Dopamine Receptor Antagonists, Radiation, and Cholesterol Biosynthesis in Mouse Models of Glioblastoma

Supplementary Methods

Cell culture

Primary human glioma cell lines were established at UCLA as described (Hemmati *et al*., PNAS 2003 [1]; Characteristics of specific gliomasphere lines can be found in Laks *et al*., Neuro-Oncology 2016 [2]). The GL261 murine glioma cell line was obtained from Charles River Laboratories, Inc., Frederick, MD. GL261 cells were cultured in log-growth phase in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, penicillin and streptomycin. Primary glioblastoma cells were propagated as gliomaspheres in serum-free conditions in T25 flasks in DMEM/F12, supplemented with B27, EGF, bFGF and heparin as described previously [3]. All cells were grown in a humidified atmosphere at 37°C with 5% CO_2. The unique identity of all patient-derived specimen was confirmed by DNA fingerprinting (Laragen, Culver City, CA). All lines were routinely tested for mycoplasma infection (MycoAlert, Lonza).

Animals

6–8-week-old C57Bl/6 mice, or NOD-*scid* IL2Rgamma^{null} originally obtained from The Jackson Laboratories (Bar Harbor, ME) were re-derived, bred and maintained in a pathogen-free environment in the American Association of Laboratory Animal Care-accredited Animal Facilities of Department of Radiation Oncology, University of California (Los Angeles, CA) in accordance to all local and national guidelines for the care of animals. Weights of the animals were recorded every day. $2x10^5$ GL261-Luc or $3x10^5$ HK-374-Luc cells were implanted into the right striatum of the brains of mice using a stereotactic frame (Kopf Instruments, Tujunga, CA) and a nano-injector pump (Stoelting, Wood Dale, IL). Injection coordinates were 0.5 mm anterior and 2.25 mm lateral

to the bregma. The needle was placed at a depth of 3.5 mm from the surface of the brain and retracted 0.5 mm for injection. Tumors were grown for 3 (HK-374), 7 (GL261) days after which successful grafting was confirmed by bioluminescence imaging.

Irradiation

Cells were irradiated at room temperature using an experimental X-ray irradiator (Gulmay Medical Inc. Atlanta, GA) at a dose rate of 5.519 Gy/min for the time required to apply a prescribed dose. The X-ray beam was operated at 300 kV and hardened using a 4 mm Be, a 3 mm Al, and a 1.5 mm Cu filter and calibrated using NIST-traceable dosimetry. Corresponding controls were sham irradiated.

Mice were irradiated using an image-guided small animal irradiator (X-RAD SmART, Precision X-Ray, North Branford, CT) with an integrated cone beam CT (60 kVp, 1 mA) and a bioluminescence-imaging unit. The X-ray beam was operated at 225 kV and calibrated with a micro-ionization chamber using NIST-traceable dosimetry. Cone beam CT images were acquired with a 2 mm Al filter. For delivery of the radiation treatment the beam was hardened using a 0.3 mm Cu filter.

During the entire procedure the interior of the irradiator cabinet was maintained at 35° C to prevent hypothermia of the animals. Anesthesia of the animals was initiated in an induction chamber. Once deeply anesthetized, the animals were then immobilized using custom 3D-printed mouse holder (MakerBot Replicator+, PLA filament) with ear pins and teeth bar, which slides onto the irradiator couch.

Cone beam CT images were acquired for each individual animal and ensured to avoid eyes and cerebellum of the mice. Individual treatment plans were calculated for each animal using the SmART-Plan treatment planning software (Precision X-Ray). Radiation treatment was applied using a square 1x1 cm collimator from a lateral field.

In-vitro sphere formation assay

Cells were seeded under serum-free conditions into untreated plates in DMEM/F12 media, supplemented with 10 ml / 500 mL of B27 (Invitrogen), 0.145 U/ml recombinant insulin (Eli Lilly, Indiana), 0.68 U/mL heparin (Fresenius Kabi, Illinois), 20 ng/ml fibroblast growth factor 2 (bFGF, Sigma) and 20 ng/ml epidermal growth factor (EGF, Sigma).

