Everolimus improves the efficacy of dasatinib in PDGFRα-driven glioma

Zachary Miklja1, Viveka Nand Yadav1, Rodrigo T. Cartaxo1, Ruby Siada1, Chase C. Thomas1, Jessica R. Cummings1, Brendan Mullan1, Stefanie Stallard1, Alyssa Paul1, Amy K. Bruzek2, Kyle Wierzbicki1, Tao Yang3, Taylor Garcia1, Ian Wolfe1, Marcia Leonard1, Patricia L. Robertson3, Hugh J.L. Garton2, Daniel R. Wahl4, Hemant Parmar5, Jann N. Sarkaria6, Cassie Kline7, Sabine Mueller7, Theodore Nicolaides8, Chana Glasser9, Sarah E.S. Leary10, Sriram Venneti11, Chandan Kumar-Sinha11,12, Arul M. Chinnaiyan11,12,13,14,15, Rajen Mody1, Manjunath P. Pai16, Timothy N. Phoenix17, Bernard L. Marini16, Carl Koschmann1*

*Correspondence to: ckoschma@med.umich.edu

This PDF file includes:
- Supplementary methods
- Supplementary figures
Supplemental materials

Supplementary methods

Neurosphere Limiting Dilution Assay

PPK cells were serially diluted in triplicate at 62, 31, 15, 7, or 3 cells/well in 96 well plates and kept for 24h in an incubator at 37°C/5%CO₂. Dasatinib and everolimus alone or in combination were added to the plates and sphere growth was monitored for 6 days. When they reached 50 µm in the control condition (DMSO), spheres above 20 µm in every well were counted and the average number plotted for each cell density originally plated.

P-gp assay

In vitro P-gp inhibitor assay was performed with the Pgp-Glo™ Assay System (Promega). Assay methods were executed as described in the supplied protocol. Briefly, the assay detected the effects of compounds on recombinant human P-gp in a cell membrane fraction. The assay utilized the ATP dependence of the light-generating reaction (firefly luciferase) of ATPase inhibition. Verapamil is a P-gp substrate that stimulates P-gp ATPase activity and, within the assay, functions as a P-gp stimulator (negative control). Sodium orthovanadate (Na₃VO₄) provides a measure of P-gp independent ATPase activity and was used as a P-gp inhibitor (positive control) within this assay. All experimental conditions were briefly rocked on a plate shaker and then put in an incubator at 37°C and 5% CO₂ for 3.5 hours. After this incubation period, all conditions were again briefly rocked on a plate shaker and incubated at room temperature for 20 minutes to allow luminescent signal to develop. Luminescent signal (ATM inhibition) was read on a Synergy HTX Multi-Mode microplate reader (BioTek).
**Human cerebrospinal fluid tumor DNA (CSF-tDNA) analysis**

Tumor DNA analysis was performed by droplet digital PCR (ddPCR), a highly sensitive PCR method that can detect low copy numbers of DNA, including ctDNA, in CSF. PCR primers and fluorescent probes were designed for the wild-type PDGFRA and mutant D842V sequences, as well as the target amplification region. An additional assay was designed for the housekeeping gene AP3B1 to be used as a reference (Bio-Rad). Patient sample CSF underwent ddPCR to document baseline CSF tDNA in UMPED52 and baseline CSF tDNA PDGFRA copy number in UMPED44. The Bio-Rad QX200 AutoDG system was used for all ddPCR work, and Bio-Rad QuantaSoft Analysis Pro was used for analysis of results.

