Targeting Glioblastoma Signaling and Metabolism with A Re-Purposed Brain-Penetrant Drug

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METHODS

Cell lines

Patient-derived GBM neurosphere lines were obtained as previously described (Buczkowicz et al., 2014; Laks et al., 2016; Nagaraja et al., 2019; Turner et al., 2017) and were cultured in DMEM/F12 medium supplemented with 1x B27, 20 ng/ ml of EGF, 20 ng/ ml of FGF, 1 µg/ ml heparin and 1x Glutamax (Gibco). U87EGFRvIII cells were established by stably expressing EGFRvIII in U87 cells, as previously described (Wang et al., 2006). U87, U87EGFRvIII, RPE1, and IMR90 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin, while normal human astrocytes (NHA) were cultured according to the manufacturer's standard protocol by using the astrocyte growth medium BulletKit (Lonza). The attached cells were maintained in 10% FBS medium and changed to 1% FBS medium for follow-up experiments as indicated in the methods. All cell lines were maintained at 37°C in a humidified incubator with 5% CO_2. More detailed information for all cell lines used in this study is listed in Table S1.

Intracranial GBM xenograft models

All mice experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Diego. Intracranial GBM xenograft models were established as described previously (Bi et al., 2019). In brief, patient-derived neurosphere cells were first engineered to express a near-infrared fluorescent protein IRFP720, and U87EGFRvIII cells were stably expressed with the turboFP635 protein. A total number of 5 x 10^4 U87EGFRvIII-FP635 cells, or 5 x 10^4 GBM39-IRFP cells, or 2.5 x10^5 HK296-IRFP cells in 5 µl PBS were intracranially injected into brains of five-week-old female athymic nude mice (Charles River Laboratories). 6~8 mice were injected for each group. For drug treatment, fluoxetine solution stocks were prepared by dissolving fluoxetine in water. Equal volumes of fluoxetine stocks or vehicle (water) were administrated to mice once daily (4.2 mg/kg, 10 mg/kg, 15 mg/kg, or 16.4 mg/kg) by oral gavage after tumors were established at day 8~10. DMSO or temozolomide resuspended in DMSO was administrated to mice once daily (5 mg/kg or 20 mg/kg) via intraperitoneal injection starting from day 8 for 5 days. For U87EGFRvIII xenograft models, 10 mg/kg fluoxetine or vehicle was administrated to mice once daily by oral gavage for 10 days starting from day 8 after tumor cells injection. Tumor growth was assessed using an FMT 2500 fluorescence tomography system (PerkinElmer), and survival dates until the onset of neurologic symptoms were recorded for survival curves. All mice were housed in a conventional barrier facility at 22°C on a 12-hour light/dark cycle with free access to water and food, and their health status was checked by following the protocols.

Gene expression and shRNA transduction

Lentivirus *SMPD1* expression plasmid was generated by cloning the full-length coding sequence of *SMPD1* into a pLVX-Puro vector (EcoRI and XbaI). Lentivirus shRNA plasmids were purchased from Sigma, and shRNA sequences are listed in the key resources table. For virus production, lentiviral shRNA or gene expression plasmids were

transfected with lentivirus packaging plasmids (Clontech) into HEK293T cells, and the supernatant containing virus was collected at 72 hours after transfection. Virus titers were measured before use, and fresh culture medium was changed to the cells after overnight infection. Infection efficiency and selection concentrations of puromycin were determined for every cell line before the follow-up experiments.

DepMap data analysis

Gene expression, genetic dependency (combined RNAi-screening from Broad, Novartis, and Marcotte projects), and drug sensitivity (PRISM Repurposing Primary Screen 19Q4) datasets (Corsello et al., 2020; Ghandi et al., 2019; McFarland et al., 2018; Nusinow et al., 2020) of Cancer Cell Line Encyclopedia (CCLE) were downloaded from the DepMap portal (https://depmap.org/portal). Mean differences of mRNA expression levels and shRNA dependency scores (DEMETER2) were calculated between glioma cell lines and other CCLE cell lines, and two-tailed Student's t-test was performed to test the significance. For drug sensitivity, the cell viability response to selected compounds (replicate collapsed log fold change values relative to DMSO) of all glioma cell lines from the DepMap dataset was analyzed by Pearson correlation and plotted as a drug-drug sensitivity matrix.