Quantitative Reverse Transcription-PCR

Total RNA was isolated using TRIZOL Reagent (Invitrogen). cDNA synthesis was carried out using the SuperScript Reverse Transcription IV (Invitrogen). Quantitative PCR was performed in the QuantStudio™ 3 Real-Time PCR System (Applied Biosystems) using the PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Carlsbad, CA, USA). C_t for each gene was determined after normalization to IPO8, TBP, PPIA and $\Delta\Delta C_t$ was calculated relative to the designated reference sample. Gene expression values were then set equal to $2^{-\Delta\Delta Ct}$ as described by the manufacturer of the kit (Applied Biosystems). All PCR primers were synthesized by Invitrogen and designed for the human sequences of cholesterol biosynthesis pathway genes (for primer sequences see **Supplementary Table1**).

Mass spectrometry

Sample Preparation: Whole blood from mice was centrifuged to isolate plasma. Quetiapine was isolated by liquid-liquid extraction from plasma: 50 µl plasma was added to 2 µl internal standard and 100 µl acetonitrile. Mouse brain tissue was washed with 2 mL cold saline and homogenized using a tissue homogenizer with fresh 2 mL cold saline. Quetiapine was then isolated and reconstituted in a similar manner by liquid-liquid extraction: 100 µl brain homogenate was added to 2 µl internal standard and 200 µl

acetonitrile. The samples were centrifuged, supernatant removed and evaporated by a rotary evaporator and reconstituted in 100 µl 50:50 water:acetonitrile.

Quetiapine Detection: Chromatographic separations were performed on a 100 x 2.1 mm Phenomenex Kinetex C18 column (Kinetex) using the 1290 Infinity LC system (Agilent). The mobile phase was composed of solvent A: 0.1% formic acid in Milli-Q water, and B: 0.1% formic acid in acetonitrile. Analytes were eluted with a gradient of 5% B (0-4 min), 5-99% B (4-32 min), 99% B (32-36 min), and then returned to 5% B for 12 min to re-equilibrate between injections. Injections of 20 µl into the chromatographic system were used with a solvent flow rate of 0.10 ml/min.

Mass spectrometry was performed on the 6460 triple quadrupole LC/MS system (Agilent). Ionization was achieved by using electrospray in the positive mode and data acquisition was made in multiple reactions monitoring mode. For quetiapine: m/z 384.2→ 253.1 and 284.2→ 279.1 with a fragmentor voltage of 140V, and collision energy of 16 and 20 eV, respectively. The analyte signal was normalized to the internal standard and concentrations were determined by comparison to the calibration curve (0.5, 5, 50, 250, 500, 2000 nM). Quetiapine brain concentrations were adjusted by 1.4% of the mouse brain weight for the residual blood in the brain vasculature as described by Dai *et al*. [4].

RNASeq

48 hours after 4 Gy irradiation or sham irradiation, RNA was extracted from HK-374 cells using Trizol. RNASeq analysis was performed by Novogene (Chula Vista, CA). Quality and integrity of total RNA was controlled on Agilent Technologies 2100 Bioanalyzer (Agilent Technologies; Waldbronn, Germany). The RNA sequencing library was generated using NEBNext® Ultra RNA

Library Prep Kit (New England Biolabs) according to manufacturer's protocols. The library concentration was quantified using a Qubit 3.0 fluorometer (Life Technologies), and then diluted to 1 ng/μl before checking insert size on an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies; Waldbronn, Germany) and quantifying to greater accuracy by quantitative Q-PCR (library molarity >2 nM). The library was sequenced on the Illumina NovaSeq6000 platform.