**Western blot and immunohistochemistry**

PDGFRα pathway expression was assessed in primary HGG cell cultures and mouse brain/tumor tissue via immunocytochemistry (1:500) and western blotting (1:1000) with PDGF Receptor α antibody (Cell Signaling, sc-3174S) and Phospho-PDGF Receptor α antibody (Cell Signaling sc-3170S). PDGFRα/MAPK pathway expression was assessed in primary mouse and human HGG cell culture via western blotting (1:500) with Phospho-Src antibody (Cell Signaling, #6943S), Src antibody (Cell Signaling, #2109S), Phospho-ERK1/2 antibody (Cell Signaling, #4695S), and ERK1/2 antibody (Santa Cruz Biotechnology, sc-514302). Expression for the mTOR pathway was assessed via western blotting using Phospho-S6 antibody (Cell Signaling, #4858S), S6 antibody (Cell Signaling, #2217S), Phospho-p70 S6 Kinase antibody (Cell Signaling, #9234T), and p70 S6 Kinase antibody (Cell Signaling, #2708T). P-glycoprotein (P-gp) expression in PDGFRα-driven HGG mouse brain and tumor tissue was assessed by
immunohistochemistry (1:50) with P-gp antibody (Santa Cruz Biotechnology, sc-390883). Phospho-Src, total Src, phospho-S6, and total S6 expression in PDGFRA-driven HGG mouse brain and tumor tissue were assessed by immunohistochemistry with Phospho-Src antibody (Cell Signaling, #6943S), Src antibody (Cell Signaling, #2109S), Phospho-S6 antibody (Cell Signaling, #4858S), and S6 antibody (Cell Signaling, #2217S), respectively.

