Perhexiline Demonstrates FYN-mediated Antitumor Activity in Glioblastoma

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ABSTRACT

Glioblastoma is the most common primary malignant brain tumor in adults. Despite aggressive treatment, outcomes remain poor with few long-term survivors. Therefore, considerable effort is being made to identify novel therapies for this malignancy. Targeting tumor metabolism represents a promising therapeutic strategy and activation of fatty acid oxidation (FAO) has been identified as a central metabolic node contributing toward gliomagenesis. Perhexiline is a compound with a long clinical track record in angina treatment and commonly described as an FAO inhibitor. We therefore sought to determine whether this compound might be repurposed to serve as a novel therapy in glioblastoma. Perhexiline demonstrated potent *in vitro* cytotoxicity, induction of redox stress and apoptosis in a panel of glioblastoma cell lines. However, the antitumor activity of perhexiline was distinct when compared with the established FAO inhibitor etomoxir. By evaluating mitochondrial respiration and lipid dynamics in glioblastoma cells following treatment with perhexiline, we confirmed this compound did not inhibit FAO in our models. Using *in silico* approaches, we identified FYN as a probable target of perhexiline and validated the role of this protein in perhexiline sensitivity. We extended studies to patient samples, validating the potential of FYN to serve as therapeutic target in glioma. When evaluated *in vivo*, perhexiline demonstrated the capacity to cross the blood–brain barrier and antitumor activity in both flank and orthotopic glioblastoma models. Collectively, we identified potent FYN-dependent antitumor activity of perhexiline in glioblastoma, thereby, representing a promising agent to be repurposed for the treatment of this devastating malignancy.

Introduction

Glioblastoma is the most common primary malignant brain tumor in adults. Despite aggressive, multimodality treatment regimens consisting of surgical resection, radiotherapy, and chemotherapy consisting of temozolomide, outcomes remain poor with a median survival of 15 months and few long-term survivors (1). Therefore, considerable effort is being made to identify novel therapies for this devastating malignancy.

In recent years, there has been a renewed interest in understanding metabolic reprogramming and its contributory role in the aggressive phenotype of cancer. Glioblastoma represents one such malignancy, which demonstrates profound metabolic plasticity that allows these cells to adapt to diverse microenvironments. On the basis of these recent discoveries, alterations in tumor metabolism have emerged as an attractive therapeutic target. Although a majority of work in this research space has focused on glycolysis, our understanding of tumor metabolism has evolved considerably, with recent investigations identifying a diverse array of additional metabolic programs that contribute toward malignancy. In glioblastoma, activation of fatty acid oxidation (FAO), which utilizes fatty acids to serve as a substrate for a variety of biologic processes, including energy, redox balance, and purines/pyrimidines for DNA synthesis (2), has been identified as a central metabolic node differentiating aggressive high-grade glioma from low-grade glioma (3, 4). On the basis of this apparent reliance on FAO, this metabolic pathway is actively being investigated as a novel anticancer strategy in glioblastoma.

Interestingly, the clinical development of FAO inhibitors has been primarily in the field of cardiology. Pharmacologic agents designed to inhibit FAO have largely been developed to target carnitine palmitoyltransferase 1 (CPT-1), the rate-limiting enzyme that converts acyl CoA to acylcarnitine. Etomoxir is a well-established CPT-1 inhibitor that was previously tested in clinical trials; however, discontinued due to hepatotoxicity (5, 6). Perhexiline represents an additional agent described as being an FAO inhibitor currently approved in Australia, New Zealand, and Asia for angina (7). Although routinely referred to as a CPT-1 inhibitor, its primary target and mechanism of action have been called into question (8). As several recent reports have identified the antitumor potential of perhexiline (9–13), in this article, we sought to both determine whether this compound had activity in glioblastoma and further investigate its underlying mechanism of action.

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Materials and Methods

Cell culture and reagents

Undifferentiated MES83 and PN19 cell lines were cultured in DMEMF/12, GlutaMAX (Gibco-Thermo Fisher Scientific) media supplemented with FGF (Prospec), Heparin (STEMCELL Technologies Inc.), B-27 (Gibco), and EGF (PeproTech). Alternatively, these lines were cultured in DMEMF/12, GlutaMAX supplemented with 10% FBS to promote differentiation. Indicated MES and PN cells were generated, authenticated, and provided by Dr. Ichiro Nakano lab at The Ohio State University (Columbus, OH; ref. 14). T98G and U251 cells were purchased from ATCC and authenticated by short tandem repeat analysis at the University of Arizona (Tucson, AZ) and were

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grown in DMEM with glucose, L-glutamine, and sodium pyruvate (Corning) supplemented with 10% FBS. Cell lines were tested for *Mycoplasma* using the MycoSensor PCR Assay Kit (Agilent Technologies). Perhexiline maleate and etomoxir sodium salt hydrate were purchased from Sigma-Aldrich. Trypan blue dye exclusion test was used to determine cell viability.

Apoptosis

Described cell lines were treated with perhexiline (5 μmol/L; 1.5 μmol/L for PN19) for 48 hours followed by Annexin V (Invitrogen) and 7AAD (Calbiochem) labeling according to manufacturer's instructions and apoptosis was assessed using a BD FACS Canto II flow cytometer (Becton Dickinson). Analysis was performed by using FlowJo V.10 Software (FlowJo, LLC).

Analysis of reactive oxygen species

Spectrophotometric assessment of reactive oxygen species (ROS) in cells following treatment with perhexiline was performed using CM-H2DCFDA (Invitrogen) according to manufacturer's instructions.

