Introduction

Diffuse intrinsic pontine glioma (DIPG) is a highly aggressive pediatric brain tumor carrying a dismal prognosis, which remained unchanged over the past decades despite major advances in the understanding of the molecular biology of this disease over the past years (1). As in adult high-grade glioma (HGG) and many other cancers, DIPG cells carry mutations in DNA damage repair genes and genes involved in signal transduction pathways, as well as amplifications in receptor tyrosine kinases (RTK) such as EGFR and PDGFRα (2–4). Recent international collaborations have uncovered several unique oncogenic mutations in this disease in the genes encoding Histone 3 (5, 6) and ACVR1 (7–9). Consequently, the new World Health Organization classification of brain tumors reclassifies DIPG and related tumors as diffuse midline gliomas carrying histone H3 mutations (10). Current efforts to develop a therapy for this devastating disease focus mainly on reversing the malignant phenotype and altered epigenetic landscape caused by these mutations (11, 12).

Concurrent with the elucidation of the genetic background of diffuse midline gliomas, the past years have seen the emergence of the first relevant in vitro and in vivo models of these tumors, leading to the first attempts to identify suitable agents for the treatment of this disease (11–15). To this day, only the HDAC inhibitor panobinostat and the IMiDS inhibitor GSKJ4 have shown single-agent therapeutic efficacy in a preclinical setting (11, 12). As such, there is a dire need for the identification of therapeutic targets and the development of new therapeutics for the treatment of DIPG.

Using publicly available datasets, we previously identified WE11 as an interesting target for DIPG treatment (13). Another highly upregulated serine/threonine kinase that emerged from this in silico analysis is maternal embryonic leucine zipper kinase (MELK). As opposed to other members of the SNF1/AMPK family of kinases, MELK can be activated by autophosphorylation.
Translational Relevance
Diffuse intrinsic pontine glioma (DIPG) is a highly aggressive pediatric brain tumor, for which no effective curative treatments are available. Consequently, there is a dire need to develop new therapeutic strategies to treat this devastating disease. This study identifies the maternal embryonic leucine zipper kinase (MELK) as a novel therapeutic target for DIPG. We show strong antitumor efficacy of the small-molecule kinase inhibitor OTSSP167—the only inhibitor targeting MELK that is currently in clinical trials—in patient-derived in vitro and in vivo models of DIPG. We further identify the glioma-associated transcription factor PPARY as a downstream effector of MELK in DIPG that can potentially be exploited for future therapeutic use. Altogether, our study demonstrates that MELK is a therapeutic target in DIPG, although other kinases may contribute to the strong efficacy of OTSSP167 in preclinical DIPG models.

With the exception of VUMC-DIPG-A, all primary DIPG cultures were maintained as neurospheres in serum-free medium. Neurospheres were cultured at 37°C and 5% CO₂ in modified Tumor Stem Medium (TSM), consisting of 48% DMEM/F12, 48% Neurobasal-A medium, 1% hEPEFS 1M, 1% MEM Non-essential amino acids, 1% 100 mmol/L sodium pyruvate, 1% GlutaMAX, supplemented with 2% B27, 1% N2 (all from Thermo Fisher Scientific), 20 ng/mL human EGF, 20 ng/mL human bFGF, 10 ng/mL human PDGF-AA, 10 ng/mL human PDGF-BB (all from Peprotech), and 5 IU/mL heparin (VU University Medical Center Hospital Pharmacy). Adherent monolayers of DIPG cells were cultured in TSM supplemented with 10% heat-inactivated FBS (Thermo Fisher Scientific). HeLa cells (ATCC) and primary astrocytes (Department of Pathology, VU University Medical Center) were cultured at 37°C and 5% CO₂ in high glucose DMEM (Sigma Aldrich), supplemented with 10% FBS.

For in vitro pharmacokinetic studies, parental LLC-PK1 cells, subclones overexpressing human MDR1 (LLC-MDR1) or murine Madr1 (LLC-Madr1a), parental MDCX cells, and sublines overexpressing human BCRP (MDCX-BCRP) or murine Bcrp1 (MDCX-Bcrp1) were generated in the Netherlands Cancer Institute by Dr. A.H. Schinkel and cultured as previously described.

All cell lines were routinely subjected to mycoplasma testing and only used for experiments when confirmed negative. In addition, routine short tandem repeat analysis was performed using the Geneprint 10 system (Promega) to ensure cell line identity. Tests were performed before all major experiments and every 3 months during maintenance of cell cultures.