Sphingolipid analysis by LC-MS

GBM cells were treated with DMSO or 5 μM fluoxetine in 1% FBS DMEM medium for 42 hours and harvested as pellets. Samples were then spiked with a set of internal standards and extracted with an organic solvent system consisting of equal parts of dichloromethane and methanol. Phase separation was achieved by the addition of an equal part of water. The organic layer was collected, the solvent was removed under argon, and the samples were reconstituted in 100 ul of isopropanol/acetonitrile (40/60, v/v). Sphingolipids were separated by liquid chromatography (LC), and the eluting metabolites were measured by mass spectrometry (MS/MS) according to methodologies established at the UCSD LIPID MAPS Lipidomics Core (http://www.ucsd-lipidmaps.org). Briefly, a Waters Acquity UPLC system (Waters Technologies, Milford, MA) with a Phenomenex Kinetex C18 column,150x2.1 mm, 1.7 μm (Phenomenex, Torrance, CA) was used for chromatographic separation. Gradient elution started at 40 % mobile phase B for 10 minutes, then increased linearly to 100 % B over 10 minutes, kept at 100 % B for 30 minutes, and the column was equilibrated with 40 % B for 8 minutes. Buffer A consisted of 100% H2O with 10mM ammonium formate and 0.1% formic acid modifiers. Buffer B consisted of isopropanol/acetonitrile (40/60, v/v) with 10mM ammonium formate and 0.1% formic acid as modifiers. The flow rate was 300 μl/minute, and 10 μl of sample was injected via autosampler.

The LC eluent was interfaced with a mass spectrometer 6500 QTrap (Sciex, Framingham, MA), controlled by Analyst v. 1.7 software, operated in Information Dependent Acquisition mode (IDA), using an Enhanced MS (EMS) scan from m/z 400-1000 at 10000 Da/s as a survey scan. Source parameters were automatically optimized using flow injection analysis into an isocratic flow of 80 % mobile phase B using individual

lipid molecules. To maximize metabolite coverage and identification, the sphingolipids were analyzed in positive and negative ion modes. The optimized source parameters of the Turbo V ion source for positive ion mode were as follows: Curtain Gas, 20; Collision Gas, High; IonSpray voltage, 5000; Temperature, 300; Gas 1, 30; Gas 2, 30, Declustering Potential, 100; Collision Energy Spread, 0; Collision Energy, 45. Source parameters for negative ion mode were: Curtain Gas, 20; Collision Gas, High; IonSpray voltage, -4500; Temperature, 300; Gas 1, 30; Gas 2, 30, Declustering Potential, -150; Collision Energy Spread, 0; Collision Energy, -10. From each survey scan, the ions exceeding a pre-set intensity threshold were chosen for Enhanced Product Ion scans (EPI). The lipid molecules were identified by molecular mass, elution time and MS/MS fragmentation patterns. At least three biological replicates were performed for each cell line per treatment. For quantitation, the MS signals of endogenous sphingolipid molecules were normalized to that of the internal standards and the cell numbers of each sample.

SMPD1 enzymatic activity assay

The enzymatic activity of acid sphingomyelinase (SMPD1/ASM) was measured by the cleavage of HMU-PC using a commercial kit (Echelon, K-3200) as described previously (van Diggelen et al., 2005). GBM cells with indicated hours DMSO or fluoxetine treatment were collected. Cell pellets were then resuspended in water with proteinase inhibitor and sonicated in an ice water bath for 10 cycles (30 seconds on and 30 seconds off). Tumor samples were first homogenized in water with proteinase inhibitor by a rotor-stator tissue homogenizer and then sonicated on ice. After 5-minutes centrifugation (10,000 x g) at 4˚C, protein concentration of each sample was determined. Equal volumes of 10 µg samples were added into each reaction and incubated at 37°C for 3 hours. After adding the stop buffer, the fluorescence of HMU was recorded on an Infinite M1000 Plate Reader (Tecan) at 360 nm excitation and 460 nm emission. Data were normalized to that of the indicated control group and plotted from four biological replicates.

