# MT1 and MT2 melatonin receptors play opposite roles in brain cancer progression

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**Running title:** The role of melatonin receptors in brain cancer

**Abstract**

**Introduction:** Primary brain tumors remain among the deadliest of all cancers. Glioma grade IV (glioblastoma), the most common and malignant type of brain cancer, is associated with a 5-year survival rate <5%. Melatonin has been widely reported as an anticancer molecule and we have recently demonstrated that the ability of gliomas to synthesize and accumulate this indolamine in the surrounding microenvironment negatively correlates with tumor malignancy. However, our understanding of the specific effects mediated through the activation of melatonin membrane receptors remains limited. Thus here, we investigated the specific roles of MT1 and MT2 in gliomas and medulloblastomas. **Methods and Results:** Using the MT2 selective antagonist DH97 we showed that MT1 activation has a positive and MT2 a negative impact on the proliferation of human glioma and medulloblastoma cell lines. Accordingly, gliomas have a decreased mRNA expression of *MT1* (also known as *MTNR1A*) and an increased mRNA expression of *MT2* (also known as *MTNR1B*) compared to normal brain cortex. Moreover, the MT1/MT2 expression ratio negatively correlates with the expression of cell cycle-related genes and is a positive prognostic factor in gliomas. Notably, we showed that dual-target drugs that simultaneously activate MT1 and inhibit MT2 exert robust anti-tumor effects in vivo and in vitro, inhibiting the energy metabolism and translation of glioma stem cells. **Discussion:** Overall, we provided the first evidence regarding the differential roles of MT1 and MT2 in brain tumor progression and highlight their relevance as therapeutic targets.

## Introduction

Despite decades of research progress, brain tumors remain among the cancers that hold the poorest prognosis. Glioma grade IV (glioblastoma), the most common and malignant type of brain cancer, is associated with a median survival of approximately 15 months in adults [1, 2]. Brain tumors are also the most common solid tumors affecting children and adolescents, and survivors often display neurocognitive impairment in adulthood due to the exposure of the developing brain to diverse medical interventions. Challenges in the treatment of such tumors include their invasive nature and cellular heterogeneity, as well as the difficulty of drug delivery across the blood-brain barrier [3]. Recently, the World Health Organization Classification of Central Nervous System Tumors has incorporated biologically and clinically relevant molecular features to the traditional histological diagnosis [4]. Diffuse gliomas in adult are now separated in three main groups with progressively worse prognosis: isocitrate dehydrogenase (IDH)-mutant, 1p/19q co-deleted tumors with oligodendroglial morphology, IDH-mutant tumors, non-1p/19q co-deleted with astrocytic morphology, and IDH-wild type glyoblastomas. New entities also include diffuse midline pediatric glioma H3 K27M–mutant; RELA fusion–positive ependymoma; medulloblastoma WNT-activated, and medulloblastoma SHH-activated [4, 5]. Melatonin synthesized by the pineal gland at night translates the environmental dark phase to the organism and ensures the synchronization of circadian and seasonal rhythms [6, 7]. The production of melatonin has also been detected in many extra-pineal tissues, including retina [8] , gastrointestinal tract [9], bone marrow [10, 11] and brain [12, 13]. Generally, extrapineal melatonin exerts local autocrine and paracrine effects [13–15], acting through biological mechanisms such as activating G protein-coupled receptors and directly scavenging of free radicals [16]. Human MT1 and MT2 melatonin receptors share 55% of amino acid sequence similarity and bind melatonin with high affinity [17] . Both are typically coupled to Gi/o proteins while MT1 can also be coupled to Gq, evoking calcium-dependent signaling [18, 19]. Melatonin receptors are widely expressed throughout the central nervous system and play a role in circadian entrainment, synaptic function and neurodevelopment [20, 21]. Interestingly, altered expression of melatonin receptors has been reported in different neurodegenerative conditions such as Alzheimer's and Parkinson’s disease [22-24].Melatonin is increasingly recognized as an anti-tumor molecule across a wide variety of malignancies [25, 26]. Accordingly, we have demonstrated that the ability of gliomas to synthesize and accumulate melatonin negatively correlates with their overall malignancy [12]. High grade