Drugs and solutions
OTSSP167 hydrochloride was purchased from Medchem Express, pioglitazone and troglitazone were from Caymanchem, and raloxifene hydrochloride was supplied by SPECS. For in vitro studies, drugs were dissolved in DMSO at >1,000x the maximum used concentration. For in vivo experiments, OTSSP167 was first dissolved in DMSO, heated and sonicated, and then mixed 1:1 with Cremophor EL (Sigma Aldrich). Directly before administration, this solution was diluted 1:4 in ddH₂O.

Lentiviral transductions
VUmc-DIPG-A was transduced with hTERT using the pLentiv6-V5-hTERT-Blast plasmid, which was kindly provided by Professor Böcker, Klinikum der Universität München. JHH-DIPG01 cells were lentivirally transduced using the pHIV-Luc-ZsGreen (Addgene #39196) and pLKO1-shMELK plasmids; shRNA sequences can be found in Supplementary Table S1.

Methods and Materials
Cell lines and culture conditions
VUMC-DIPG-A (15), VUMC-DIPG-F, VUMC-DIPG-08, VUMC-DIPG-10, and VUMC-DIPG-11 were established at the VU University Medical Center. VUMC-DIPG-A and -F were established from pretreatment biopsy samples of DIPG patients, whereas VUMC-DIPG-08, -10, and -11 were established from (posttreatment) autopsy specimens. HSJD-DIPG-07, HSJD-DIPG-08, and HSJD-DIPG-12 cell lines were kindly provided by Dr. Montero Carcaboso (Hospital Sant Joan de Déu Barcelona, Spain). The JHH-DIPG-01 cell line (14) was a kind gift from Dr. Raabe (Johns Hopkins Hospital, Baltimore). The SF761 cell line (35) was generously provided by Dr. Hashizume (Northwestern University, Chicago, IL). All cell lines carry the characteristic mutation in H3F3A resulting in a K27M substitution in the Histone 3.3 protein. The only exception is VUMC-DIPG-10, which possesses no mutations in genes encoding Histone 3.

Cell viability assays
For neurosphere viability assays, cells were plated at a density of 3,000 cells/well in F-bottom 96-well plates with cell-repellent surface (Greiner Bio-one, #650971). For adherent monolayers, cells were seeded at 1,500 cells/well in regular F-bottom 96-well plates. After 24 hours, drugs were dispensed with a Tecan D300e Digital Dispenser at different concentrations and incubated at 37°C and 5% CO₂ for 96 hours. CellTiter-Glo 3D Luminescent Cell Viability Assay (Promega) was used as a method to determine the number of viable cells in culture following the manufacturer’s protocol. Luminescence was measured using a Tecan Infinite 200 reader using iControl 1.10 software.
RNA sequencing

Neuroospheres from the JHH-DIPG-01, HSJD-DIPG-07, HSJD-DIPG-12, and SF7761 cell lines were treated with 20 nmol/L OTSSP167 for 24 hours prior to RNA extraction.

RNA was isolated using the mirVana miRNA isolation Kit without phenol (Ambion/Life Technologies), supplemented with Acid-Phenol:Chloroform, pH 4.5 (with IAA, 125:24:1). RNA quality was analyzed on an Agilent 2100 Bioanalyzer using the Agilent RNA 6000 Nano Kit according to the manufacturer’s instructions. The NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs) was used to process the samples according to the manufacturer’s instructions. The quality and yield after sample preparation were measured using the Fragment Analyzer. The size of the resulting fragments was measured using the Fragment Analyzer. The quality and yield after sample preparation were measured using the Fragment Analyzer.

Parametric Analysis of Gene Expression (PAGE), the KEGG geneset collection was used. Geneset maps of PPAR target genes were generated using Ingenuity Pathway Analysis (IPA; Qiagen Inc.; ref. 37).

Western blot analysis

Cells were harvested under optimal growth conditions for all Western blots, or after 48-hour exposure to OTSSP167 when stated. Cells were immediately lysed with lysis buffer (0.1% Triton X-100, 20 mmol/L Tris HCl, pH 7.6, 100 mmol/L NaCl, 50 mmol/L β-glycerophosphate, 1 mmol/L DTT, 1x Complete Mini EDTA-Free Protease inhibitor Cocktail (Roche), and 1 mmol/L Na3VO4 for 30 minutes in agitation at 4°C. Protein concentration was measured using BioRad protein assay in Biochrom Genecuant Pro UV/Vis Spectrophotometer (Amersham Biosciences).

