM1 compared with NP1 cells as indicated by the differential 13C label enrichment of dihydroxyacetone phosphate (Fig. 4C), glyceraldehyde 3-phosphate (Fig. 4D), glycerol 3-phosphate (Fig. 4E), phosphoenolpyruvate (Fig. 4F), and lactate (Fig. 4G). An unlabeled metabolite is detected as the molecular ion (M0) in the mass spectrum. Each additional 13C-carbon atom introduced to the specific molecule gives rise to an increase in mass of 1 (M1, M2, M3, and so forth). The incorporation of the 13C label into the M2 isotopologs of TCA cycle intermediates citrate (Fig. 4H), succinate (Fig. 4I), and fumarate (Fig. 4J), and malate (Fig. 4K) significantly decreased in KHS101-treated GBM1 cells (P < 0.05), whereas enrichment of these metabolites remained unchanged in NP1 cells, indicating a selective perturbation of label enrichment through glycolysis and pyruvate dehydrogenase-initiated TCA cycle reactions. After 24 hours of KHS101 treatment, total cellular adenosine triphosphate (ATP) levels were reduced (≥50%) in GBM1 compared with NP1 cells (Fig. S3B). In addition, we noted a higher fractional enrichment of the M1 isotopologs of citrate (Fig. 4H), succinate (Fig. 4I), fumarate (Fig. 4J), and malate (Fig. 4K) in control GBM versus NP1 cells, which was selectively reduced by KHS101 in GBM1 cells. Relative enrichment of the M3 isotopologs of TCA cycle intermediates was elevated in GBM1 versus NP1 cells (Fig. 4, H to K). Increased labeling of the M3 aspartate (a proxy for oxaloacetate), through the action of pyruvate carboxylase, was observed in KHS101-treated GBM cells (Fig. 4L). In summary, these findings indicate that KHS101 selectively impairs aerobic glycolysis, mitochondrial respiration–dependent, and malic enzyme–dependent biosynthetic pathways in GBM cells.

**KHS101 interacts with HSPD1 in GBM cells**

Mitochondrial dynamics are important mediators of tumorigenesis and cancer.
stem cell phenotypes (23, 25). To elucidate the cellular target(s) underlying the reduced mitochondrial and metabolic capacity in KHS101-treated GBM cells, we investigated the physical interaction of KHS101 with potential cellular protein(s) using an established affinity-based target identification protocol (17). The photoaffinity probe KHS101-BP (a KHS101 derivative containing a benzophenone moiety and an alkyne substituent) and KHS101 showed similar bioactivity in GBM cells (fig. S5). A distinct KHS101-BP–protein complex of ~60 kDa (isoelectric point, ~5.7) appeared reduced by >50% in the presence of a 50-fold excess of unlabeled KHS101 and was therefore used for examination of KHS101-interacting protein (Fig. 5A). Proteomics analysis revealed that the KHS101-BP–bound protein corresponded to the mitochondrial 60-kDa heat shock protein family D member 1 (HSPD1).

A specific interaction between KHS101 and HSPD1 was further observed by in vitro pull-down experiments using human recombinant HSPD1 protein (Fig. 5B). Cellular fractionation followed by Western blot analysis showed that HSPD1 was overexpressed in GBM1 cells and predominantly localized to the mitochondria as indicated by a marked increase in the mitochondrial-to-cytoplasmic ratio compared with NP1 cells (P < 0.01; Fig. 5C). Reduction of HSPD1 expression by lentiviral short hairpin RNA (shRNA) in GBM1 cells by 50 to 65% (P < 0.01; fig. S6A) was associated with an increase in mRNA expression of stress-inducible chaperone heat shock protein family A member 1 (Hsp70) member 1A (HSPA1A; >5-fold; P < 0.05), which has been linked to mitochondrial proteostasis in cancer cells (fig. S6B) (39). In agreement with a reported role for HSPD1 in glioma cell line proliferation (40), a decline in mitochondrial activity/OXPHOS (~50%; fig. S6C), as well as a significant decrease in proliferation (~50%; P < 0.0001), was observed in the HSPD1 shRNA-harboring (low HSPD1-expressing) GBM1 cells (note that KHS101 addition further reduced the growth of HSPD1 knockdown GBM1 cells; fig. S6D). KHS101 altered neither HSPD1 protein levels nor HSPD1 mRNA expression (Fig. 5, D and E, and fig. S6E), suggesting that the KHS101–GBM cytoxicity is independent of HSPD1 mRNA/protein down-regulation.