Cell viability assay

Cell viability was assessed using a CellTiter-Glo luminescent cell viability assay kit (Promega). Attached cells or GBM neurosphere cells were seeded into each well of 384-well plates with DMEM medium supplemented with 1% FBS and 1% penicillin/streptomycin or DMEM/F12 medium supplemented with 1/4x B27, 20 ng/ ml of EGF, 20 ng/ ml of FGF, 1 µg/ ml heparin, and 1x Glutamax respectively. Equal volumes of vehicles or drugs diluted with the medium were added into the wells the next day, and the cells were cultured for 72 hours. After 15 minutes of incubation with CellTiter-Glo reagent at room temperature, the luminescent was recorded using an Infinite M1000 Plate Reader (Tecan). Four biological replicates were performed for each cell line per treatment. The area under the curve (AUC) was calculated by the AUC function in the DescTools R package with the “spline” method, which results in the area under the natural cubic spline interpolation.

Cell death and Annexin V-positive cell analysis

Annexin V-positive cells were determined by flow cytometry using a FITC Annexin V Apoptosis Detection Kit (BD Biosciences). In brief, cells were treated with DMSO or fluoxetine and cultured for 72 hours in DMEM medium with 1% FBS (attached cells) or DMEM/F12 medium supplemented with 1/4x B27, 20 ng/ ml of EGF, 20 ng/ ml of FGF, 1 μg/ ml heparin and 1x Glutamax (neurosphere cells). For shRNA experiments, cells after shRNA lentivirus infection were reseeded and cultured for 72 hours. Cells were then collected for Annexin V/ PI staining and analyzed by using a BD LSRII flow cytometer (BD Biosciences). For cell death trypan blue assay, cells were seeded in 6-well plates (attached cells) or 25 cm^2 flasks (neurosphere cells) and cultured for five days after shRNA lentivirus infection or with drug treatment. Dead cells and live cells were counted by trypan blue assay using a TC10 automatic cell counter (Bio-Rad). At least three biological replicates were performed for each cell line per treatment.

Soft-agar colony formation assay

For each well of 12-well plates, 2000 GBM cells were mixed with 0.4% low-gelling-temperature agarose (Sigma) in growth medium and immediately plated onto a solidified bottom layer containing 1% low-gelling-temperature agarose in the growth medium. Four biological replicates were performed for each treatment, and Cells were treated and fed with fresh growth medium every three days for 3 weeks. Colonies were then stained with 0.005% crystal violet, imaged by a ChemiDoc MP imaging system (Bio-Rad), and counted by ImageJ.

Crystal violet clonogenic assay

24 hours after shRNA or gene expression lentivirus infection, 2000 GBM cells were reseeded into each well of 6-well plates and cultured in 2 ml growth medium for 2 weeks. The medium was refreshed every three days and removed before crystal violet staining. Colonies were fixed with 80% methanol in ddH_2O for 10 minutes and stained with 0.05% crystal violet for 20 minutes. After washed with water, plates were imaged on a ChemiDoc MP imaging system (Bio-Rad), and colony density was quantified by ImageJ.

RNA extraction and qRT-PCR

Total RNA extraction was performed using an RNeasy mini kit (QIAGEN) and the SuperScript IV VILO master mix (Invitrogen) was used for reverse transcription. Samples are mixed with primers and SYBR Green Supermix and amplified on a CFX96 real-time PCR detection system (Bio-Rad). The results were processed by the $\Delta\Delta C_t$ method, and expression levels were normalized to the reference gene and indicated control group.

Immunofluorescence staining

Cells were seeded in laminin-coated chamber slides and treated with DMSO or 5 μM fluoxetine for 42 hours. After washed twice with PBS, cells were fixed in 4% PFA for 15 minutes, permeabilized with 0.2% Triton X-100 in PBS for 15 minutes and then blocked