Note that 30 μg of total protein per sample was separated using 10% SDS-PAGE gel and transferred onto a miniFormat 0.2 μmol/L nitrocellulose membrane using a TrAns-Blot Turbo transfer system (all from Biorad). Membranes were blocked in 10 ml. blocking buffer for fluorescent Western blotting (Rockland) for 2 hours and incubated overnight at 4°C with either 1:500 anti-MELK polyclonal antibody (Cell Signaling Technology; #2274), 1:500 anti-(phospho Ser112) PPARy antibody (Abcam; ab195925), or 1:500 PPARα antibody (Abcam; ab209350). In addition, membranes were incubated with 1:5,000 anti-Actin mouse monoclonal antibody (Millipore) as a loading control. The next day, membranes were washed and incubated with 1:20,000 secondary goat anti-rabbit antibody IRDye 800CW (LI-COR) and/or 1:10,000 secondary goat anti-mouse antibody IRDye 680CW (LI-COR). Protein detection was performed using a LI-COR Odyssey fluorescent imager (Model 9120; Surplus Solutions, LLC). Quantification of bands was performed using ImageJ software.

Immunofluorescence

HeLa cells were seeded onto coverslips at a density of 1 × 10^5 cells and treated with 0 or 20 nmol/L OTSSP167 for 24 hours. After treatment, cells were fixed with 4% paraformaldehyde for 15 minutes and permeabilized using 0.25% Triton-X100 for 10 minutes at room temperature. Nonspecific binding was blocked by incubating with blocking buffer (PBS 1X, 0.1% TritonX-100, 0.05% Azide, and 10% FBS) for 30 minutes at room temperature. Primary antibody incubation was performed overnight at 4°C using a 1:200 anti-PPARy antibody (Abcam; ab209350) diluted in PBS 1X BSA (PBS 1X, 0.1% TX-100, 0.05% Azide, and 0.5% BSA). Then, cells were washed with PBS 1X BSA and incubated with secondary Anti-Rabbit IgG H&L (Alexa Fluor 488) antibody (Abcam; ab150077) for 2 hours at room temperature. After an additional washing step, samples were incubated with Phalloidin-iFluor 594 Conjugate (AAT Bioquest) following the manufacturer’s protocol in PBS-1% BSA for 30 minutes and counterstained with DAPI diluted 1:50,000 in PBS for 5 minutes. Finally, cells were mounted on slides using VectaShield mounting medium for imaging under the Leica-DM6000 microscope using the Leica DM Control Software.

Animals

All animal experiments were approved by local animal experimental committees and carried out according to national and institutional guidelines. Animals were provided food and water ad libitum for the entire duration of the experiments.

In vitro pharmacokinetic studies

Two types of transport assays were carried out. As described before, monolayer permeability of OTSSP167 was assessed using conventional transport assays, whereas Concentration Equilibrium Transport Assays (CETA) were carried out to analyze transporter affinity (36). All transport assays were conducted using 100 nmol/L OTSSP167; 5 μmol/L L-800 (MDR1 inhibitor) or elacridar (dual MDR1/BCRP inhibitor) was used to block transport. MDCK or LLC cells were seeded onto Transwell microporous polycarbonate membrane filters (3.0-μm pore size; Costar Corning) and allowed to grow into a monolayer. The experiment was started by replacing medium in each compartment by medium containing OTSSP167 or blank medium, and samples were taken at specific time points. Monolayer integrity was assessed by translocation of Carboxyl-[14C]-inulin. Wells demonstrating leakiness exceeding 1.5% per hour were excluded from LC-MS/MS analysis.

In vivo pharmacokinetic studies

Wild-type (WT), Bcrp1−/− (Abcg2−/−), Mdr1a/b−/− (Abcb1a/b−/−), and Bcrp1:Mdr1a/b−/− (Abcg2−/−; Abcb1a/b−/−) FVB mice were used for brain penetration analysis. OTSSP167 was administered i.v. at a dose of 5 mg/kg in a formulation consisting of DMSO:Cremophor EL:saline containing 0.001 mol/ L HCl (1:1:8). Blood was collected 1 hour after administration by cardiac puncture under isoflurane anesthesia, and brains were subsequently collected. Plasma was obtained by centrifugation (5 minutes, 5,000 rpm, 4°C). Tissues were homogenized after weighing using a FastPrep-24 (MP- Biomedicals) in 1% (w/v) BSA in water.

LC-MS/MS analysis

In vitro medium samples (1+3) and plasma and tissue homogenates (1+6) were mixed with acetonitrile: formic acid...
Statistical analysis

In vitro cell survival percentages and combination indices were statistically compared using the independent t-test. Drug synergy was calculated using the combination index formula as described by Chou and Talalay (38).

For in vivo pharmacokinetic experiments, one-way ANOVA with post hoc Bonferroni correction was performed. Statistical analysis of the CETAs was done using a general linear model for repeated measurements. Multivariate significance tests were performed to determine whether the apical–basal concentration differences were significantly increased over time. Biomimetic data and growth curves were analyzed longitudinally by multilevel regression analysis. Kaplan–Meier curves were generated, and significance of survival differences was determined using the log-rank (Mantel–Cox) test.