Cellular bioenergetics

Perhexiline- and etomoxir-dependent change in oxygen consumption rate (OCR) was measured after the addition of compounds using the Seahorse XF24 platform (Agilent). Briefly, 35,000 T98G cells were seeded overnight in DMEM complete media. The next day, before performing the Seahorse assay, DMEM complete media was replaced with XF media. Similarly, 70,000 MES83 cells were seeded in poly-D lysin pretreated XF plate prior to running the experiment. XF media used for the assay was supplemented with an equimolar concentration of glucose, pyruvate, and glutamine, found in individual cell lines' regular growth media and cells were incubated in XF media for 45 minutes in a non-CO_2 incubator before performing the assay. After basal OCR was measured, perhexiline (5 μmol/L) or etomoxir (40 μmol/L) were added through the port, OCR was monitored in real-time, and data were normalized to cell number.

Acylcarnitine quantification

Acylcarnitines were quantified using the Absolute IDQ p180 Kit (Biocrates Life Sciences AG) according to manufacturer's instructions. Briefly, MES83 cells were treated with perhexiline (5 μmol/L; 8 hours) followed by washing with ice-cold PBS. Cells were lysed in phosphate buffer (10 mmol/L) and evaluated according to manufacturer's instructions. Medium and long-chain acylcarnitine data above the limit of detection were quantified and reported as fold change compared with control.

Transient knockdown using siRNA

siRNA targeted against FYN and c-SRC or scrambled sequence was used for knockdown (Thermo Fisher Scientific) according to manufacturer's protocol using Lipofectamine 2000 (Life Technologies).

Publicly available database analysis

Transcriptional expression data of PN, MES, T98G, and U373 cells were downloaded from Geo Expression Omnibus (GEO; accession number GSE67089). The Affymetrix data of normal and glioblastoma tissue along with molecular subtypes of glioblastoma were downloaded from The Cancer Genome Atlas (TCGA) UCSC Xena browser (http://xena.ucsc.edu/). Differential expression of FYN among the various human cancer types was obtained from cBioPortal. Differential FYN expression based on specific anatomic regions in glioblastoma was downloaded from Ivy Glioblastoma Atlas Project (15, 16). SwissTargetPrediction was used to estimate the most probable macromolecule targets of perhexiline (17).

Western blot analysis

Expression of protein was evaluated using Western blot analysis as described previously (18). Antibodies against FYN and SRC were obtained from Santacruz Biotechnology, and Phospho-FYN (Tyr530) was obtained from Invitrogen. Antitubulin (CP-06) was purchased from Calbiochem and antiactin was from Abcam. Anti-EGFR was purchased from Cell Signaling Technology. Horseradish peroxidase–conjugated secondary antibodies were from Sigma-Aldrich. Membrane was developed on X-ray film using ECL Western blotting substrate purchased from Thermo Fisher Scientific.

FYN kinase activity assay

FYN kinase activity was determined using a FYN A Kinase Enzyme System purchased from Promega and performed following manufacturer's instruction.

Detection of perhexiline in brain tissue

Described tissue (10 mg) was homogenized in 300 μL of methanol and supernatant was collected after centrifugation. Supernatants were further filtered using 0.22 μm Corning Costar Spin-X centrifuge tube filters (Corning Incorporated) at 10,000 g for 5 minutes. Qualitative analysis of perhexiline was performed using a Dionex 3000 UHPLC coupled with an Orbitrap Elite. Briefly, a ACQUITY BEH C18, 1.7 μm, 2.1 × 100 mm column (Waters Technologies Ireland Ltd) with mobile phase of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) was used. A flow rate of 0.4 mL/minute, the ESI+ gradient was (time in minutes, %B): (0, 1), (2.5, 1), (16, 99), (18, 99), (18.11, 1), (20, 1) and [M+H]+ ion using a m/z of 278.2842 for perhexiline.

Animal handling

Animal experiments were conducted according to guidelines of the Institutional Animal Care and Use Committee. Flank and orthotopic MES83 tumor xenografts were established in nu/nu mice (Charles River Laboratories), as described previously (14). Tumor growth in the flank was measured with calipers, and orthotopic tumors were measured 10–12 days posttumor implant with MRI as described previously (18).

Statistical analysis

ANOVA with Tukey test or *t* test was performed using Origin Pro 2019 software (Origin Lab Corporation). The log-rank test was used to compare survival.

Results

Perhexiline demonstrates potent cytotoxicity in patient-derived glioblastoma stem cells

FAO has emerged as a central metabolic node in glioblastoma (3, 4), suggesting this metabolic pathway may serve as a therapeutic target. As perhexiline has been described as a FAO inhibitor and has a long clinical track record, we evaluated the potential for this agent to be repurposed for the treatment of glioblastoma. As an initial investigation, we evaluated the antitumor activity of perhexiline in subtype-specific glioblastoma stem cells (4, 14, 19) and established cell lines. Perhexiline showed dose-dependent cytotoxicity in both proneural (PN19) and mesenchymal (MES83) glioblastoma stem cells (**Fig. 1A**).