Downstream analysis was performed using a combination of programs including STAR, HTseq, and Cufflink. Alignments were parsed using the program Tophat and differential expressions were determined through DESeq2. Reference genome and gene model annotation files were downloaded from genome website browser (NCBI/UCSC/Ensembl) directly. Indexes of the reference genome were built using STAR and paired-end clean reads were aligned to the reference genome, using STAR (v2.5). HTSeq v0.6.1 was used to count the read numbers mapped of each gene. The FPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene.

Differential expression analysis between irradiated and control samples (three biological replicates per condition) was performed using the DESeq2 R package (2_1.6.3). The resulting *p*-values were adjusted using the Benjamini and Hochberg's approach for controlling the False Discovery Rate (FDR). Genes with an adjusted *p*-value of <0.05 found by DESeq2 were assigned as differentially expressed. To identify the correlations between differentially expressed genes, we generated heatmaps using the hierarchical clustering distance method with the function of heatmap, SOM (Self-organization mapping) and k-means using the silhouette coefficient to adapt the optimal classification with default parameters in R. Gene Ontology enrichment analysis of differentially expressed genes using the Gene **O**ntology 5nalysis5t 5nalysis and vizua**L**iz**A**tion tool [5, 6]. GO terms with corrected *p*-values less than 0.05 were considered significantly enriched by differential expressed genes.

Shotgun lipidomics analysis

Samples were analyzed on the Sciex Lipidyzer Platform and quantified with normalization to cell number. This platform has been described elsewhere [7]. Prior to collection, cell number was assessed by Automated Cell Counter (Invitrogen Countess 3). After removal of culturing media, 1 mL of ice-cold PBS was added into each well and cells were scraped with cell lifters and spun down in glass tubes at 365 $\times g$ for 5 min at 4°C. Two wells of cells were combined as a single replicate. A modified Bligh and Dyer extraction [8] was carried out on samples. Prior to biphasic extraction, a 13-lipid class Lipidyzer Internal Standard Mix is added to each sample (AB Sciex, 5040156). Following two successive extractions, pooled organic layers were dried down in a Genevac EZ-2 Elite. Lipid samples were resuspended in 1:1 methanol/dichloromethane with 10 mM Ammonium Acetate and transferred to robovials (Thermo 10800107) for analysis. Samples were analyzed on the Sciex Lipidyzer Platform for targeted quantitative measurement of 1100 lipid species across 13 classes. Differential Mobility Device on Lipidyzer was tuned with SelexION tuning kit (Sciex 5040141). Instrument settings, tuning settings, and MRM list available upon request. Data analysis performed on Lipidyzer software. Quantitative values were normalized to cell counts. For informatics analysis, heat maps and PCA plots were generated with Clustvis [9]. Rows were centered and unit variance scaling is applied to rows (Z score). Rows were clustered using correlation distance and complete linkage. For PCA, unit variance scaling was applied to rows, Nipals PCA is used to calculate principal components. X and Y axis show principal component 1 and principal component 2 that explain percentage of the total variance, respectively. Prediction ellipses are such that with probability 0.95, a new observation from the same group will fall inside the ellipse.

References

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Supplementary Table 1. Primer list