Supplementary Figures
Figure S1: Characterization of IUE PPK generated tumors and primary cell culture A) Representative coronal plane image of mouse brain with IUE PPK tumor (80-90% cortex effaced) (left). Tumor images for IUE PPK generated tumors depicting high-grade features including necrosis and vascular proliferation (right). B) IHC for IUE PPK tumors demonstrating increased total PDGFRα, tumor-specific positive staining for H3K27M, and negative staining for H3K27me3. C) Sequencing of PPK cells for genes DNp53, PDGFRA D842V and H3.3K27M. D) Quantification of p-PDGFRα and total PDGFRα expression in western blot of PPK cells with PDGFα/β ligand supplementation. E) Growth response of human HGG tumor cells with PDGFRA amplification (UMPED58) after supplementation with or without ligands PDGF α and β (** ** P ≤ 0.00005 by two-tailed Welch’s t-test). Data represent mean ± SEM. Scale bars: A(top): left 200 µm, right 100 µm; (bottom) up 200 µm, down 50 µm.
Figure S2: Sensitivity of human primary cell cultures to treatment with dasatinib A) Dasatinib dose-response curve for various cancer types (data from the Sanger Institute – Genomics of Drug Sensitivity). Most low-grade glioma and glioblastoma multiforme cell cultures were sensitive to dasatinib within 0.01 to 1 μM ranges (red region).
Figure S3: Effect of dasatinib and everolimus monotherapy or co-administration on RTK/MAPK/mTOR pathways A) Western blot data assessing the effect of dasatinib...
monotherapy, everolimus monotherapy, or co-administration of both drugs in vitro on RTK/MAPK and mTOR pathways in mouse PDGFRA-mutant HGG cell line (left) or human PDGFRA-enhanced HGG cell line (right). Expression of p-PDGFRα/β (Y849/Y857), p-Src (Y416), p-ERK (Thr202/Tyr204), p-S6 (S235-236), p-p70 S6 Kinase (Thr389), and Alpha-Tubulin (control) were analyzed. In the co-administration condition, drugs were administered at equal doses (doses indicated in text above figure).
Figure S4: Quantification of western blot data shown in Supplemental Figure 3 A) Western blot data quantification showing that when compared to dasatinib or everolimus monotherapy, co-treatment of both drugs resulted in reduced expression of p-PDGFRα/β (Y849/Y857) in mouse PDGFRA-mutant glioma (PPK) cells at 1 and 10 uM doses. Co-treatment also resulted in reduced expression of p-S6 (S235-236) at 0.1 uM in PPK cells and at all administered concentrations in human PDGFRA-enhanced HGG (XIII-P) cells.
Figure S5: Dasatinib and everolimus efficacy in PPK neurospheres and P-gp inhibition over time. A) Representative PPK neurosphere images (n=3 per group) after treatment with dasatinib and everolimus monotherapy or in combination. B) P-gp inhibition by varying concentrations of everolimus over time using in vitro assay (n=5 technical replicates). [Data represent mean ± SEM].
Figure S6: Pharmacokinetic (PK) analysis of dasatinib concentrations in non-tumor mouse model A) Schematic of PK mouse studies (tail vein injection (TVI) and oral gavage (OG)). B) Dasatinib plasma concentration (ng/mL) over time for treatment with dasatinib alone and
Dasatinib with everolimus. No statistical significance was found between treatment groups at any time points by two-tailed Welch’s t-tests. **C)** Dasatinib cortex concentration (ng/mL) over time for treatment with dasatinib alone and dasatinib with everolimus. Statistical significance was found between treatment groups at the 4 hour time point (**P ≤ 0.05 by two-tailed Welch’s t-test). No other statistical significance was found. **D)** Dasatinib brainstem concentration (ng/mL) over time for treatment with dasatinib alone and dasatinib with everolimus. No statistical significance was found between treatment groups at any time points by two-tailed Welch’s t-tests. **E)** Dasatinib concentration (ng/mL) for plasma, cortex, and brainstem over time when treated with dasatinib alone. **F)** Dasatinib concentration (ng/mL) for plasma, cortex, and brainstem over time when treated with dasatinib and everolimus. [Data represent mean ± SEM for B-F].
Figure S7: PK analysis of everolimus blood and brain concentration in non-tumor mouse model. **A)** Everolimus plasma concentrations (ng/mL) over time after treatment with everolimus [n=3 replicates]. **B)** Everolimus brain concentrations (ng/g) over time after treatment with everolimus [n=3 replicates]. [Data represent mean ± SEM for A and B].
Figure S8: Immunohistochemistry of mouse tumors co-treated with dasatinib and everolimus immediately prior to processing. A) P-gp expression in the treated condition was shown to be reduced when compared to the untreated condition. Red arrows represent positive staining cells. Scale bars (from left to right): 200 µm; 200 µm; 50 µm. B) IHC quantification for P-gp expression shown in part A (*** $P \leq 0.0005$ by two-tailed Welch’s t-test) [n=6 replicates for untreated group and n=5 replicates for everolimus+dasatinib group]. [Data represent mean ± SEM].
Figure S9. Effect of dasatinib and everolimus co-treatment or monotherapy on p-Src and p-S6 expression levels. A) Representative IHC staining images [n=3 mice per treatment]
Whole brain images with tumors outlined in red and 20x magnification tumor images are shown for p-Src, total Src (T-Src), p-S6, and total S6 (T-S6) staining. Dasatinib and everolimus co-treatment displayed the lowest staining for p-Src and p-S6. B) IHC quantification data for p-Src, p-S6, total Src, and total S6, showing the greatest reduction in expression levels in the co-treatment group when compared to the untreated group [n=3 animals per treatment group, 4 images per animal]. Statistical significance (see Figure 3) was found between the untreated group and co-treatment group for p-Src and p-S6 expression (**** $P \leq 0.0001$ by Dunnett’s multiple comparisons test). [Data represent mean ± SEM.]
Figure S10: Pharmacokinetic (PK) analysis of dasatinib concentrations in plasma and CSF in patients A) Time course depicting when MRI images and dasatinib plasma + CSF PK analyses were performed during treatment of two human patients with PDGFRα-driven glioma (UMPED44 and UMPED52). B) Comparison of plasma dasatinib concentrations for dasatinib alone and dasatinib and everolimus co-treatment for UMPED44 and UMPED52. C) Comparison of CSF dasatinib concentrations for dasatinib alone and dasatinib and everolimus co-treatment conditions for UMPED44.
Figure S11: Molecular attributes and tumor imaging of PDGFRA-mutated tail patient (UMPED52) A) UMPED52 copy number profile with estimated tumor content. B) UMPED52 somatic variants with a PDGFRA variant allele fraction of 34%. C) UMPED52 baseline CSF tDNA documenting PDGFRA D824V allele fraction. D) Serial UMPED52 MRI (axial FLAIR T2) at baseline, pre cycle 3, and pre cycle 4.
Figure S12: PedcBioPortal (adult HGG) P-gp RNA-seq data A) No statistical significance was found between P-gp expression and PDGFRA status, age at diagnosis, or MGMT status within these adult data sets. Data represent mean ± SEM.