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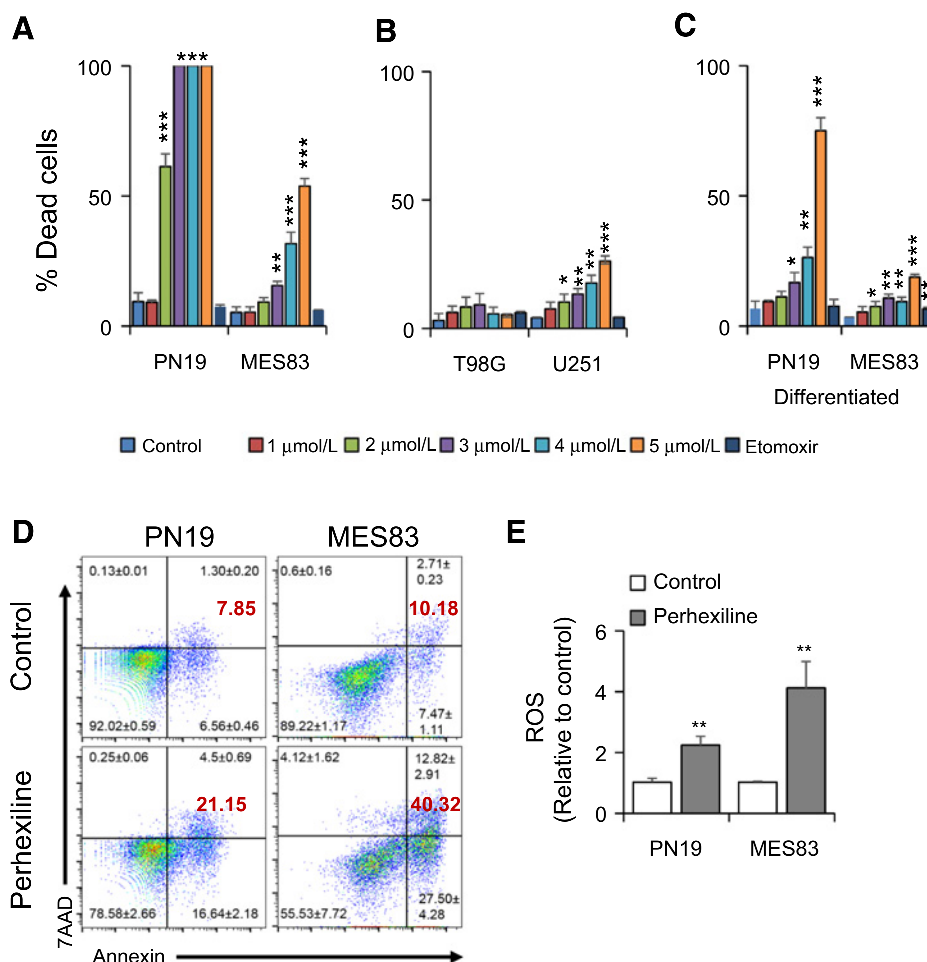

Figure 1.

Perhexiline induces cell death in patient-derived glioblastoma stem cells. Cell lines were treated with the indicated concentration of perhexiline or etomoxir (40 µmol/L) for 48 hours. **A–C**, Dead cells were counted using Trypan blue dye. **D,** Apoptosis was analyzed by flow cytometer after staining with Annexin V/7AAD in perhexiline-treated PN19 (1.5 µmol/L; 48 hours) and MES 83 (5 µmol/L; 48 hours) cells. The percentage of apoptotic cells (early and late) are highlighted in red. **E,** Cellular ROS was measured using CM-H2DCFDA following treatment with perhexiline after 2 hours. Data are representative or average ± SD of three or more independent experiments (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$).

Differential activity was observed when extended to established glioblastoma cell lines. T98G cells displayed resistance to perhexiline, while U251 cells demonstrated cytotoxicity, although, were less sensitive when compared with the glioblastoma stem cell lines (**Fig. 1B**). As perhexiline demonstrated considerably more antitumor activity toward undifferentiated glioblastoma stem cells when compared with differentiated, established lines, we hypothesized that cellular differentiation may modulate sensitivity to this agent. To test this, we cultured glioblastoma stem cell lines in differentiation conditions consisting of 10% FBS, leading to growth in monolayer from undifferentiated tumor spheroids (14, 20). Intriguingly, differentiation influenced perhexiline-induced cytotoxicity in both lines (**Fig. 1C**). Most notably, differentiated mesenchymal cells displayed resistance to perhexiline. Similarly, proneural cells were relatively resistant to perhexiline; however, cytotoxicity was still observed at higher concentrations in this line. Interestingly, the antitumor profile of perhexiline in these glioblastoma cell models was very different when compared with the established FAO/CPT-1 inhibitor etomoxir. Specifically, treatment with etomoxir did not result in cytotoxicity using doses established to inhibit FAO in any of the globalstoma models tested (**Fig. 1A**–**C**), and only antiproliferative effects were observed in the MES83 and T98G cell lines (Supplementary Fig. S1), suggesting an alternate mechanism of action of perhexiline.

We extended investigations to define mechanisms driving perhexiline-induced cytotoxicity in the sensitive glioblastoma stem cell lines. Robust increases in apoptosis was observed in perhexiline-treated cells, as determined by Annexin V labeling, along with increases in oxidative stress, suggesting ROS may serve as a mediator of perhexiline induces cytotoxicity (**Fig. 1D** and **E**).

Perhexiline does not inhibit FAO in glioblastoma cells

Although described as an FAO inhibitor, we observed that the toxicity profile of perhexiline was distinct when compared with the well-established CPT-1/FAO inhibitor etomoxir in our glioblastoma model systems. We therefore sought to determine the capacity of perhexiline to modulate FAO in these cell lines. As an initial investigation, we analyzed the OCR of cells following FAO inhibition using the Seahorse XF platform. During FAO, short to long-chain fatty acids are catabolized in the mitochondria through sequential removal of two carbons by oxidation at the β-carbon position of the fatty acyl CoA molecule (2, 21). This results in the production of acetyl CoA, acyl CoA two carbon atoms shorter than the initial substrate, and one mole of FADH2/NADH in each round of β-oxidation. If fully oxidized, the acetyl CoA results in the production of an additional three moles of NADH and one mole of FADH2. The generated NADH and FADH2 provide the electron gradient required for ATP synthesis during oxidative phosphorylation, with oxygen serving as the final electron acceptor. Therefore, a reduction in OCR following FAO inhibition would correspond to FAO-dependent cellular respiration. As expected, a >30% reduction in OCR following FAO inhibition with