Fig. 2. KHS101 selectively induces an autophagic and proapoptotic cell fate across a spectrum of GBM cell models. (A) EM and immunocytochemistry [phase-contrast (Phc); anti-LC3B; 4′,6-diamidino-2-phenylindole (DAPI)] images (scale bars, 5 and 25 μm, respectively) of GBM1 and NP1 cells 12 hours after KHS101 (7.5 μM) or DMSO (0.1%) treatments. (B) Immunocytochemistry (phase-contrast; anti-LC3B; DAPI) images (scale bar, 30 μm) of indicated cell models 12 hours after KHS101 (7.5 μM) or DMSO (0.1%) treatments. (C) Quantification of the LC3B-positive cytoplasmic area (% of cellular area 12 hours after treatment with KHS101 (at the indicated concentrations) or DMSO (D; 0.1%) using the specified cell models. (D) Quantification of CYTO-ID–positive GBM1 cells 12 hours after treatment with DMSO (D; 0.1%) or KHS101 (at the indicated concentrations). (E) Kinetics of caspase 3/7 activation in GBM1 cells treated with KHS101 (7.5 μM) or DMSO (0.1%; data were normalized to t0). (F) Relative caspase 3/7 activation (at the 48-hour time point) in response to DMSO (D; 0.1%) or KHS101 (K; 7.5 μM) in the specified cell models. N, negative control (K; 7.5 μM + pan-caspase inhibitor Z-VAD-FMK; 2 μM); P, positive control (staurosporine; 1 μM). Data are means ± SD of three biological replicates; **P < 0.01, Student’s t test (two-tailed).

KHS101 aggregates HSPD1 and metabolic enzymes in GBM cells, promoting their metabolic exhaustion

To investigate whether KHS101 directly inhibits HSPD1 function, HSPD1/heat shock protein family E (Hsp10) member 1 (HSPE1) chaperonin complex activity was assessed upon KHS101 addition in vitro. A concentration-dependent inhibition of HSPD1-dependent substrate refolding was readily detected in the presence of KHS101 (IC$_{50}$, 14.4 μM) (Fig. 6A). Refolding activity remained unaffected upon the addition of a structurally closely related KHS101 analog (HB072; Fig. 6B and file S3), which was phenotypically inactive in GBM cells (fig. S7A). In contrast, the mitochondrial HSPD1-targeting natural product myrtucommulone (MC) elicited a concentration-dependent decrease in HSPD1/HSPE1 refolding activity (Fig. 6B).

Moreover, MC and KHS101 shared mitochondrial and bioenergetic...
Fig. 4. KHS101 impairs relative incorporation of glucose-derived carbon through glycolysis and the TCA cycle in GBM cells. (A) Gas chromatography–mass spectrometry stable isotope analysis of methoximation and silylation-derivatized metabolites extracted from NP1 and GBM1 cells after a 4-hour treatment with KHS101 (7.5 μM) or DMSO (0.1%) in medium containing U-13C glucose. Graph shows the fractional enrichment (percentage) in the isotopologs of glucose. (B to L) Fractional enrichments of fructose 6-phosphate (F6P) (B), dihydroxyacetone phosphate (DHAP) (C), glyceraldehyde 3-phosphate (GAP) (D), glycerol 3-phosphate (G3P) (E), phosphoenolpyruvate (PEP) (F), lactate (G), citrate (H), succinate (I), fumarate (J), malate (K), and aspartate (L). The x axis indicates the mass isotopomers (which are designated as M0, M1, M2…Mn, where n is the number of labeled atoms in the molecule) in the specified metabolites (corrected for 13C natural abundance; lactate M2 is not shown, as enrichment above natural abundance was not detected). Data are means ± SEM of three biological replicates. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001, one-way ANOVA (Tukey post hoc test).

Fig. 5. KHS101 interacts with mitochondrial HSPD1. (A) Two-dimensional SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting of GBM1 cell lysates (20 to 40% ammonium sulfate–precipitated fraction) detecting KHS101-BP–labeled protein in the presence or absence of unlabeled KHS101 (as specified) after photo-crosslinking (30 min) and biotin-tag labeling (click chemistry reaction using biotin-azide). Asterisk: 60 kDa. Right inlay shows the relative reduction of candidate compound-protein complex signal (%; spots 1–4) in the presence of unlabeled KHS101. Median of three technical repeats (back dots) is shown. Spot 1 corresponded to HSPD1 (identified by proteomics analysis after protein spot excision). (B) Specific in vitro binding of recombinant human HSPD1 with biotinylated KHS101 (KHS101-bio) was detected by silver staining of SDS-PAGE gels in the presence/absence of unlabeled KHS101, precipitated with streptavidin-conjugated agarose beads. Asterisk, 60 kDa. (C) HSPD1 mitochondrial (M)–to–cytoplasmic (C) ratio in GBM1 and NP1 cells as assessed by immunoblot quantification. Black dots represent biological replicates (median ± SD is shown). **P < 0.01, Student’s t test (two-tailed, equal variance). (D) Relative mitochondrial HSPD1 protein expression (percentage; normalized to control values as assessed by immunoblot analysis) 6 hours after DMSO (D; 0.1%) or KHS101 (K; 7.5 μM) treatment in GBM1 cells. (E) HSPD1 mRNA expression (fold changes) in GBM1 cells treated with DMSO (D; 0.1%) or KHS101 (K; 7.5 μM). SD of three biological repeats (black dots) is shown.