with 2% BSA in PBS for 45 minutes. Primary antibodies (anti-LAMP1, #9091, Cell Signaling at 1: 200 dilution; anti-phospho-histone H2A.X (Ser139), 05-636, Millipore at 1:200 dilution) in PBS with 0.02% Triton X-100 and 0.5% BSA was applied to cells and incubated overnight at 4°C. After four washes with PBS, cells were then incubated with fluorescent secondary antibody Alexa Fluor anti-Rabbit 546 (A11010, Invitrogen) or Alexa Fluor anti-Mouse 488 (A11017, Invitrogen) at a dilution of 1:1000 in PBS at room temperature for 1 hour. For EGFR staining, an EGFR antibody conjugated with Alexa Fluor 488 (#5616, Cell Signaling) was used at 1:200 dilution in overnight incubation. For LysoTracker staining, after 42 hours treatment of DMSO or 5 μM fluoxetine, cells were washed with PBS and incubated with 50 nM LysoTracker (L7528, Invitrogen) at 37 °C for 1 hour. Cells were then washed with PBS, fixed in 4% PFA for 15 minutes. After four washes with PBS, cells were mounted with antifade reagent with DAPI (Life Technologies) for imaging on an Olympus FV1000 confocal microscope. Fluorescent intensity was quantified by ImageJ.

Drug and lipid treatment

For western blot, cells were collected after 48 hours of treatment unless otherwise indicated. GBM neurosphere cells were seeded in DMEM/F12 medium supplemented with 1/4x B27, 20 ng/ ml of EGF, 20 ng/ ml of FGF, 1 μg/ ml heparin and 1x Glutamax, cultured overnight, and treated with DMSO or fluoxetine for indicated hours before harvesting for enzymatic assay or western blots. In proteasome and lysosome inhibitors experiments, cells were treated with DMSO or fluoxetine for 42 hours and incubated for additional 6 hours in the absence or presence of 10 μM MG132 or 50 μM Chloroquine before collecting. For lipid treatment, 10 μM of sphingolipids conjugated with BSA or an equal amount of BSA solution was added into the growth medium and mixed on a shaker at room temperature for 30 minutes before treating cells. Cells were then cultured with the lipid or vehicle-adding medium for 48 hours and applied for further western blot analysis or immunofluorescence staining. In cell viability assay, GBM cells were firstly cultured with lipid or vehicle -adding medium overnight and then incubated with fluoxetine or DMSO for 72 hours before CellTiter-Glo assay.

Membrane lipid order imaging of live cells

Membrane lipid order imaging of live GBM cells was performed as described previously (Owen et al., 2011). Briefly, GBM cells in glass-bottom dishes were treated with 5 μM fluoxetine for 40 hours in 1% FBS DMEM medium and then stained with 5 μM Laurdan (D250, Invitrogen) for 3 hours in serum-free medium at 37°C in a humidified incubator with 5% CO2. Cells were then imaged on a Leica SP5 Confocal/Multiphoton system with the excitation at 800 nm and the emission at 400–460 nm and 470–530 nm). Pseudocolored generalized polarization (GP) images were achieved by using an ImageJ plug-in as described (Owen et al., 2011). GP values at the plasma membrane region of at least 60 cells were quantified by ImageJ and plotted as histograms.

Western blot analysis

Cells were washed with cold PBS and lysed with 1x RIPA lysis buffer containing 1x protease and phosphatase inhibitor cocktail on ice for 30 minutes. Tumor samples were homogenized on ice with cold PBS supplemented with protease and phosphatase inhibitor cocktail and then lysed with an equal volume of 2x RIPA buffer on ice for 30 minutes. BCA protein assay kit (Thermo Scientific) was used to determine the protein concentration. Equal amounts of protein samples were mixed with Laemmli sample buffer, boiled at 100°C for 5 minutes, electrophoresed using 4%–12% NuPAGE Bis-Tris mini gels, and then transferred onto nitrocellulose membranes by a Trans-Blot Turbo transfer system (Bio-Rad). Membranes were blocked with 5% BSA in TBST buffer and incubated with corresponding primary antibodies at 4°C overnight, followed by incubation with HRP-conjugated secondary antibodies at room temperature for 1 hour. After washing, the blots were developed with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) and imaged using Image Lab software on a ChemiDoc MP imaging system (Bio-Rad).