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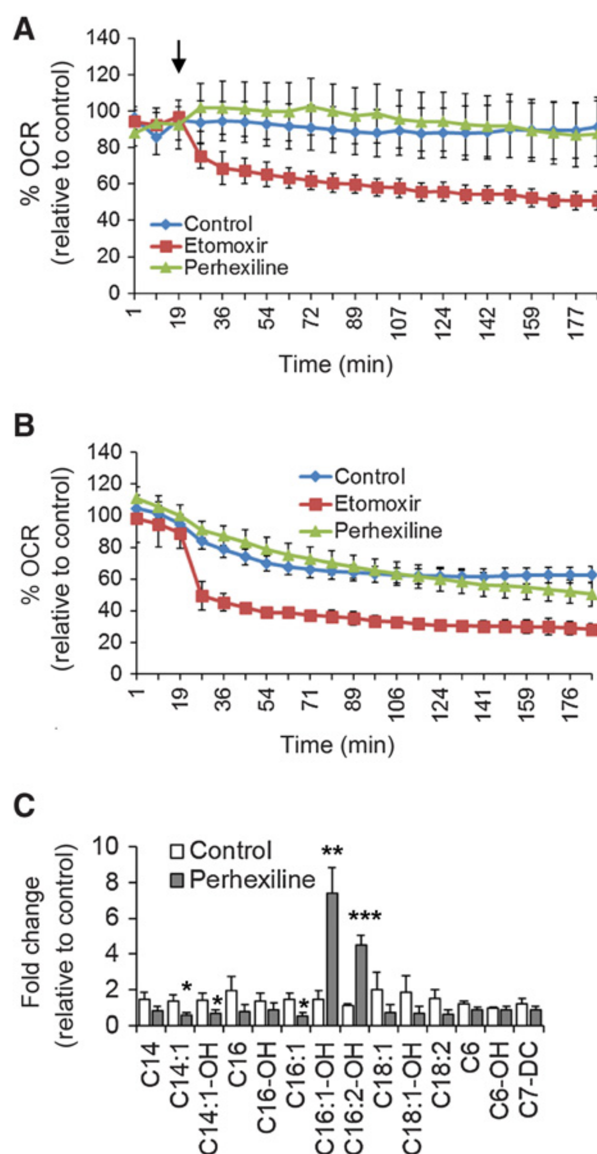

Figure 2.

Perhexiline does not inhibit FAO in glioblastoma. OCR was measured in MES83 (**A**) and T98G (**B**) using the Seahorse platform. Arrow indicates the time of addition of etomoxir (40 μmol/L) or perhexiline (5 μmol/L) to the cells through the port. Data shown are mean ± SD of 3–5 technical replicates and presented as the percent change in OCR relative to untreated control. Data are representative of at least two independent experiments. **C,** Cellular acylcarnitines was measured in MES83 cells treated with perhexiline (5 μmol/L; 8 hours). Data represent average ± SD of at least three independent experiments (*, $P < 0.05$; **, $P < 0.005$).

etomoxir was observed in MES83 and T98G cells (**Fig. 2A** and **B**), which represents the two cell lines sensitive to the antiproliferative effects of this agent (Supplementary Fig. S1). Conversely, no changes in OCR were observed when these cells were treated with perhexiline, further supporting that this agent does not inhibit FAO in these glioblastoma models. To extend this line of investigation, targeted metabolomics was performed to quantify levels of acylcarnitines in cells following treatment with etomoxir and perhexiline. Medium and long-chain fatty acids destined for FAO are impermeable to the outer mitochondrial membrane. Therefore, these fatty acids are first converted to acylcarnitines by CPT-1 (2). Carnitylated fatty acids or acylcarnitines can then enter the mitochondria and undergo FAO (2). As expected, we have observed treating glioblastoma cells with the established CPT-1/FAO inhibitor etomoxir resulted in reductions of both medium and long-chain acylcarnitines (S. Kant; unpublished data). However, consistent with the above findings, perhexiline treatment resulted in a very different acylcarnitine profile, with a majority of these fatty acids being unchanged, along with two long-chain acylcarnitines actually demonstrating 4–7-fold increases with this agent (**Fig. 2C**). Collectively, these findings confirm that perhexiline does not inhibit CPT-1 or modulate FAO in the tested glioblastoma models.

The antitumor activity of perhexiline is mediated by FYN

As we demonstrated that the cytotoxic activity of perhexiline in glioblastoma cells was independent of CPT-1/FAO, we next sought to identify the target of this promising compound. As an initial investigation, we utilized an *in silico* approach to estimate the most probable macromolecule targets of this agent using canonical SMILES of perhexiline analyzed on Swiss Target Prediction software (17). This resulted in the identification of 10 possible targets having a high probability (probability of 1) in *Homo sapiens* (**Fig. 3A**) that comprised several different classes, included the kinases EGFR and FYN and four family AG protein–coupled receptors. Next, we evaluated for the differential expression of these proteins in our glioblastoma cell lines. Of these 10 proteins, FYN expression was high in the perhexiline-sensitive cell lines and low in the perhexiline-resistant T98G cells (**Fig. 3B**), suggesting this nonreceptor, membrane-associated Src family tyrosine kinase may play a role in the cytotoxic activity of perhexiline. We first validated differential expression of FYN by Western blot analysis (**Fig. 4A**). Intriguingly, when these studies were extended to differentiated glioblastoma stem cells, we observed FYN expression was lost in the mesenchymal line, which was also resistant to perhexiline. As EGFR was identified as a possible target of perhexiline with known implications in gliomagenesis (1), we evaluated its expression in our models. As EGFR expression was not detectable in the perhexiline-sensitive proneural line, this suggests that this oncoprotein does not have a direct role in perhexiline-mediated antitumor activity. To more definitively establish FYN as a mediator of perhexiline-induced cytotoxic, we knocked down FYN expression using RNAi in the MES line, which resulted in resistance to perhexiline (**Fig. 4B** and **C**). We next evaluated the potential of perhexiline to directly inhibit FYN activation. Autophosphorylation at Y419 results in FYN activation, while phosphorylation at Y530 inactivates this kinase (22, 23). As antibodies against phospho-Y419 FYN are currently not available, we evaluated phospho-Y530 status of FYN following treatment with perhexiline in our models. A time-dependent increase in phospho-Y530 FYN was observed in PN19, MES83, and U251 cells, indicating perhexiline inhibits FYN activation **Fig. 4D**. This was further validated by directly evaluating FYN kinase activity following perhexiline treatment (Supplementary Fig. S2). Knockdown of c-SRC, another putative target of perhexiline (22) did not rescue cells from cytotoxic effect of perhexiline (Supplementary Fig. S3). Collectively, these findings support the contributory role of FYN in mediating perhexiline-induced cytotoxicity.