stress-promoting activities as observed by reduced mitochondrial respiration capacity, up-regulation of DNA damage-inducible transcript 3 (DDIT3) and HMOX1 mRNA, and ATP depletion (fig. S7, B to D). Consistently, MC recapitulated the KHS101-induced autophagy and cell death phenotype in GBM cells (fig. S7, E and F).
We next asked whether KHS101 disrupts mitochondrial HSPD1 function in a cell-based in vitro system. To this end, we quantified protein aggregation by fractionation of detergent-insoluble mitochondrial proteins in GBM1 compared with NP1 cells upon compound addition. Silver staining indicated that aggregated proteins (pellet) were enriched by about fourfold \( (P < 0.01) \), whereas soluble protein enrichment was not significantly affected in GBM1 compared with NP1 cells 1 hour after KHS101 treatment (Fig. 6C). Proteomics analysis determined that HSPD1 and enzymes with functions in glycolysis [aldolase, fructose–bisphosphate A (ALDOA)], TCA cycle [dihydrolipoyltransacetylase (DLST)], OXPHOS [ATP synthase F1 subunit alpha (ATP5A1)], and mitochondrial integrity [Lon peptidase 1, mitochondrial (LONP1) (42)] were specifically enriched in the aggregated protein fractions of GBM1 compared to NP1 cells (table S2). Consistently, the aggregated proteins readily integrated into a predicted HSPD1-centered protein-protein interaction network using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; Fig. 6D) (43). In addition to mitochondrial fractionation, a quantitative global proteomics analysis of KHS101–treated GBM1 cells [data deposited at the Proteomics Identifications (PRIDE) database, identifier PXD009429] showed that the following aggregated proteins of the predicted HSPD1 interaction network were significantly down-regulated 1 hour after KHS101 treatment \( (P < 7.5 \times 10^{-7}) \): DLST, ATP5A1, solute carrier family 25 member 3 (SLC25A3), ALDOA, pyruvate kinase M1/2 (PKM2), phosphoenolpyruvate carboxykinase 2, mitochondrial (PKC2), serine hydroxymethyltransferase (SHMT2), phosphoglycerate carboxykinase 2, mitochondrial (PGK2), heat shock protein 90 alpha family class B member 1 (HSP90AB1), and LONP1 (Fig. 6E and file S3). Changes in the KHS101-regulated GBM proteome were time-dependent as indicated by PCA and differential peptide abundances (fig. S8, A and B). Global protein abundance was decreased by KHS101 at both the 1- and 12-hour time points. Compared with \( t_0 \), 9500 and 9607 polypeptides were significantly down-regulated \( (P \leq 0.05) \), whereas only 93 and 16 proteins were markedly up-regulated at the 1- and 12-hour time points, respectively (Fig. 6E, fig. S8C, and file S3). Protein set enrichment analysis indicated that mitochondrial rather than endoplasmic reticulum pathways were affected (Fig. 6E, fig. S8C, and file S3). In summary, these findings corroborate a link between the KHS101-mediated disruption of mitochondrial HSPD1 activity and metabolic stress in GBM cells.

**KHS101 attenuates tumor growth and invasion in vivo**

To investigate the potential pharmacological effects of KHS101 on GBM in vivo,
GBM is a devastating cancer with limited treatment options and correspondingly poor patient outcomes. We began our investigation with the hypothesis that GBM tumor stem cell–like cells might be eradicated by a small molecule–mediated prodifferentiation phenotype (12). In this context, the compound KHS101 shows BBB penetrability as well as nontoxic neuronal differentiation properties by targeting TACC3 (17, 47). However, instead of a “forced” prodifferentiation phenotype that reduces GBM tumorigenicity in a cell death–independent manner (14, 29), we observed a cytotoxic lethal GBM cell fate, characterized by autophagy-driven cellular self-destruction.