The statistical analyses were performed with Microsoft Excel, Graphpad Prism (version 6), and SPSS (version 22), and a P value < 0.05 was considered statistically significant.

Results

Primary DIPG cultures with high MELK expression are sensitive to OTSSP167

Analysis of publicly available datasets of mRNA expression profiles using the online bioinformatics tool R2 (http://r2.amc.nl) revealed overexpression of MELK mRNA in DIPG patient samples (2), compared with normal brain and cerebellar tissue, making it an attractive therapeutic target (Fig. 1A). This low expression of MELK in normal central nervous system (CNS) tissue was confirmed by data from the Brainspan developmental transcriptome dataset (http://www.brainspan.org) that showed high expression of MELK mRNA in all CNS tissues during the first trimester of embryonic development, with expression levels rapidly declining during later stages of fetal development and reaching background levels around birth (Supplementary Fig. S1).

Next, we confirmed the expression of MELK protein in nine primary DIPG cultures by Western blot (Fig. 1B). Based on these results, we exposed nine DIPG cultures to 5, 10, or 20 nmol/L OTSSP167 for 96 hours to determine the antiproliferative effect of MELK inhibition in these cells (Fig. 1C). Relative cell numbers after 96-hour exposure to 20 nmol/L OTSSP167 were reduced by more than 70% in seven of nine cell lines, compared with vehicle-treated control cells. Primary astrocytes, which were used as a normal control, were less sensitive to OTSSP167 than these seven DIPG cell lines, with a relative cell number of nearly 50% at 20 nmol/L compared with untreated cells. The two cell lines with the lowest baseline expression of MELK, HSID-DIPG-07, and VUMC-DIPG-A were relatively resistant to OTSSP167 with IC50 well above 20 nmol/L. To determine if the efficacy of OTSSP167 was related to MELK expression, we downregulated MELK expression by use of RNA interference, shRNA-mediated MELK knockdown by two independent constructs in JHH-DIPG-01 cells showed a strong decrease in proliferation (Fig. 1D) and a higher sensitivity to treatment with OTSSP167 than their parental cells (Supplementary Fig. S2).

MELK inhibition causes an upregulation of PPARγ target genes in DIPG cells

To elucidate the molecular mechanisms by which MELK inhibition exerts its effects on DIPG cells, we performed RNA sequencing on four primary DIPG cultures treated with 20 nmol/L OTSSP167 or vehicle for 24 hours. We performed a PAGE on these samples using R2. This revealed a significant upregulation of the PPAR signaling pathway geneset in two of the four cell lines after treatment (FDR P < 0.05, Fig. 2A). As the PPAR family of proteins functions as transcription factors, we then determined the effect of MELK inhibition on the expression of PPAR target genes. Therefore, we performed an analysis of upstream regulators of differentially expressed genes using IPA, which revealed a significant differential regulation of PPARγ target genes in all four DIPG cultures treated with OTSSP167 (P < 0.001; Fig. 2B, Supplementary Fig. S3). To determine if treatment with OTSSP167 resulted in an upregulation of PPARγ target genes, we then generated a heatmap of known PPARγ target genes from the Broad Institute's geneset collection. Ordering the PPARγ target genes expressed in our dataset according to Spearman rank correlation coefficient revealed an overall upregulation of PPARγ target genes (Fig. 2C). Given these results, and as within the PPAR family of transcription factors PPARγ has been implicated most frequently in cancer, and in glioblastoma in particular (39–41), we focused on the connection between MELK and PPARγ activity.

MELK inhibition decreases inhibitory phosphorylation of PPARγ at Ser112 in DIPG cells

PPARγ undergoes multiple posttranslational modifications that alter its activity, either by changing the cellular localization or stability of the protein or by altering the recruitment of...
cofactors to the transcription factor complex (41–43). Most notably, the transcriptional activity of PPARγ is inhibited by phosphorylation on Ser112 (41–43). Western blot analysis of DIPG cells treated with OTSSP167 for 48 hours revealed a concentration-dependent decrease in protein levels of MELK and in phosphorylation of PPARγ at Ser112, whereas total levels of PPARγ were less affected by the treatment (Fig. 3A). To eliminate the possibility that this effect was due to an off-target effect of OTSSP167, we knocked down MELK by two different shRNA constructs in JHH-DIPG-01 cells and confirmed decreased MELK expression and phosphorylation of PPARγ at Ser112 in these cells (Fig. 3B).