We next sought to determine the potential significance of FYN in patient-derived brain tumors. As an initial investigation, we evaluated FYN expression using a pan-cancer TCGA dataset. Of the diverse types of cancers included in this database, low-grade glioma and

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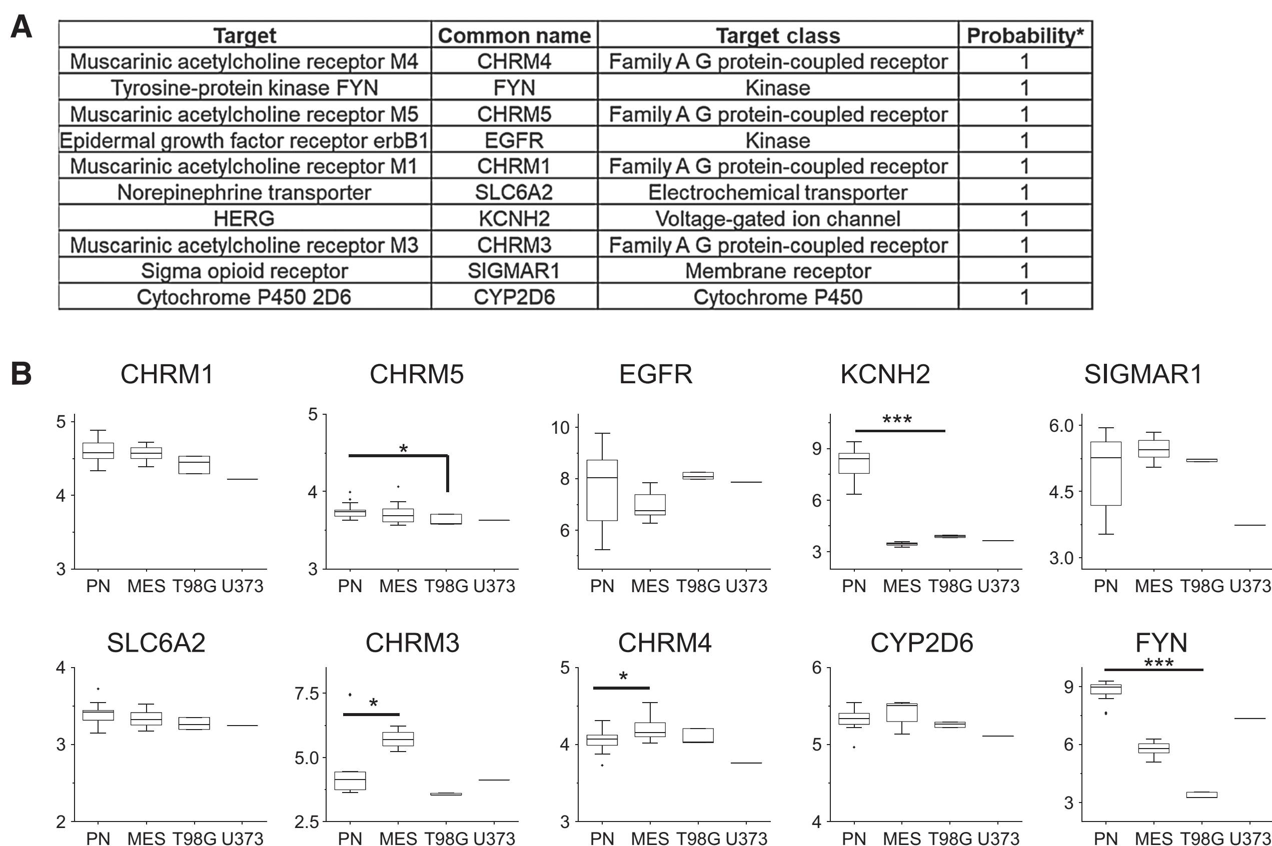

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Target	Common name	Target class	Probability*
Muscarinic acetylcholine receptor M4	CHRM4	Family A G protein-coupled receptor	1
Tyrosine-protein kinase FYN	FYN	Kinase	1
Muscarinic acetylcholine receptor M5	CHRM5	Family A G protein-coupled receptor	1
Epidermal growth factor receptor erbB1	EGFR	Kinase	1
Muscarinic acetylcholine receptor M1	CHRM1	Family A G protein-coupled receptor	1
Norepinephrine transporter	SLC6A2	Electrochemical transporter	1
HERG	KCNH2	Voltage-gated ion channel	1
Muscarinic acetylcholine receptor M3	CHRM3	Family A G protein-coupled receptor	1
Sigma opioid receptor	SIGMAR1	Membrane receptor	1
Cytochrome P450 2D6	CYP2D6	Cytochrome P450	1

Figure 3.