gliomas have a decreased expression of N-acetylserotonin O-methyltransferase (*ASMT*), the final enzyme of melatonin biosynthesis, combined with a high expression of cytochrome P450 1B1 (*CYP1B1*), the main enzyme of melatonin extra-hepatic metabolism. Importantly, the content of melatonin in the tumor microenvironment, as predicted by the ASMT:CYP1B1 expression index, was a positive prognostic factor, independent of glioma grade and histological subtype. [12]. Additionally, high concentrations of melatonin (mM range) have been shown to impair the invasion and migration of human glioma cell lines and reduce the viability of glioma initiating cells, while lower concentrations did not produced significant results [27–29]. MT1 and MT2 bind melatonin with nM affinity [19]; thus effects observed only at the mM range likely involve receptor-independent mechanisms. Few studies have assessed the role ofMT1 in brain cancer, while no data is available regarding MT2. In glioma stem cells, MT1 activation has been shown to inhibit the expression of nestin, p-c-Myc(S62), and c-Myc, suppressing neurosphere formation and inducing G2/M arrest [30]. Moreover, in gliomas with increased expression of MT1, melatonin treatment exerts a more pronounced impairment of cell growth both in vitro and in vivo [31]. Thus, despite previous efforts, the relevance of melatonin receptors as potential targets for the treatment of brain cancer remains largely unexplored. To provide a framework for the rational use of melatonin and other melatonergic compounds in brain cancer therapy, here we dissected the distinct effects of MT1 and MT2 activation in human gliomas a medulloblastomas.

Using a MT2-selective antagonist we showed that MT1 impairs, while MT2 promotes, the proliferation of glioma and medulloblastoma cell lines. Accordingly, patients expressing high *MT1* receptors (also known as *MTNR1A*) and low *MT2* (also known as *MTNR1B*) receptor levels presented a significantly better prognosis. Finally, we show that functional selective drugs displaying MT1 agonist and MT2 antagonist properties exert robust anti-tumor effects in vitro and in vivo and interfere with the proliferation and metabolism of glioma stem cells.

## Materials and methods

### Cell lines

Human glioma cell lines HOG, T98G, U87MG, U87MG-luc (expressing a luciferase reporter gene), and human medulloblastoma cell line DAOY were cultured in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific), 100 IU/mL penicillin (Thermo Fisher Scientific), and 100 μg/mL streptomycin (Thermo Fisher Scientific). T98G, U87MG and DAOY were purchased from ATCC, HOG was kindly provided by Dr. Suely K. N. Marie (University of Sao Paulo, Sao Paulo, Brazil); and U87MG-luc was kindly provided by Dr. Andrew L. Kung (Memorial Sloan Kettering Cancer Center, NY, USA). Cancer stem cell-enriched cultures

MGG23 and MGH143 were derived from glioblastoma specimens at the Massachusetts General Hospital and kindly provided by Dr. Mario Suvà. Cell lines were grown as neurospheres and maintained in neurobasal medium (Thermo Fisher Scientific) supplemented with L-glutamine (Thermo Fisher Scientific), B27 supplement (Thermo Fisher Scientific), N2 supplement (Thermo Fisher Scientific), 100 IU/mL penicillin (Thermo Fisher Scientific), and 100 μg/mL streptomycin (Thermo Fisher Scientific), 20 ng/mL EGF (Sigma), and 20 ng/mL FGF2 (Peprotec). All cell lines were kept at 37°C in a humidified atmosphere of 5% $CO_2$

**Flow cytometry**

Cells were detached with 0.2% EDTA in PBS (10 min, room temperature), fixed with 2% PFA in PBS (20 min, on ice), permeabilized with 0.1% TritonX (10 min, room temperature), blocked with 2% BSA in PBS (1h, room temperature) and incubated overnight at 4°C with goat anti-MT1 (1:100, sc-13186, Santa Cruz Biotechnology) or goat anti-MT2 (1:100, sc-13177, Santa Cruz Biotechnology) primary antibodies. Cells were washed twice with 2% BSA before incubation with FITC-conjugated anti-goat IgG antibody (1:200, sc-2777, Santa Cruz Biotechnology) for 1 h at room temperature. Data were acquired on a Amnis FlowSight flow cytometer (Merck Millipore), and analyses were carried on using the IDEAS software (Merck Millipore) and FlowJo v9.