Immunohistochemistry analysis

Formalin-fixed, paraffin-embedded tissue sections were performed by the Tissue Technology Shared Resource (TTSR)-Histology Core at UCSD. Standard staining protocols were followed. In brief, the antigen was retrieved by boiling slides in 0.01 M of sodium citrate (pH 6.0) for 15 minutes. Tissue sections were then incubated with primary antibodies overnight at 4°C, followed with 30 minutes incubation with biotinylated secondary antibodies at room temperature. Tissue sections for TUNEL staining were incubated with TdT/ dUTP at 37°C for 30 minutes and then with HRP-conjugated anti-digoxigenin at room temperature for another 30 minutes. Stained slides were imaged on an Olympus BX43 microscope and quantified in a double-blind fashion using Visiopharm image analysis software.

Cell surface EGFR and internalization analysis

The intensity of cell surface EGFR was determined by flow cytometry with an EGFR antibody that recognizes the extracellular domains of both wild-type EGFR and EGFRvIII proteins as described previously (Lu et al., 2007; Luwor et al., 2001). In brief, GBM39 and HK296 cells were first chilled on ice for 20 minutes and then incubated with a primary EGFR antibody (GR01, Millipore, mAb528, 1:20) on ice for one hour. After gently washed once with 10 ml cold PBS, the cells were incubated with an Alexa Fluor 488 goat anti-mouse second antibody (A11017, Invitrogen, 1:500) on ice for an additional hour. The cells were then gently washed once with 10 ml cold PBS and analyzed on a BD LSRFortessa X-20 flow cytometer (BD Biosciences). For EGFR internalization assay, GBM39 cells treated with 5 μM fluoxetine or DMSO for 48 hours were chilled on ice and incubated with the EGFR antibody (GR01, Millipore, mAb528, 1:40) on ice for one hour. Primary antibody-stained cells were either kept on ice or moved to 37 ℃ for 15 or 30 minutes to allow internalization. Following internalization, the cells were washed and incubated with Alexa Fluor 488 goat anti-mouse second antibody (Cat#A11017, Invitrogen, 1:500) for another hour before analyzing by flow cytometer. Internalized EGFR level was defined as the decreased signal of surface EGFR after incubation at 37 ℃.

Three to four biological replicates were performed for each treatment. A total of 10, 000 events for each sample was recorded and analyzed.

Density gradient fractionation

The detergent-free density gradient fractionation was performed as previously described (Cizmecioglu et al., 2016; Macdonald and Pike, 2005). In brief, GBM39 cells treated with 5 μM fluoxetine or DMSO for 48 hours were pelleted at 250 g for 5 minutes, washed once with cold PBS, and resuspended in 1 ml of cold homogenization buffer (20 mM Tris-HCl, pH7.8, 0.250 M sucrose, 1 mM $CaCl_2$ and 1 mM $MgCl_2$) with protease and phosphatase inhibitors. Homogenates were then passed through a 23 g needle for 20 times followed by centrifugation at 4°C at 1000 g for 10 min. 1 ml of supernatants were collected, mixed with 1 ml of 50% Opti-Prep solution (Sigma), and placed in the bottom of a 5 ml Ultra-Clear centrifuge tube (Beckman Coulter). 400 μl each of 20%, 17.5%, 15%, 12.5%, 10%, 7.5% and 5% Opti-Prep solutions were then poured onto the top. After ultracentrifugation at 100, 000 g for 2 hours at 4°C using an SW-55Ti rotor in a Beckman ultracentrifuge, equal volumes of six fractions were collected from the top layer to bottom layer and loaded for further western blot analysis. Lipid rafts fractions were characterized by the non-lipid rafts marker Calnexin and lipid rafts markers Flotilin-1 and Gα(q). The percentages of protein level in fraction 1 were calculated by dividing the amount of protein in fraction 1 by the total amount of protein in all six fractions and plotted from three independent experiments.