In silico identification of FYN as a probable target of perhexiline. **A,** Probable perhexiline targets as identified by Swiss target prediction webserver are represented in tabulated form with only highest probability (probability 1). **B,** The expression data of cell lines (MES, $n = 12$; PN, $n = 18$; T98G, $n = 3$; U373****, $n = 1$) were downloaded from publicly available GEO transcription database and analyzed and represented as box plot. Statistical significance was calculated with respect to PN cells. *, $P < 0.05$; ***, $P < 0.0005$. ****Although these data have been deposited as the cell line U373, according to ATCC, genomic similarities were observed between U251 and U373, suggesting these are the same cell lines.

glioblastoma emerged as tumors with the highest expression of FYN (**Fig. 4E**). Furthermore, in glioblastoma, a significant increase in FYN expression was observed when compared with normal brain (**Fig. 4F**), further supporting this protein may serve as a molecular target in this malignancy. Although FYN is overexpressed in glioblastoma, considerable heterogeneity was observed. We therefore sought to better understand factors contributing toward this heterogeneity. Continuing to utilize the TCGA database, we observed that FYN is enriched in the proneural subtype (**Fig. 4G**), which is consistent with our cell line data demonstrating these lines to be particularly sensitive to the antitumor activity of this agent (**Fig. 1A**). Furthermore, we sought to determine whether FYN is differentially expressed within an individual tumor using the Ivy Glioblastoma Atlas Project (Ivy GAP) database, which contains region-based expression profiles within an individual tumor (16). Interestingly, overexpression of FYN was observed in the cellular and infiltrative regions of glioblastoma (**Fig. 4H**), suggesting FYN may provide a biologic advantage in this unique microenvironment.

Perhexiline demonstrates antitumor activity in glioblastoma *in vivo*

We went on to evaluate the antitumor activity of perhexiline in glioblastoma *in vivo*. As an initial investigation, we performed pharmacokinetics of perhexiline to determine the penetrance of this agent in the brain. Although not quantitative, clear accumulation of perhexiline was detected using LC/MS in cerebrum and cerebellum of mice following treatment with perhexiline (80 mg/kg via oral gavage × 3 days; Supplementary Fig. S4), suggesting the capacity of this agent to cross the blood–brain barrier. Next, we extended investigations to a mouse flank model, confirming antitumor activity of perhexiline in glioblastoma (**Fig. 5A**). On the basis of these promising findings, we extended studies to an orthotopic, intracranial model. MRI was performed 7 days following treatment, which demonstrated a significant reduction in tumor size when compared with control (**Fig. 5B** and **C**), confirming the antitumor activity perhexiline, which resulted in an improvement in overall survival (**Fig. 5D**).

Discussion

Perhexiline was developed and marketed in the 1960-70s as an antianginal agent. One of the first studies showing antitumor activity of perhexiline was by Ramu and colleagues, which demonstrated the capacity of perhexiline to reverse anthracycline and vinblastine resistance in leukemia cells (12). Initial studies identifying the enzyme CPT-1, which represents a rate-limiting enzyme of FAO, as the primary target of perhexiline were in the mid-1990s. These studies

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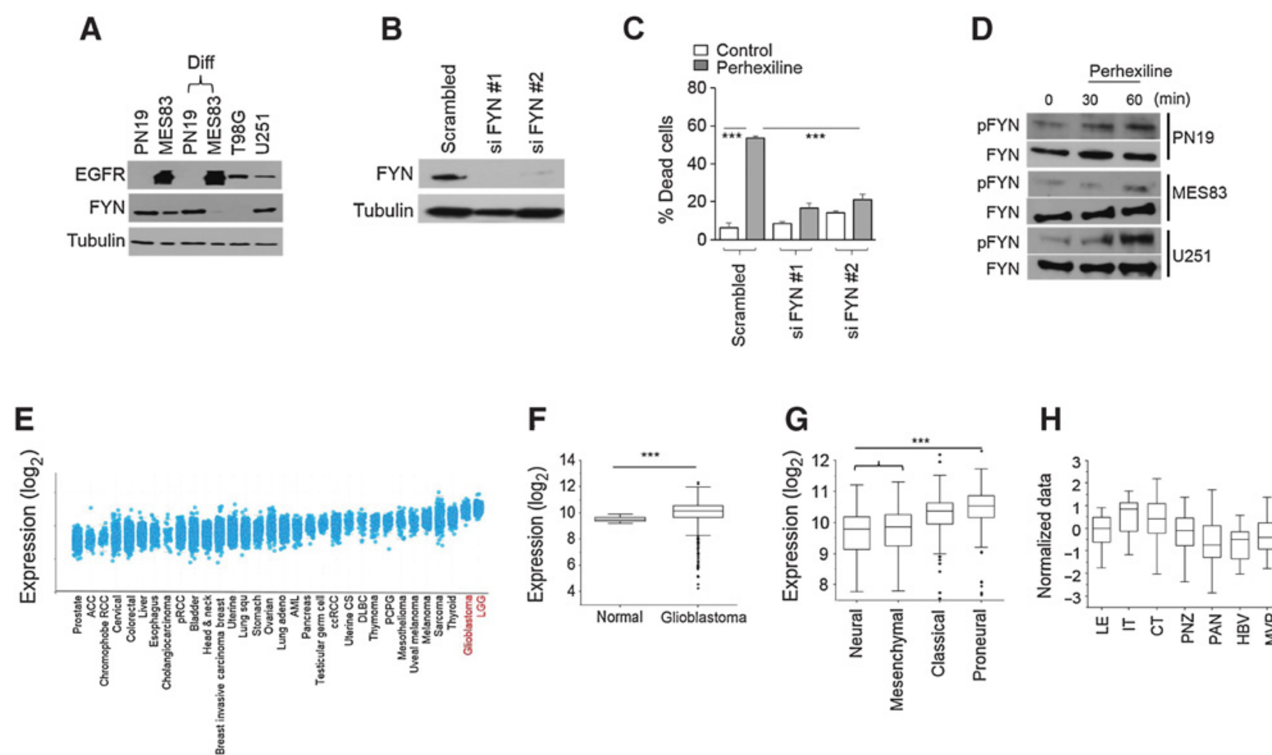

Figure 4.