**MTT proliferation assay**

Cells (4 x $10^3$ per well) were seeded on 96-well plates, left to attach overnight and treated with DH97 (3 x $10^{-10}$ – $10^{-6}$ M), 5-HEAT ($10^{-9}$ – $10^{-6}$ M), UCM799 ($10^{-9}$ – $10^{-6}$ M), or the appropriate vehicle for 48 h. Culture media was then replaced with a MTT solution (0.5 mg/mL in PBS, Sigma) and cells were maintained in the incubator for 4 hr. The reduced MTT (formazan crystals) were dissolved in isopropanol:DMSO (1v:1v) for 20 minutes at room temperature. Absorbance was measured at 570 nm, with background subtraction at 690 nm, in a SpectraMax 250 spectrophotometer (Molecular Devices).

**U87MG-luc orthotopic xenograft model**

U87MG-luc cells ($5 \times 10^5$) suspended in 5 μL PBS were injected into the right striatum of female/male 8–10-week-old Balb/C nude mice (Charles River International) using a 10 μL Hamilton syringe attached to a Harvard 22 syringe pump (Harvard Apparatus), as previously described [32, 33] Two weeks after tumor implantation, animals were randomly assigned to four experimental groups: vehicle (0.2% DMSO, n = 7), $10^{-4}$ M melatonin (n = 5), $10^{-4}$ M 5-HEAT (n = 5) and $10^{-4}$ M UCM799 (n = 5). Treatments were continuously infused (0.25 μl/hr) into the right striatum of mice for 14 days using ALZET mini osmotic pumps (model 1002) and the ALZET brain infusion kit 3 (DURECT Corporation). Prior to implantation, pre-filled pumps were primed in sterile 0.9% saline overnight at 37°C, according to manufacturer's instructions. Given the pump infusion rate, r = 0.25 μl/h, and the average volume (v = 35 μL) and production rate (p = 18 μL/h) of cerebrospinal fluid in mice [34], the expected equilibrium concentration of compounds in the tumor microenvironment is approximately $10^{-6}$ M, which corresponds to the concentrations of 5-HEAT and UCM799 that exerted maximal inhibition in the in vitro dose-response assays (Fig. 4a–b)

For in vivo bioluminescence imaging, animals were anesthetized with isoflurane, injected intraperitoneally with D-luciferin (50 μg/g, PerkinElmer) and placed into an In Vivo FX PRO imaging system (Bruker). Analyses were performed using the MI Software (Bruker). Fourteen days post treatments mice were euthanized by deep anesthesia and encapsulated tumors were resected. Tumor volume ($mm^3$) was determined using width (a) and length (b) measurements ($V = (a^2 \times b)/2$, where $a \leq b$). Institutional guidelines for animal welfare and experimental conduct were followed. The study was approved by the Animal Ethics Committee of A. C. Camargo Cancer

Center (process 076/17), and by the Animal Ethics Committee of the Institute of Bioscience of the University of Sao Paulo (process 284/2017).

**TCGA and GTEx data**

The Cancer Genome Atlas (TCGA) RNA-seq and clinical data from 662 primary gliomas (509 lower grade gliomas and 153 glioblastomas) and Genotype-Tissue Expression (GTEx) RNA-seq data from 283 normal brain cortex were downloaded from the UCSC XENA Browser (Goldman et al., 2018). RNA-seq data were generated using the Illumina HiSeq 2000 RNA sequencing platform and quantified by RSEM. Estimated counts were upper quartile normalized, $\log_2(normalized\ counts + 1)$ transformed and converted to z-scorefor calculating the MT1/MT2 expression ratio.

**Survival analysis**

We evaluated the association between the MT1/MT2 expression ratio and patient 10-year survival using tumors expressing MT1 and/or MT2 (331 lower grade gliomas and 91 glioblastomas). Cutoffs used for patient dichotomization were defined using a log-rank test-based approach that identifies the most statistically significant data split, as previously described [36]. Univariate analyses of survival were performed using Kaplan-Meier curves and the log-rank test. Multivariate analyses of survival adjusting for clinically significant parameters were performed using Cox proportional hazards regression. Hazard ratios (HRs) including 95% confidence intervals were calculated.