Inhibition of MELK by OTSSP167 induces nuclear translocation of PPARγ

Studies investigating the interaction of other kinases, such as MEK and ERK, with PPARγ have revealed that besides altering phosphorylation of Ser112, these kinases can also alter the intracellular localization of PPARγ via different mechanisms (44, 45). Therefore, to further elucidate the mechanism by which MELK inhibition increases transcription of PPARγ target genes, we investigated the influence of treatment of cells with OTSSP167 on the intracellular localization of PPARγ. For this purpose, HeLa cells, which possess a relatively high expression of MELK, were exposed for 24 hours to 20 nmol/L OTSSP167 before fixation. Immunofluorescent staining of these cells for PPARγ revealed an increased nuclear localization of PPARγ in OTSSP167-treated cells compared with controls (Fig. 3C). This observation indicates that the mechanism by which MELK inhibition increases PPARγ transcriptional activity not only includes altered phosphorylation on Ser112, but changes in intracellular localization of PPARγ as well.

Treatment of DIPG cells with PPARγ agonists potentiates the effect of OTSSP167

Based on the demonstrated relation between MELK and PPARγ activity, we set out to determine if this interaction could be used to enhance the efficacy of OTSSP167 treatment of DIPG cells. Therefore, we treated DIPG cells with combinations of OTSSP167 and low levels of the PPARγ agonists pioglitazone and troglitazone to determine their impact on survival of DIPG cells in vitro. Although the PPARγ agonists themselves had no

Figure 1.
MELK is a therapeutic target in DIPG. A, mRNA expression levels of MELK in normal brain, cerebellum, and biopsy samples of DIPG patients (GSE 13564, 3526, and 26576). Image was generated using R2 (http://r2.amc.nl). B, Western blot showing expression levels of MELK protein in a panel of primary DIPG cultures. Actin was used as a loading control. Red arrow indicates band corresponding to 74 kDa full-length MELK protein. C, Relative cell numbers of primary DIPG cultures after 96-hour exposure to varying concentrations of the MELK inhibitor OTSSP167. Error bars represent ±SEM (n = 5). **, P < 0.01; ***, P < 0.001 (independent t test compared with primary astrocytes). D, Growth curves of JHH-DIPG-01 neurospheres after shRNA-mediated knockdown of MELK by two different constructs. Error bars represent ±SEM (n = 12). ***, P < 0.001 (multilevel regression analysis).
impact on survival or proliferation of DIPG cells (Fig. 4A), both agents did significantly enhance the efficacy of OTSSP167 in three of four primary DIPG cultures tested. Figure 4B shows a heatmap of combination indices resulting from combined treatment of these cultures with varying concentrations of OTSSP167, pioglitazone, and troglitazone. Using this heatmap, we determined the optimal concentrations, on average for all four DIPG cultures tested, for synergy with OTSSP167 to be 200 nmol/L pioglitazone and 5 μmol/L troglitazone. Treating JHH-DIPG-01 and HSJD-DIPG-12 cells with these concentrations of the PPARγ agonists and IC50 concentrations of OTSSP167 resulted in a significant decrease in cell survival compared with treatment with OTSSP167 alone (Fig. 4C). These results indicate that the increased activity of PPARγ upon treatment with OTSSP167 is at least partially responsible for its therapeutic effect on DIPG cells.

Brain distribution of OTSSP167 is limited by the activity of multidrug transporter proteins

As the blood–brain barrier (BBB) is commonly seen as one of the main reasons for treatment failure in brain tumors (46), we determined the BBB permeability of OTSSP167. For this, we used previously described CETAs, in which the apical-to-basal and basal-to-apical transport across a monolayer of BCRP/Bcrp1- and MDR1/Mdr1a-expressing cells (LLC or MDCK) is measured. Assays were performed in the presence of zosuquidar (MDR1 inhibitor) and/or elacridar (MDR1/BCRP inhibitor), to demonstrate the specificity of the observed influence of...
overexpression of the respective multidrug transporters (36). These assays revealed that OTSSP167 is a substrate for both MDR1 and BCRP, predicting limited brain bioavailability (Fig. 5A). To determine the effect of these transporters on the BBB penetration of OTSSP167 in vivo, we measured the brain penetration of OTSSP167 in different knockout mouse strains lacking important multidrug transporters on the BBB using mass spectrometry (36). Therefore, we administered 10 mg/kg OTSSP167 intravenously to WT, Mdr1a/b−/−, Bcrp1−/−, and Mdr1a/b−/−;Bcrp1−/− mice. After 1 hour, mice were sacrificed, and blood and brain tissue was collected. Mass spectrometry analysis of brain homogenate and plasma revealed a brain:plasma ratio of <0.02 in WT and Bcrp1−/− mice, corresponding to background levels, due to residual blood in the brain homogenate. The brain–plasma ratio was slightly elevated in Mdr1a/b−/− mice (0.033; P < 0.05). Relevant brain concentration of OTSSP167 was seen only in the group of mice with a Mdr1a/b−/−;Bcrp1−/− double knockout, reaching brain concentrations of roughly 1,300 pmol/g with a blood:plasma ratio of 0.078 (P < 0.0001), indicating that brain bioavailability of OTSSP167 is restricted by the activity of both MDR1 and BCRP (Fig. 5B).