The antitumor activity of perhexiline is mediated by FYN. **A,** EGFR and FYN expression were determined in the described cell lines by Western blot analysis. **B,** MES83 cells were treated with scrambled and FYN (siFYN) siRNA and knockdown validated with Western blot analysis. **C,** After 48 hours of siRNA treatment, MES83 cells were treated with perhexiline (5 μmol/L; 48 hours), and cytotoxicity was evaluated using Trypan blue dye. Results are average ± SD from at least three independent experiments. ***, $P < 0.0005$. **D,** Undifferentiated PN19, MES83, and standard U251 cells were treated with perhexiline (5 μmol/L; 1.5 μmol/L for PN19) for the indicated time and phosphorylation status of Y530 of FYN was analyzed by Western blot analysis. **E,** FYN expression in various human cancers was sorted on the basis of median value and downloaded using the cBioPortal and TCGA database. **F,** The expression of FYN was analyzed in normal ($n = 10$) and gliobastoma ($n = 529$) tissue using TCGA. **G** and **H,** The relative expression of FYN was evaluated in the context of molecular subtypes of glioblastoma using TCGA (neural, $n = 87$; mesenchymal, $n = 158$; classical, $n = 145$; proneural, $n = 139$) and anatomic structure within an individual tumor using the Ivy-GAP database (***, $P < 0.0005$).

included the potential for perhexiline to reduce FAO in a rat heart model (24) and Kennedy and colleagues went on to describe its activity to be specific to CPT-1 (25). Intriguingly, the connection between perhexiline and FAO was likely further perpetuated by verbiage used in patents filed in the context of drug development efforts in heart failure rather than clear experimental data (8). Binding studies confirming the interaction between perhexiline and CPT-1 were not performed in these early investigations, and further, perhexiline is structurally a very different compound than the well-established CPT-1 inhibitor etomoxir, raising concerns as to its mode of action. Accordingly, a more in-depth evaluation of CPT-1 and modulators of its activity concluded that perhexiline was indeed a very weak CPT-1 inhibitor (26). Despite this, perhexiline has been reflexively deemed and utilized as an established CPT-1/FAO inhibitor in a variety of preclinical investigations over the last several decades, including numerous recent publications (9–11, 13). It would be worthwhile revisiting and directly measuring the ability of perhexiline to inhibit FAO in the various model systems used in these previous investigations, as targets and mechanism(s) may vary considerably based on a given model. On the basis of the specific glioblastoma models we used in these studies, we determined that CPT-1 inhibition is not a primary mode of action of perhexiline. This is supported by our data demonstrating (i) clear differences in antitumor activity when compared with the well-established CPT-1 inhibitor etomoxir, (ii) the inability of perhexiline to inhibit mitochondrial respiration, and (iii) its inability to modulate intracellular lipid dynamics, as observed with etomoxir.

Guided by *in silico* target prediction software, we identified a contributory role of FYN in perhexiline-mediated antitumor activity. FYN is a nonreceptor, membrane-associated Src family tyrosine kinase involved in T-cell and neuronal signaling (23). FYN has been reported to be aberrantly expressed in a wide variety of cancers, contributing to multifaceted signaling that regulates several aspects of tumorigenesis, including the cell cycle, migration, transformation, and cell motility (23, 27, 28). For example, in cholangiocarcinoma, FYN negatively regulates AMPK activity, thereby promoting cell migration and invasion through AMPK/mTOR-mediated signaling (27). In breast cancer, FYN has been demonstrated to play a role in maintaining a mesenchymal phenotype through STAT5/NOTCH2 signaling (28). In addition to cellular signaling, FYN has been shown to play a functional role in modulating redox stress. Specifically, in cardiomyocytes, FYN has been shown to interact with the NAPD oxidase NOX4 and negatively regulate NOX4-induced ROS production and apoptosis (29). In glioblastoma, FYN has been reported to be overexpressed; however, the therapeutic efficacy of targeting FYN in preclinical models has shown mixed results, suggesting the role FYN plays is cell line specific (30, 31). For example, Lu and colleagues showed that FYN

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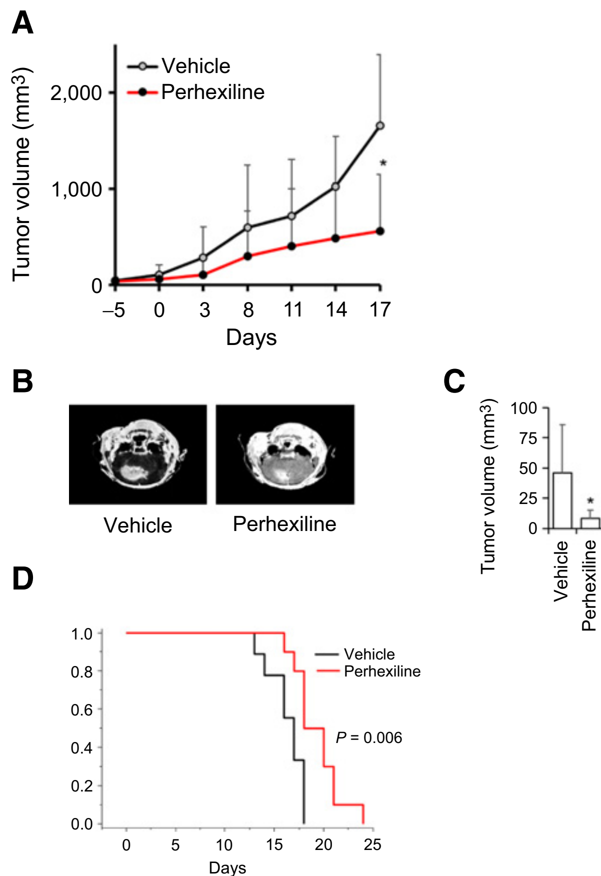

Figure 5.