**The cancer cell line encyclopedia data**

RNA-seq and drug response data from 23 brain cancer cell lines expressingMT1 and/or MT2 were downloaded from the CCLE Depmap Portal (https://depmap.org/portal/) [37]. RNA-seq data were generated using the Illumina HiSeq 2000 RNA sequencing platform, quantified with RSEM, TPM-normalized and log2(TPM + 1) transformed. Expression levels of MT1 and MT2 were converted to zscore for calculating the MT1/MT2 expression ratio. Drug response data were originally generated by the Broad Institute CTD2 Center (CTRP v2) [38] using CellTiter- Glo assays and represent the area under 16-point curves (AUC). We only considered compounds tested in at least 15 brain cancer cell lines.

**RNA-seq**

MGH143 and MGG23 cells (1,000 per well) were seeded in 96-well plates, left to rest overnight and treated with 5-HEAT ($10^{-6}$ M) or vehicle (2 x $10^{-3}$ % DMSO) for 48 h. Culture media was removed after centrifugation and 1 µL RNasin lysis buffer (SMART-Seq V4 Ultra Low Input RNA Kit; Clontech) was added to each well. Samples were incubated for 5 min at room temperature and transferred to −80°C. Samples were thawed, and reverse transcription and cDNA amplification (12 cycles) of the full transcriptome were performed with the SMART-Seq V4 Ultra Low Input RNA Kit according to the manufacturer's protocol. Following Agencourt Ampure XP beads cleanup (Beckman Coulter), 200 pg of amplified DNA were used for library preparation, as previously described [39]. Individual barcodes were ligated to each sample to allow multiplexing. Between 10 and 12million single-end reads were sequenced per sample using an Illumina HiSeq 2500 v4 instrument.

Single-end reads were aligned to the GHCh38/hg38 human genome using Bowtie and expression values were quantified using RSEM. Data are presented as log2(TPM +1).

**GSEA**

For the TCGA RNA-seq data analysis, we selected tumors expressing MT1 and/or MT2 (331 lower grade gliomas and 91 glioblastomas), and used Pearson's correlation coefficient with the MT1/MT2 expression ratio as the ranking metric. For the RNA-seq data analysis of glioma stem-like cells, genes were ranked according to the average log fold change observed across samples treated with 5-HEAT compared to the vehicle group. GSEA was performed using the GSEA desktop application v3.0 [40] and Reactome pathways [41]. Enrichment scores (ES) were calculated based on a weighted Kolmogorov–Smirnov-like statistic and normalized (NES) to account for gene set size. *P*-values corresponding to each NES were calculated using 1,000 gene set permutations and corrected for multiple comparisons with the false discovery rate (FDR) procedure. Adjusted *P*-values < 0.1 were considered statistically significant. The Cytoscape plug-in EnrichmentMap was used to generate network-based enrichment maps of significantly enriched gene sets (Merico, Isserlin, Stueker, Emili, & Bader, 2010).

**Additional statistical analysis**

We used two-sided Student's t test to perform two-group comparisons and Pearson's correlation to assess associations between continuous variable. Where specified, p values were corrected for multiple comparisons using the false discovery (FDR) procedure and combined using Fisher's method. Sample size and number of experimental and technical replicates are reported in Figure Legends. P-values < 0.05 were considered statistically significant. Analyses were performed with GraphPad Prism 6 and R (www.r-project.org).