OTSSP167 inhibits growth of primary DIPG xenografts in Mdr1a/b−/−;Bcrp1−/− mice

Based on our in vitro results, we investigated the potential of MELK inhibition for the treatment of DIPG in relevant in vivo models. As OTSSP167 did not penetrate the BBB in WT mice, we equipped Mdr1a/b−/−;Bcrp1−/− athymic mice with orthotopic xenografts by injecting patient-derived DIPG cells in the pontine region of the brain. For these experiments, we used the previously described JHH-DIPG-01 xenograft model (14), transduced to express luciferase to enable BLI, and the newly developed, pretreatment biopsy-derived VUMC-DIPG-F xenograft model. MELK expression was confirmed in VUMC-DIPG-F cells by Western blot, albeit at lower levels than JHH-DIPG-01 cells (Supplementary Fig. S4). BBB integrity of these xenograft models was demonstrated by the lack of contrast enhancement on T1 MRI after establishment of brainstem tumors (Fig. 6A). Two or 4 weeks after injection of VUMC-DIPG-F and
Figure 4.
Synergistic effect of combined treatment of primary DIPG cultures with OTSSP167 and PPARγ agonists. A, Dose-response curves of JHH-DIPG-01, HSJD-DIPG-12, SF7761, and HSJD-DIPG-07 cultures treated with the PPARγ agonists pioglitazone and troglitazone. Results are shown as fitted mean ± SEM (n = 4). B, Heatmap of combination indices (SI) of combined treatment with OTSSP167 and different concentrations of the PPARγ agonists pioglitazone and troglitazone. Marked in red are the concentrations of the PPARγ agonists that produce the strongest synergy on average across the four cell lines. C and D, Cell viability assays of JHH-DIPG-01 (C) and HSJD-DIPG-12 (D) cultures treated with OTSSP167 and either pioglitazone or troglitazone at concentrations producing optimal synergy. Data are shown as relative survival ± SEM (n = 4); *, P < 0.05 and **, P < 0.01 (independent t test).
JHH-DIPG-01-Fluc tumor cells, respectively, oral treatment with 5 mg/kg OTSSP167 daily was initiated and continued for 6 weeks. In the JHH-DIPG-01-Fluc xenograft–bearing mice, weekly BLI showed significant inhibition of tumor growth with four of nine mice achieving stable disease or even remission after 2 weeks of treatment. Two of these mice displayed long-term stable disease (>100 days) despite cessation of treatment after 6 weeks (Fig. 6B). Moreover, survival of mice bearing VUMC-DIPG-F xenograft tumors was significantly prolonged by treatment with OTSSP167 [median survival, 228 vs. 166 days, \( P < 0.01 \) (log-rank test), Fig. 6C], confirming the therapeutic potential of MELK inhibition for the treatment of DIPG.

Figure 5.
BBB penetration of OTSSP167 is limited by multidrug transporters. A, In vitro CETAs using monolayers of MDCK and LLC cells overexpressing MDR1 or BCRP or their murine equivalents Mdr1a or Bcrp1. Results show OTSSP167 as a substrate for MDR1, BCRP, and their murine equivalents Mdr1a and Bcrp1. The MDR1 inhibitor zosuquidar and dual MDR1/BCRP inhibitor elacridar were used to demonstrate specificity of multidrug transporters for OTSSP167. \( * \), \( P < 0.05 \); \( ** \), \( P < 0.01 \); \( *** \), \( P < 0.001 \); \( **** \), \( P < 0.0001 \) (multivariate GLM for repeated measures). B, Brain bioavailability of OTSSP167 in WT, Mdr1a/b\(^{-/-} \), Bcrl/b\(^{-/-} \), and Mdr1a/b\(^{-/-}\)/Bcrl/b\(^{-/-} \) mice, measured by LC-MS/MS 1 hour after intravenous administration of 10 mg/kg OTSSP167. \( * \), \( P < 0.05 \); \( ** \), \( P < 0.01 \); \( *** \), \( P < 0.001 \); and \( **** \), \( P < 0.0001 \) (one-way ANOVA).
Discussion

The present study identifies MELK as a highly expressed kinase and putative therapeutic target in DIPG that can be targeted effectively by the kinase inhibitor OTSSP167, which we show to be selectively toxic to malignant cells of the CNS. Furthermore, the selective expression of MELK in early embryonal CNS structures provides clues to the developmental origin of DIPG, which has previously been described (47, 48). We demonstrate high levels of MELK protein in our primary DIPG cultures and xenografts, which is in line with the high levels of MELK mRNA found in gene expression profiles of patient samples (2). This result validates our DIPG cultures and xenografts as a model to study the function of MELK and the therapeutic potential of MELK inhibition for DIPG. Therefore, we selected OTSSP167, the only MELK inhibitor that is currently in clinical trials (http://www.clinicaltrials.gov NCT02795520 and NCT02926690).