RNA-seq analysis

Neurosphere cells were treated with DMSO or 5 μM fluoxetine for 42 hours and collected for RNA extraction. RNA sequencing was performed by Novogene. RNA-Seq reads were aligned to the human reference transcriptome (GRCh38 release-98) and quantified using the Salmon software (Patro et al., 2017). The --gcBias flag was used to estimate a correction factor for systematic biases commonly present in RNA-seq data. The differential expression analysis was performed using the likelihood ratio test (LRT) in DESeq2(Love et al., 2014). The LRT examines two nested models for the read counts, a full model where gene expression was explained by fluoxetine treatment and cell lines and a reduced model, in which only cell lines were considered. The test determines if fluoxetine treatment contributed significantly to the gene expression beyond the expected expression level due to cell lines. Gene Set Enrichment Analysis (GSEA) was performed on all genes ranked by likelihood ratio test statistic against MSigDB v7.1 (Subramanian et al., 2005). Enriched terms, including EGFR signaling inhibitor down and up signatures (Kobayashi et al., 2006) (https://www.gsea-msigdb.org/gsea/msigdb/cards/KOBAYASHI_EGFR_SIGNALING_24HR_DN and https://www.gsea-msigdb.org/gsea/msigdb/geneset_page.jsp?geneSetName=KOBAYASHI_EGFR_SIGNALING_24HR_UP), were visualized using ClueGO (Bindea et al., 2009).

TCGA data analysis

TCGA GBM RNA-seq dataset was used in patient survival study. In survival association group analysis, we used the “lifelines” package in python to fit Cox proportional hazard models (Andersen, 1982). *P* values were calculated by log likelihood ratio tests. To evaluate whether a gene’s expression provides additional prognostic information beyond the baseline survival probability due to age at diagnosis, we compared the likelihood of two nested models: a full model with gene expression and age of patients and a reduced model, in which only age was considered. Proportional hazards model and log-rank test were applied to assess the prognostic significance of individual genes. Overall survival of GBM patients with the top 25% and bottom 25% of *SMPD1* expression in the TCGA GBM cohort (RNA-seq) was statistically compared by Log-rank test. Cox proportional hazard ratios were calculated. *P* values and numbers of patients of each cohort were indicated in the figure. The Gene Expression Profiling Interactive Analysis (GEPIA) web-server(Tang et al., 2017) was used to analyze the expression of metabolic genes between GBM tumors and normal brains based on TCGA and GTEx RNA-seq data. The genetic alterations of *EGFR*, *SMPD1*, and *IDH1* in the merged cohort of LGG and GBM TCGA (PanCancer Altas) datasets were assessed using cBioPortal for Cancer Genomics (http://www.cbioportal.org) (Cerami et al., 2012; Gao et al., 2013). Gene set enrichment analysis was performed to characterize genes differentially expressed in GBM clinical samples (TCGA GBM, HGU133A) with high or low *SMPD1* expression. The median of the GBM cohort was chosen as the cutoff for high and low *SMPD1* expression groups.

Evaluation of Fluoxetine in Overall Survival of GBM Patients in Electronic Medical Records

Complete details of the database used, data overview, use of ICD9 and ICD10 codes, data extraction pipeline, exclusion criteria, enrichment for GBM patients, statistical analyses, and full results are presented in Supplemental Document S1.

QUANTIFICATION AND STATISTICAL ANALYSIS

Unless otherwise indicated, all statistical analysis was performed using GraphPad Prism 8 software. Unpaired two-tailed Student’s t-test was applied to compare two experimental groups, and one-way or two-way ANOVA followed by multiple comparisons test was used to assess differences between three or more experimental groups. Paired two-tailed Student’s t-test was only performed in comparing the percentage of protein intensity in paired samples. Log-rank (Mantel-Cox) test was used in survival analysis. Fisher’s exact test was applied for mutual exclusivity analysis of genetic alterations in TCGA clinical samples and tumor recurrence analysis of PDX mice models. Bar graphs show mean ± SD or SEM as indicated in the legends, and p values less than 0.05 are defined to be significant. Numbers of samples, statistical tests, and p values analyzed in each experiment are reported in the respective figure legends or methods.

SUPPLEMENTAL ITEM TITLES AND LEGENDS

Document S1. Evaluation of Fluoxetine in Overall Survival of GBM Patients in Electronic Medical Records from The IBM Marketscan Dataset.

Table S1. Information of Cell Lines Used. It includes major clinical and genomic features, such as age, gender, tumor category, and prior treatments, of 18 patient-derived adult GBM and DIPG neurosphere lines that were utilized in this study.

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