## Results

### Melatonin receptors MT1 and MT2 differentially control the proliferation of glioma and medulloblastoma cell lines

To explore the biological role of melatonin receptors in brain tumors, we first showed the expression of MT1 and MT2 in three human glioma cell lines (HOG, an oligodendroglioma grade III, T98G, a glioblastoma, and U87MG, a tumorigenic glioblastoma, **Fig. 1A**). The less aggressive cell lines HOG and T98G synthesize and accumulate significant amounts of melatonin in their microenvironment [12]. Using luzindole, a non-selective antagonist of melatonin receptors, we have previously demonstrated that this glioma-synthesized melatonin exerts an autocrine anti-proliferative effect in a receptor-dependent manner [12]. Thus, to elucidate the specific roles of MT1 and MT2, we treated cells with the MT2 antagonist DH97, which displays a 89-fold selectivity over MT1. Surprisingly, the selective blockage of MT2 by $10^{-8}$ M DH97 significantly decreased the proliferation of HOG and T98G (**Fig. 1B**). This effect was reverted, in a concentration dependent manner (DH97 $10^{-7}$ – $10^{-6}$ M), probably by the concomitant inhibition of both melatonin receptors, suggesting opposite roles for MT1 and MT2 (**Fig. 1B**). Indeed, the treatment with $10^{-6}$ M DH97 mimicked the effects of the non-selective antagonist luzindole and stimulated the proliferation of HOG and T98G [12]. Similar results were obtained using the medulloblastoma cell line DAOY, which expresses MT1, MT2, and the enzymes involved in melatonin synthesis AANAT, its active form pAANAT and ASMT, and accumulates significant amounts of melatonin (9.8 ± 0.7 pg/mL, n = 6) in the culture media (**Fig. S1A-B**). No difference was observed for the glioma cell line U87MG (**Fig. 1A**), which produces low levels of melatonin, and is also unaffected by luzindole [12].

**Clinical relevance of MT1 and MT2 expression in glioma**

Analysis of TCGA and GTEx RNAseq data revealed that lower grade gliomas and glioblastomas have a decreased expression of MT1 and an increased expression of MT2 compared to normal brain cortex (**Fig. 2A**). Moreover, gliomas simultaneously expressing high MT1 and low MT2 (high MT1/MT2 ratio) were associated with significantly better 10-year survival **(Fig. 2B).** Such effect was especially relevant in gliomas predicted to synthesize and accumulate more melatonin, for which the MT1/MT2 ratio was a positive prognostic factor independent of age, gender, IDH mutation and 1p/19q co-deletion (**Table 1**). Next, in a way to assess whether a high MT1/MT2 ratio translates into enhanced MT1-induced Gq activation, using GSEA we showed that the ratio positively correlates with the expression of gene sets associated with calcium-dependent signaling (**Fig. 2C, Table S1**). Moreover, in accordance with the in vitro results suggesting that MT1 has a negative and MT2 a positive impact on glioma growth, the MT1/MT2 ratio also negatively correlated with the expression of cell cycle-related gene sets (**Fig. 2C, Table S1**),while no significant results were observed for genes involved in apoptosis (Fig. 3a and S2A). Tumors with a high MT1/MT2 ratio presented a significantly decreased expression of cell cycle regulators such as the DNA replication factor PCNA, the cyclin CCNA2, and the cyclin-dependent kinases CDK2 (Fig. 3a). Accordingly, in CCLE brain cancer cell lines [37], the MT1/MT2 ratio negatively correlated with the sensitivity of these cells to inhibitors of cyclin dependent kinases (Fig. 3b–c). Altogether, these results are highly consistent with the decreased proliferation associated with a high MT1/MT2 ratio.

**Functional selective drugs that simultaneously activate MT1 and inhibit MT2 receptors exert robust anti-tumor effects**

Given the opposite roles of melatonin receptors suggested by our data, we reasoned that drugs able to activate MT1 while inhibiting MT2 would have promising therapeutic potential. We tested two high affinity compounds that act as agonists of MT1 and antagonists of MT2: 5-HEAT, that bears a hydroxyethoxy group on the C5-indole position of melatonin [43], and UCM799, which is a N-anilinoethylamide derivative carrying a benzyl substituent on the aniline nitrogen [44]. Both compounds inhibited the in vitro proliferation of all four cell lines analyzed (**Fig. 3A-B, S1C**). We also tested the therapeutic efficiency of such compounds in vivo using the U87MG-

luc orthotopic xenograft model. We expect 5-HEAT and UCM799 to be effective regardless of the content of melatonin in the tumor microenvironment. For instance, given the limited capacity of U87MG cells to produce and accumulate melatonin, we postulate that in vivo, during the day, such compounds would act mainly by activating MT1, while at night they would also antagonize the binding of pineal melatonin to MT2.