We show that OTSSP167 has strong antiproliferative effects on DIPG cell lines at low nanomolar concentrations, whereas normal primary astrocytes were less susceptible, demonstrating a selectivity of this treatment toward malignant cells.

The sensitivity of cell lines to OTSSP167 seems to correlate to the baseline expression level of MELK protein, with the two relatively resistant cell lines having the lowest expression levels. A notable exception was HSJD-DIPG-08, being highly sensitive to OTSSP167 despite relatively low baseline levels of MELK. No conclusions can be drawn regarding a potential relationship between Histone 3 mutations and MELK expression, as the publicly available gene expression dataset on DIPG does not contain any information regarding Histone 3 mutational status. However, VUMC-DIPG-10, the only primary DIPG culture tested that does not possess the typical H3.3 K27M mutation, was intermediate both in expression of MELK protein and OTSSP167.

Figure 6.

Therapeutic efficacy of OTSSP167 in two primary patient-derived DIPG xenograft models. A, MRI of the pontine region of the brain of mice bearing JHH-DIPG-01 and VUMC-DIPG-F xenografts at 63 and 155 days, respectively, after injection of tumor cells. The lack of gadolinium contrast enhancement (right plots) indicates maintenance of an intact BBB in these tumors. B, BLI of JHH-DIPG-01-Fluc xenograft-bearing mice treated for 6 weeks with 5 mg/kg/d OTSSP167 reveals significantly decreased average tumor growth in treated mice (left plot), with partial remissions seen in four of nine mice after 15 days of treatment (right plot, red arrow). Red areas indicate treatment period. **, P < 0.05 (multilevel regression analysis). C, Survival analysis of VUMC-DIPG-F xenograft-bearing mice treated for 6 weeks with 5 mg/kg/d OTSSP167 showing significantly increased survival of the treated mice. Red area indicates treatment period. **, P < 0.01 (log-rank test).
efficacy, suggesting that the effects of MELK inhibition are independent of H3 mutational status.

The molecular mechanisms by which MELK exerts its oncogenic functions have not yet been elucidated completely, but multiple downstream targets seem to play an important role. First of all, MELK seems to control the activity of the transcription factors FOXM1 (21, 29, 30), ZPR9, and NIP1 (16), thereby leading to increased transcription of oncogenes such as CTNNB1, VEGF, MMP2, and EZH2 (30). In addition, MELK has been shown to be involved in DNA damage repair and radioresistance via its effects on p53 and ATM activity and transcription of double-strand break repair genes (21, 28, 30). Thirdly, MELK is involved in the regulation of the cell cycle, among others via phosphorylation of CDC25B (23) and altered transcription of Aurora and polo-like kinases (21, 26). Finally, MELK has been shown to control the expression and activity of central antiapoptotic genes such as MCL1 and BCL-G (19, 25).

Using next-generation sequencing techniques, we further showed that treatment with OTSSP167 increases the transcriptional activity of PPARY, an effect that can be explained both by the decreased phosphorylation of PPARY on Ser112 and its nuclear translocation after MELK inhibition. The observed effects of MELK inhibition on PPARY phosphorylation were confirmed by shRNA-mediated knockdown of MELK in DIPG cells.

Phosphorylation of PPARY at Ser112 by different mechanisms has previously been associated with a decrease in its transcriptional activity (41–43). In addition, it has previously been shown that other kinases from the SNF1/AMPK family are capable of phosphorylating PPARY at this site (42). Furthermore, interactions of different kinases with PPARY are known to induce cytoplasmic localization of PPARY, with their inhibition leading to increased nuclear localization via mechanisms independent of Ser112 phosphorylation (44, 45). We therefore propose a mechanism in which MELK inhibits the transcriptional activity of PPARY in DIPG cells by causing an increase in phosphorylation of PPARY at Ser112 and decreasing its nuclear localization, either directly or indirectly.