One challenge for GBM target discovery and validation is to incorporate the ever-changing composition of molecularly and phenotypically diverse tumor cell populations (26, 27, 48) into preclinical disease modeling. To this end, transcriptional diversity among and within our six patient-derived models was revealed by microfluidic single-cell qRT-PCR analysis. We adapted computational approaches that robustly indicated classical, pronuclear, and mesenchymal GBM subtype compartments in our GBM models, which were independent of GBM subtype features, prodifferentiation signaling (14, 29), low-oxygen culture conditions, and parental tumor origin (primary versus recurrent GBM, and MGMT methylation status).

Consistent with previous work (18), KHS101 treatment decreased endogenous TACC3 protein in GBM cells over time. The resulting TACC3 degradation kinetics (onset, ≥18 hours) was not involved in the more rapidly evolving autophagy and cytoplasmic degradation processes in GBM cells. However, the KHS101-mediated decrease in TACC3 levels may have contributed to the alterations in GBM cell cycle and mitotic pathways as indicated by microarray gene expression analysis 24 hours after KHS101 addition. KHS101–induced gene expression changes (observed for SLC2A1, HK2, HMOXI, and DDIT3) suggested a yet unexplored KHS101 bioactivity with regard to metabolic and mitochondrial pathway perturbation. Consistent with the notion that altered energy metabolism is a hallmark of cancers and a potential tumor cell vulnerability (24), the GBM cell models consistently exhibited elevated bioenergetic demands compared with NP and astrocyte cell lines. Both intracellular and extracellular metabolic phenotyping indicated that KHS101 disrupted GBM cell energy metabolism. Glucose transporter gene expression (SLC2A1 and SLC2A3) was increased in GBM cells after a 24-hour treatment period. Moreover, HK2 expression increased after KHS101 treatment in vitro and in vivo. HK2 is a key enzyme for enhanced glucose turnover and may contribute to ATP availability (32), which was reduced in KHS101-treated GBM cells at the 24-hour time point. Fractional enrichment of glucose-derived carbon through glycolysis and the TCA cycle was impaired in the GBM cells, and the latter was indicated by a reduction in $^{13}$C label incorporation into the M2 isotopologs of TCA cycle intermediates. The M1 isotopologs of TCA cycle intermediates were likely derived from the generation of M1-labeled pyruvate, by the action of malic enzyme 1 (ME1), which subsequently reenter the TCA cycle either by the action of pyruvate carboxylase or the reverse ME1 reaction. ME1 is overexpressed in cancer cells to meet redox balancing and lipid biosynthetic demands, and its inhibition has been suggested to impair cancer cell growth (49). Increased
labeling of M3 aspartate (a proxy for oxaloacetate) may represent a compensatory carbon entry into the TCA cycle in KHS101-treated GBM cells via increased, pyruvate carboxylase–mediated, pyruvate anaplerosis, which has previously been described in lung metastases (50). Consistent with bioenergetic changes, stress response genes [DDIT3 and HMOX1 indicating mitochondrial stress in glioma depending on the experimental context (51, 52)] were up-regulated by KHS101. Moreover, a NAD(P)H cytoplasmic accumulation [indicating mitochondrial/redox anomalies (44, 53)] was observed in GBM cells and xenograft tumors upon KHS101 treatment. It is plausible that, concomitant with KHS101-induced advances in bioenergetic insufficiencies, cellular stress evolved and spread across the GBM cell organelle system, for example, via the mitochondrial unfolded protein response [as indicated by the up-regulation of DDIT3 and TRIB3 mRNA expression in treated GBM1 cells (54, 55)].

In agreement with a KHS101 interference with mitochondrial dynamics, affinity-based target identification suggested a physical interaction between KHS101 and the mitochondrial chaperone HSPD1 in GBM cells. The reduction of HSPD1 using shRNAs expectedly affected GBM cell growth and mitochondrial capacity (40), and a potential compensation for reduced HSPD1 expression via HSPA1A mRNA up-regulation was observed. However, KHS101 did not affect cellular HSPD1 mRNA and protein levels, highlighting the importance of recognizing differences between genetic and small-molecule target inhibition [reviewed in (56)]. As affinity-based target identification provides important, but not sufficient, evidence for small-molecule mechanisms of action investigations (9), we sought to address whether KHS101 disrupted HSPD1 function. The activity of the HSPD1/HSPE1 complex was reduced by KHS101 in a concentration-dependent fashion in vitro. A structurally related KHS101 analog that did not affect cellular HSPD1 mRNA and protein levels, recapitulated the KHS101-induced GBM cell cytotoxicity.