Notably, continuous brain infusion of 5-HEAT and UCM799 for 14 days reduced tumor growth by approximately 75% compared to vehicle (**Fig. 3C**). Cancer stem cells are a subpopulation within tumors that has enhanced tumorigenicity and is capable of self-renewal and differentiation [45]. In glioblastomas, the failure of current gold-standard therapies to eliminate tumor stem cells has been considered a major factor contributing to the inevitable tumor recurrence [46]. Thus to better understand the mechanism of action of the functional selective melatonergic compounds, we used glioma stem cell-enriched neurosphere cultures MGG23 and MGH143, previously shown to maintain primary tumor phenotype and genotype [447, 48]. Neurospheres were treated with vehicle or 5-HEAT $10^{-6}$ M for 48 h and profiled by RNA-seq. We then used GSEA to explore gene sets differentially expressed upon treatment in both cell lines. We identified 73 gene sets negatively enriched compared to the vehicle group, whereas no gene sets were positively enriched (**Fig. 4A, Table S2**). As expected, in both cell lines 5-HEAT inhibited the expression of cell cycle genes including CCND1, CDK4, CKD9, regulators of DNA replication RPA2, GINS2 and RFC2/5, as well as tubulins (**Fig. 4B, Table S3**). 5-HEAT also impaired the expression of multiple RNA processing and translation genes, and important regulators of cellular metabolism such as the glycolysis enzymes GAPDH and ENO1, translocases of inner mitochondrial membrane TOMM22, TIMM17B/22/50 and CYC1, which encodes a subunit of the cytochrome bc1 complex, the third complex in the electron transport chain of the mitochondrial (**Fig. 4B, Table S3**).

## Discussion

The detailed characterization of the biological roles of melatonin receptors is essential for a rational clinical application of melatonin and other melatonergic compounds in the treatment of brain tumors. Despite the widespread interest in using melatonin as an adjuvant anticancer therapy,

the specific effects mediated through the activation of MT1 and MT2 receptors remain poorly understood. Although accumulating studies support the anticancer properties of melatonin in different tumor types, experiments often involve millimolar concentrations of this indolamine [25], which likely trigger diverse receptorindependent mechanisms, masking the impact of MT1 and MT2 activation. Here we demonstrated that in glioma and medulloblastoma, the receptor-dependent anti-proliferative effect of melatonin is mediated by the activation of MT1, whereas MT2 seems to play a pro-tumor role and has a significantly higher mRNA expression in tumors compared to the normal brain cortex. Accordingly, the ratio between the expression of MT1 and MT2 is a positive prognostic factor of patient survival, being particularly relevant when considering tumors with higher local production and accumulation of melatonin. Finally, we also showed the potential of MT1 and MT2 receptors as druggable targets, as the simultaneous activation of MT1 and inhibition of MT2 promote a decrease in the expression of genes related to cell cycle, metabolism, and protein translation in glioma stem-like cells, besides impairing tumor growth in vitro and in vivo. MT1 has often been recognized as the mediator of receptordependent anti-tumor actions of melatonin [25]. Studies with melanoma and breast cancer cell lines demonstrated that MT1 overexpression potentiates the growth suppressive effects of melatonin [49–51]. In estrogen receptor positive ductal breast carcinomas, MT1 protein levels decrease with tumor grade and positively correlate with patient overall survival [52]. Moreover, mRNA expression of MT1 is significant decreased in colorectal cancer compared to the adjacent mucosa [53]. In contrast, activation of MT2 receptors in the brain has been linked to the neuroprotection conferred by melatonin following ischemic strokes [54, 55]. Treatment with melatonin enhances endogenous neurogenesis and cell proliferation in the peri-infarct regions in a MT2-dependent manner, improving survival rates and the neural functioning of mice [54]. Under high glucose conditions, melatonin has also been shown to prevent neuronal cell apoptosis via a MT2/Akt/NF-κB pathway [56]. Importantly, in Alzheimer's disease patients, the hippocampal expression of MT1 is upregulated and of MT2 is downregulated [23, 24]. Moreover, MT2 activation prevents the disruption of dendritic complexity and spine induced by amyloid β in hippocampal neuron cultures [57]. Altogether, these findings corroborate the idea that MT1 and MT2 have opposite roles controlling cell proliferation in the brain, a pattern that seems to be preserved in brain cancer cells, as revealed by our data. Recent studies using subtype selective receptor ligands and knockout mice suggest that MT1 and MT2 also play differential roles in processes such as the control of