Gene	Forward	Reverse
DHCR7	GCTGCAAAATCGCAACCCAA	GCTCGCCAGTGAAAACCAGT
FDFT1	CCACCCCGAAGAGTTCTACAA	TGCGACTGGTCTGATTGAGATA
HMGCR	TGATTGACCTTTCCAGAGCAAG	CTAAAATTGCCATTCCACGAGC
HMGCS1	CATTAGACCGCTGCTATTCTGTC	TTCAGCAACATCCGAGCTAGA
INSIG1	CCTGGCATCATCGCCTGTT	AGAGTGACATTCCTCTGGATCTG
LIPG	GATGGACGATGAGCGGTATCT	CGCATCCGTGTAAAGCTGG
MSMO1	TATGCTGGTTCTCGGCATCAT	CCAAAAATTCGATCCCACCATGT
MVD	CGTGGCATCGGTGAACAACT	GTGTAGGCTAGGCAGGCATA
SCD	TCTAGCTCCTATACCACCACCA	TCGTCTCCAACTTATCTCCTCC
SQLE	GATGATGCAGCTATTTTCGAGGC	CCTGAGCAAGGATATTCACGACA
ANGPT1	AGCGCCGAAGTCCAGAAAAC	TACTCTCACGACAGTTGCCAT
PDK4	GACCCAGTCACCAATCAAAATCT	GGTTCATCAGCATCCGAGTAGA
KRT18	TCGCAAATACTGTGGACAATGC	GCAGTCGTGTGATATTGGTGT
CIITA	CCTGGAGCTTCTTAACAGCGA	TGTGTCGGGTTCTGAGTAGAG
KCNK2	ATAGTGGCAGCAATAAATGCAGG	TTCTGTGCGTGGTGAGATGTT
PLK2	CTACGCCGCAAAAATTATTCCTC	TCTTTGTCCTCGAAGTAGTGGT
THBS1	AGACTCCGCATCGCAAAGG	TCACCACGTTGTTGTCAAGGG
ANKRD1	AGTAGAGGAACTGGTCACTGG	TGTTTCTCGCTTTTCCACTGTT
KRT14	GGCCTGCTGAGATCAAAGACTAC	CACTGTGGCTGTGAGAATCTTGTT
EDN1	AAGGCAACAGACCGTGAAAAT	CGACCTGGTTTGTCTTAGGTG
TBP	TGCACAGGAGCCAAGAGTGAA	CACATCACAGCTCCCCACCA
PPIA	ATGCTGGACCCAACACAAAT	TCTTTCACTTTGCCAAACACC
IPO8	CGAAGTTGCGGATTGCAG	GAATTCCACATGGTCAGAGACT
HSD17B7	AACATGAGCAAGGCAGAAG	TCTTGAAAAGAGGCCAAAGA
ELOVL6	CCTGGATGTAGCTGAGAGG	GCTTCATTCTCGTTGAACTG
POR	GCTTCATTCTCGTTGAACTG	AGCATTTCTTGGGGATGTAG
APOE	GTGGAGCAAGCGGTG	TCAACTCCTTCATGGTCTCG
G6PD	CTTCCATCAGTCGGATACAC	CAAAGAAGTCCTCCAGCTT
SC5D	CATACGTGTATCCAGCCAC	AAGAACAGTGCAACAGTAAGA
TM7SF2	GTAATGGGGCAGAGAGATG	CAGTCGACACCATAAATCCC
NSDHL	CCTACGGACGGAAAAGAAA	TTGACAGCATATCCTCTTGC
FASN	GCGCCCTCACCAGAG	GGGTGGACTCCGAAGAA
CYP51A1	GGCCAGGCGATGGAG	TGCCCAAGGAATGGAATTG
JUND	ACCCTTCTACGGCGAT	GTCAGCGCGTCCTTCT