Perhexiline decreases tumor growth and improves survival in an orthotopic glioblastoma mouse model. **A,** MES83 cells were grown in mice as flank tumors and treated with vehicle ($n = 6$) or perhexiline ($n = 8$; 80 mg/kg via oral gavage, 5 days a week) and tumor growth was measured using a caliper. **B** and **C,** Mice bearing orthotopic MES83 tumors were treated using a similar regimen (vehicle, $n = 9$; perhexiline, $n = 10$) and imaged and tumor volume calculated 7 days after treatment. **D,** Kaplan–Meier survival plot of mice with orthotopic tumors treated with perhexiline (median 19 days) or vehicle control (median 17 days). Data represent mean ± SD (*, $P < 0.05$).

is a mediator of EGFR signaling, playing a role in cell motility (30), while Lewis-Tuffin and colleagues showed that short hairpin RNA–-mediated knockdown of FYN did not affect the growth of glioblastoma tumor xenografts (31).

Our findings suggest that rather than serving as an FAO inhibitor, the antitumor activity of perhexiline is mediated through FYN. For example, cytotoxicity following exposure to perhexiline was directly dependent on baseline FYN expression and rapid inhibition of FYN activity was observed in a panel of perhexiline-treated glioblastoma cell lines. Intriguingly, when cultured in media conditions promoting cellular differentiation, the mesenchymal tumor stem cell line MES83 lost both FYN expression and sensitivity to perhexiline, which we went on to validate using RNAi. Despite clearly linking the antitumor activity of perhexiline with FYN, the specific mechanism by which this leads to cell death remains unclear. For example, simply inhibiting FYN using RNAi in a FYN expressing line did not lead to cytotoxicity. In addition, formal binding studies were not performed in this study to more definitively establish a direct interaction between perhexiline and FYN. Therefore, further work designed to understand how perhexiline interacts with FYN and its subsequent downstream signaling is warranted. However, the biologic consequence of this interaction does appear to involve a rapid induction of redox stress, which is consistent with previous reports (29). Alternate mechanisms contributing toward the antitumor activity of perhexiline have been described previously, including its potential to promote HER3 ablation in breast cancer lines (32). This further supports the complexity of this compound and its capacity to differentially interact with a variety of mediators is dependent on a given cell line.

Perhexiline is also interesting from a drug development perspective. This compound was initially developed in the late 1960s and marketed in the 70s as an antianginal agent. However, a small number of cases of severe hepatotoxicity and neurotoxicity led to a rapid decline in its use. Interestingly, some of the adverse effects of this compound related to the central nervous system, including cerebellar dysfunction, were presumed because this compound crossed the blood–brain barrier, which is consistent with our findings. Many countries removed this compound from the market; however, perhexiline use was continued in Australia and New Zealand based on its efficacy. During this time, it was discovered that this compound is metabolized by CYP2D6, and approximately 10% of the population have mutations in this gene (7). These mutations lead to poor metabolism and high plasma concentrations of perhexiline and subsequent toxicity. Identification of these poor metabolizers and dose modifications through plasma monitoring has significantly diminished any significant side effects of this agent and has led to the redevelopment of this compound clinically (31). In the United States, perhexiline has been actively examined in a phase 3 clinical trial for the treatment of hypertrophic cardiomyopathy and moderate to severe heart failure (NCT02431221). Collectively, this long clinical track record supports the possibility of repurposing perhexiline as a cancer therapy, and with its potential to cross the blood–brain barrier, a promising agent to be tested in glioblastoma.

We and others (31) have demonstrated aberrant expression of FYN in glioblastoma, suggesting FYN expression may be used as a predictive marker to identify patients with tumors that would likely be sensitive to the antitumor activity of perhexiline. Although we have shown clear antitumor activity of perhexiline in glioblastoma in both a flank and intracranial mouse models, durable, long-term control of disease was not observed in any of the mice, limiting it likelihood of clinical activity when used as a single agent. We hypothesize that the disconnect between the potent cytotoxicity observed *in vitro* and modest activity *in vivo* is likely attributed to intratumoral heterogeneity, which represents an archetypal phenotype of this malignancy (15). Specifically, at least in our mesenchymal glioblastoma cell model, we observed that sensitivity to perhexiline is lost upon tumor stem cell differentiation. As recent work in glioblastoma, along with several other malignancies, suggest that undifferentiated tumor stem cells represent a small population of cells within a tumor that contribute to therapeutic resistance (14, 33, 34), rational combinatorial strategies designed to target these different cell populations within a tumor by combining perhexiline with established therapies in glioblastoma, including radiation and/or the chemotherapeutic temozolomide, may serve as a promising approach to extend these findings clinically.

In summary, we identified potent FYN-dependent antitumor activity of perhexiline in glioblastoma. As this agent has a long, clinical track record and potential to cross the blood–brain barrier, it represents a promising agent to be repurposed for the treatment of glioblastoma. Further investigations are warranted to determine the potential of perhexiline to enhance radiation and/or temozolomide response, with

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promising findings providing the framework for early phase clinical trials in this otherwise fatal malignancy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S. Kant, P. Chinnaiyan
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Kant, P. Kesarwani, A.R. Guastella, P. Kumar, S.F. Graham, K.L. Buelow, P. Chinnaiyan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Kant, P. Kesarwani, A.R. Guastella, P. Chinnaiyan
Writing, review, and/or revision of the manuscript: S. Kant, P. Kesarwani, P. Chinnaiyan
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Kant, P. Kesarwani, K.L. Buelow, I. Nakano, P. Chinnaiyan
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