sleep and body temperature [58, 59]. Activation of MT1 receptors seems to be implicated in the regulation of rapid eye movement (REM) sleep, whereas MT2 receptors selectively increase non-REM (NREM) sleep [58]. MT1 knockout mice have an increase in NREM sleep and a decrease in REM sleep, while MT2 knockout mice have a decrease in NREM sleep. Regarding thermal regulation, administration of the MT1-selective partial agonist UCM871 and the MT2selective partial agonist UCM924 impact body temperature at different times of the dark phase and with opposite magnitude. UCM871 enhances body temperature just after the light-dark transition, and UCM924 decreases body temperature just before the dark-light transition [59]. The current mainstay treatment of glioblastomas (i.e., maximal surgical resection, concurrent chemoradiation, and adjuvant chemotherapy) offers only palliation and is normally followed by tumor recurrence [60]. In this regard, the clinical significance of glioma stem cells is supported by studies showing their ability to promote radioresistance by preferential activation of the DNA damage responses [61] and to propagate tumor growth after chemotherapy [62]. Single-cell RNA-seq characterization of different types of glioma, including glioblastomas, has also shown that cycling cells within human tumors are enriched in stem-like subpopulation [47, 63, 64]. Notably, the MT1 agonist and MT2 antagonist 5HEAT suppressed the expression of multiple genes involved in cell cycle, protein translation, and energy metabolism in stem cell-enriched cultures, a mechanism that likely contributes to the robust growth suppressive effect of 5-HEAT observed in vivo. Additionally, the ability of 5-HEAT to downregulate the expression of both glycolysis enzymes and mitochondrial proteins might be specially beneficial given the capacity of glioblastoma stem cells to rely on both oxidative and non-oxidative glucose metabolism, depending on the environment conditions [65, 66]. Overall, here we provided the first evidences regarding the differential role of MT1 and MT2 in brain tumor progression, supporting further investigations of their specific signaling pathways [67]. Our findings suggest that melatonin anti-tumor effects mediated by MT1 can be counterbalanced by the protumor MT2 activation that could be especially relevant in tumors expressing low MT1 and high MT2. More importantly, we highlight the clinical potential of selective melatonergic compounds, 5-HEAT and UCM799, that activate MT1 and/or inhibit MT2 receptors in brain cancer therapy. Both compounds might be of particular clinical interest as their amphiphilic molecular structure [43, 44] allows them to cross the blood-brain barrier and penetrate the central nervous system following systemic administration, overcoming the drug delivery challenge that is often faced in neuropharmacology.

The detailed characterization of the biological roles of melatonin receptors is essential for a rational clinical application of melatonin and other melatonergic compounds in the treatment of brain tumors. Despite the widespread interest in using melatonin as an adjuvant anticancer therapy, our understanding of the specific effects mediated through MT1 and MT2 receptor activation remains poorly understood. Although accumulating studies support the anticancer properties of melatonin in different tumor types, in vitro and in vivo animal models experiments often involve millimolar concentrations of this indolamine (Li et al., 2017), which likely trigger diverse receptor-independent mechanisms, masking the impact of MT1 and MT2 activation. Here we demonstrated that in glioma and medulloblastoma the receptor-dependent anti-proliferative effect of melatonin is mediated by the activation of MT1 whereas MT2 seems to play a pro-tumor role and has a significantly higher mRNA expressionin tumors compared to the normal brain cortex. Accordingly, the ratio between MT1/MT2 expression is a positive prognostic factor of patient survival, being particularly relevant when considering tumors with higher local production and accumulation of melatonin Finally, we also showed the potential of MT1 and MT2 receptors as druggable therapeutic targets, as the simultaneous activation of MT1 and inhibition of MT2 promotes a decrease in the expression of genes related to cell cycle, metabolism, and protein translation in glioma stem-like cells, besides impairing tumor growth in vitro and in vivo.