The PPARY family of transcription factors and, most notably, PPARY has been implicated in the development and progression of several cancers, including adult HGG (49). Polymorphisms in the PPARG gene have been associated with a predisposition to develop GBM. A retrospective analysis revealed both a reduced incidence of HGG and a trend toward improved survival in adult HGG patients receiving PPARY agonists for the treatment of diabetes (49). Based on this observation and the availability of BBB-penetrable (50), clinically approved PPARY agonists such as pioglitazone and troglitazone, we investigated the effects of combined treatment with OTSSP167 and these PPARY agonists on DIPG cells in vitro. The combined treatment with OTSSP167 and either pioglitazone or troglitazone resulted in a synergistic increase in the antiproliferative effect of the MELK inhibitor, suggesting that the increase in PPARY activity after MELK inhibition is at least partially responsible for the reduction in DIPG cell proliferation seen with OTSSP167. Furthermore, our results suggest the possibility to use these readily available, BBB-penetrable PPARY agonists as an adjuvant therapy in future MELK inhibition–based therapeutic strategies for DIPG.

To investigate the clinical potential of OTSSP167 for the treatment of DIPG, we determined the pharmacokinetics of OTSSP167, and especially the concentration of the drug that can be achieved in the brain. These experiments showed that OTSSP167 does not cross the BBB in Bcrp1;Mdr1a/b WT mice due to the activity of both MDR1 and BCRP1 multidrug transporters. Furthermore, in silico analysis of the structure of OTSSP167 shows that, under physiologic conditions, it is mainly present in its charged, hydrophilic form, limiting its BBB penetration and explaining why even in Bcrp1;Mdr1a/b KO only 8% of the plasma levels are reached in the brain. Nonetheless, we designed an experimental setup to demonstrate the therapeutic potential of MELK inhibition for DIPG. Therefore, immunodeficient athymic mice lacking the murine equivalents of MDR1 and BCRP on their BBB were equipped with JHH-DIPG-01-Fluc or VUMC-DIPG-F xenografts. After 4 weeks, robust engraftment of JHH-DIPG-01-Fluc tumors was seen on BLI, and treatment with OTSSP167 commenced, resulting in a strong growth inhibition of the tumors; after 2 weeks, four of nine treated mice even showed partial remissions of the tumors, and two of these displayed long-term stable disease. In a second model, survival of mice bearing VUMC-DIPG-F xenografts was significantly improved by treatment with OTSSP167. These results demonstrate the therapeutic potential of pharmacologic MELK inhibition in both a posttreatment autopsy-derived (JHH-DIPG-01) and a pretreatment biopsy-derived (VUMC-DIPG-F) DIPG model. The predictive value of MELK levels for the in vivo therapeutic response of DIPG xenografts to OTSSP167 was not as prominent as observed in cell cultures, though, as VUMC-DIPG-F xenografts responded at least as well as JHH-DIPG-01, while possessing lower MELK expression. Therefore, although possibly relevant for the prediction of clinical response to MELK inhibitors, our study provides insufficient arguments for a predictive value of MELK expression for OTSSP167 efficacy.

Considering that, even in these knockout mice, only 8% of the plasma level of OTSSP167 was achieved in the brain, and that we used a lower dose of OTSSP167 than the 10 mg/kg/d dose that has been used successfully in in vivo studies of breast, prostate, pancreatic, and gastric cancer (22, 24), the effect of OTSSP167 on the growth of the DIPG xenografts was remarkably strong.

Recently, several research papers have cast doubt on the suitability of MELK inhibition for the treatment of cancer in general, showing that the therapeutic effect of OTSSP167 in multiple cancer models is independent of MELK and due to off-target effects (33, 51, 52). Although these off-target effects may certainly play a role in the therapeutic effect of OTSSP167 in DIPG, the observation that MELK knockdown strongly reduces the proliferation of DIPG cells, and the increased sensitivity to OTSSP167 of DIPG cells carrying a stable knockdown of MELK, implies that MELK inhibition is at least partially responsible for its efficacy. The dependency of DIPG...

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cells on MELK contrasts with the recent findings that MELK is dispensable in other types of cancer. Possibly, this is related to the cell of origin of DIPG, as it is currently thought to arise during embryonic development of the CNS, and MELK is selectively expressed in CNS tissues at this stage (Supplementary Fig. S1). Furthermore, analysis of all known off-target effects of OTSSP167 in a publicly available database, at the concentrations used in this study, reveals no known therapeutic targets in DIPG and no kinases known to interact with PPARY (http://www.proteomicsdb.org, Supplementary Table S2). Furthermore, these MELK knockdown cells displayed the same effects on PPARY phosphorylation, proving that this mechanism is indeed MELK dependent, and not due to off-target effects of OTSSP167. Nonetheless, research into the off-target effects of OTSSP167 may reveal novel therapeutic targets for the treatment of DIPG that contribute to the efficacy of this inhibitor.

Altogether, our results demonstrate that pharmacologic MELK inhibition may represent a future therapeutic strategy for the treatment of DIPG that warrants further investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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