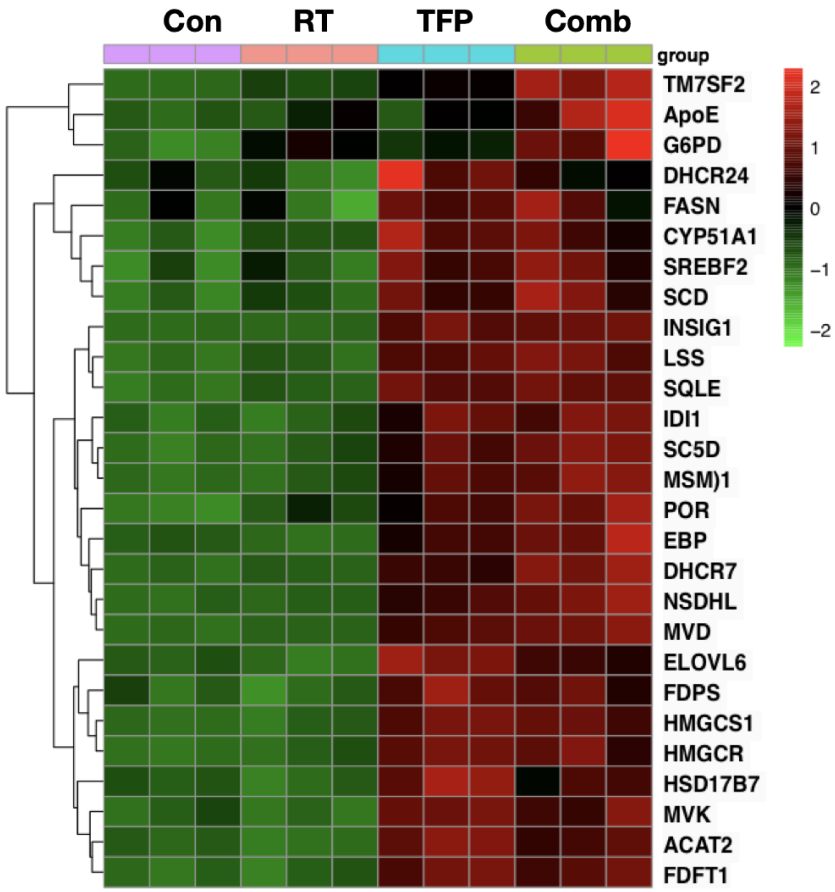
EGR1	GGGCTCCCCAGTTCC	GAGAGATGTCAGGAAAAGACT
BHLHB2	CAGCAAGGAGACCTACAAAT	TGCTGCTGATCAATTAGGTT
ZBTB7B	CCCGACGGCTGAGAG	GCTTCTTGAAGTAGTGGCT
JUNB	ACCAGGCACCCAGTC	TTTCAGGAGTTTGTAGTCGT
FOSB	CCCGGTTCCTTCGTG	TGGTAGTTCCGCTGGT

Supplementary Table 2. D_0 values for the *in vitro* survival curves

Cell line	RT	RT+QTP 5 µM	RT+ATR 50 nM	RT+QTP 5 µM +ATR 50 nM
HK-374	2.5	2.31	2.69	2.59
HK-382	3.44	3.12	3.407	N/A
HK-308	3.11	3.037	3.15	2.395
HK-157	2.633	2.697	2.66	N/A

Supplementary Figures

A



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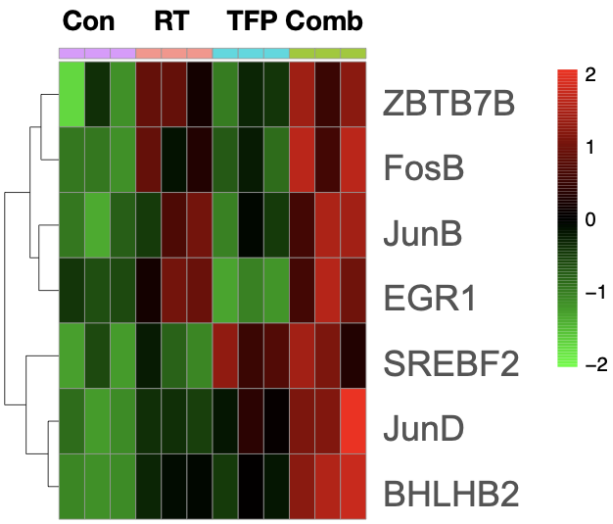

Supplementary Figure 1: Genes involved in the biosynthesis of cholesterol are upregulated in HK-374 cells, 48 hours after treatment with radiation (4 Gy) and quetiapine (**G**) or radiation and trifluoperazine (TFP) **(A)**. Genes of the first-level regulator network of SREBF2, the master regulator of cholesterol biosynthesis are upregulated in HK-374 cells, 48 hours after treatment with radiation (4 Gy) trifluoperazine (**B**).