A selective aggregation of HSPD1 and its potential client proteins was observed in GBM cells 1 hour after KHS101 addition. The acute deregulation of a predicted HSPD1-centered enzymatic network, including ALDOA (regulating glycolysis), DLST (regulating TCA cycle), ATP5A1 (regulating OXPHOS), and the chaperone LONP1 [regulating mitochondrial integrity in cancer cells (42)], provides an explanation for the rapidly evolving metabolic stress that was not uniquely mitochondrial. Mitochondrial fractionation of aggregated ALDOA and PKM2 proteins suggests close association of these proteins with the HSPD1 complex in GBM cells.
enzymes with GBM cell mitochondria, a phenomenon reported for HK2 in the cancer cell context (32).

Selective effects of KHS101 toward brain cancer cells were observed throughout our study at the protein, metabolite, mRNA, and organelle levels. Cellular self-degradation processes were markedly pronounced in GBM but not NP cells 12 hours after KHS101 treatment. The loss of stem cell–like features and the significant increase in apoptotic cell death over time (>24 hours) indicate that GBM cells failed to compensate for the KHS101-mediated impairment of critical metabolic and mitochondrial fitness and ATP production. Together, these results support a causative relationship between the KHS101-induced HSPD1 disruption and a lethal GBM cellular phenotype.

KHS101 activity has not been associated with toxicity in noncancer contexts in vitro and in vivo (17, 18, 47, 58). KHS101 showed favorable in vivo properties including accelerated neuronal differentiation in adult rats [without affecting apoptosis of brain cells (17, 47)]. Consistent with the specific KHS101 cytotoxicity in GBM compared with NP cells, the compound markedly decreased the progression of established xenograft tumors, whereas adverse effects (liver toxicity) were not observed in mice after prolonged (10-week) systemic administration.

There are some limitations to our study. KHS101 is an experimental/preclinical compound that may require chemical and pharmacological optimization before KHS101–like bioactivities can be tested in clinical applications. The exact molecular nature of the KHS101–HSPD1 interaction and the role of HSPD1 in the metabolic reprogramming that drives brain tumorigenesis remain to be further investigated.

In summary, this experimental small-molecule phenotype and target profiling study identifies HSPD1 enzymatic function as a specific molecular vulnerability linked to energy metabolism in GBM cell models. A lethal GBM cell fate can be selectively triggered in a heterogeneous spectrum of GBM cells by a single agent. These findings highlight the potential for using KHS101–like compounds for therapeutic developments.

MATERIALS AND METHODS

Study design

Our objective was to characterize the effect of the synthetic small-molecule KHS101 in the GBM cellular context in vitro and in vivo. The control and treatment groups and the number of biological replicates (sample sizes) for each experiment are specified in the figure legends. For in vivo tumor xenograft studies, no power analysis was performed to predetermine the sample size, and animals were randomly allocated to the control and treatment groups and housed together to minimize environmental differences and experimental bias. Analysis of endpoint readouts was carried out in a blinded fashion.

Statistical analysis

A minimum of three independent biological repeats were analyzed using the Student’s t test (two-tailed, equal variance),
one-way or two-way ANOVA (Tukey post hoc test), or Benjamini-Hochberg procedure (for false discovery rates) as specified in the figure legends. Data were expressed as means ± SD. Three biological repeats comprised a minimum of three technical replicates. Approximate normal distribution of data was assumed. For xenograft tumor analysis, the Mann-Whitney U test was used (one-tailed). For Kaplan-Meier xenograft tumor analysis, the significance was calculated using the log-rank test. For all other materials and methods, see Supplementary Materials.

**SUPPLEMENTARY MATERIALS**

[Website link to supplementary materials]

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**REFERENCES AND NOTES**


KHS101 disrupts energy metabolism in human glioblastoma cells and reduces tumor growth in mice


Sci Transl Med 10, eaar2718.
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A serendipitous metabolic target for glioblastoma

Glioblastoma (GBM), one of the most aggressive brain cancers, is associated with poor prognosis and low survival rate. GBM stem cells contribute to aggressive GBM growth. Polson et al. hypothesized that promoting neural differentiation could have therapeutic effects. While testing the therapeutic properties of the small-molecule KHS101, previously shown to promote neural differentiation, the authors made the serendipitous discovery that KHS101 exerted cytotoxic activity in multiple patient-derived GBM cell lines by disrupting cell metabolism and promoting autophagy. In vivo administration of KHS101 reduced tumor growth and prolonged survival in patient-derived xenograft mouse models of GBM. The authors suggest that targeting cell metabolism using small molecules might be effective for treating GBM.