MT1 has often been recognized as the mediator of receptor-dependent antitumor actions of melatonin[25]Studies with melanoma and breast cancer cell lines demonstrated that MT1 overexpression potentiates the growth suppressive effects of melatonin (Collins et al., 2003; Kadekaro et al., 2004; Yuan, Collins, Dai, Dubocovich, & Hill, 2002).. In estrogen receptor positive ductal breast carcinomas, MT1 protein levels decrease with tumor grade and positively correlates with patient overall survival (Jablonska et al., 2013). Moreover, mRNA expression of MT1 is significant decreased in colorectal cancer compared to the adjacent mucosa (Nemeth et al., 2011). In contrast, activation of MT2 receptors in the brain has been linked to the neuroprotection conferred by melatonin following ischemic strokes (Chern, Liao, Wang, & Shen, 2012; C. H. Lee et al., 2010). Treatment with melatonin enhances endogenous neurogenesis and cell proliferation in the peri-infarct regions in a MT2-dependent manner, improving the survival rates and neural functioning of mice (Chern et al., 2012). Under high glucose conditions, melatonin has also been shown to prevent neuronal cell apoptosis via an $MT_2$ /Akt/NF-κB pathway (Onphachanh et al.,

2017). More importantly, in Alzheimer's disease patients, the hippocampal expression of MT1 is up- and of MT2 is down-regulated (Savaskan et al., 2005, 2002). Moreover,MT2 activation prevents the disruption of dendritic complexity and spine induced by amyloid β in hippocampal neuron cultures (Tang et al., 2019). Altogether, these findings corroborate the idea that MT1 and MT2 have opposite roles controlling cell proliferation in the brain, a pattern that seems to be preserved in brain cancer cells, as revealed by our data. Recent studies using subtype selective receptor ligands and knockout mice suggest that MT1 and MT2 also play differential roles in processes such as the control of sleep and body temperature [58, 59]. Activation of MT1 receptors seems to be implicated in the regulation of rapid eye movement (REM) sleep, whereas MT2 receptors selectively increase non-REM (NREM) sleep [58]. MT1 knockout mice have an increase in NREM sleep and a decrease in REM sleep, while MT2 knockout mice have a decrease in NREM sleep. Regarding thermal regulation, administration of the MT1-selective partial agonist UCM871 and the MT2selective partial agonist UCM924 impact body temperature at different times of the dark phase and with opposite magnitude. UCM871 enhances body temperature just after the light-dark transition, and UCM924 decreases body temperature just before the dark-light transition [59]. The current mainstay treatment of glioblastomas (i.e., maximal surgical resection, concurrent chemoradiation, and adjuvant chemotherapy) offers only palliation and is normally followed by tumor recurrence [60]. In this regard, the clinical significance of glioma stem cells is supported by studies showing their ability to promote radioresistance by preferential activation of the DNA damage responses [61] and to propagate tumor growth after chemotherapy [62]. Single-cell RNA-seq characterization of different types of glioma, including glioblastomas, has also shown that cycling cells within human tumors are enriched in stem-like subpopulation [47, 63, 64]. Notably, the MT1 agonist and MT2 antagonist 5HEAT suppressed the expression of multiple genes involved in cell cycle, protein translation, and energy metabolism in stem cell-enriched cultures, a mechanism that likely contributes to the robust growth suppressive effect of 5-HEAT observed in vivo. Additionally, the ability of 5-HEAT to downregulate the expression of both glycolysis enzymes and mitochondrial proteins might be specially beneficial given the capacity of glioblastoma stem cells to rely on both oxidative and non-oxidative glucose metabolism, depending on the environment conditions [65, 66]. Overall, here we provided the first evidences regarding the differential role of MT1 and MT2 in brain tumor progression, supporting further investigations of their specific signaling pathways [67]. Our findings suggest that melatonin anti-

tumor effects mediated by MT1 can be counterbalanced by the protumor MT2 activation that could be especially relevant in tumors expressing low MT1 and high MT2. More importantly, we highlight the clinical potential of selective melatonergic compounds, 5-HEAT and UCM799, that activate MT1 and/or inhibit MT2 receptors in brain cancer therapy. Both compounds might be of particular clinical interest as their amphiphilic molecular structure [43, 44] allows them to cross the blood-brain barrier and penetrate the central nervous system following systemic administration, overcoming the drug delivery challenge that is often faced in neuropharmacology.

**Authors' contributions** G.S.K., L.H.O., S.M.M., T.G.S., R.P.M., andP.A.F. designed the study. G.S.K., L.H.O., P.A.C.R., and R.C. performedthe experiments. S.M.M., I.T., S.R., V.R.M., T.G.S., R.P.M., and P.A.F.contributed with reagents, material, and analysis tools. G.S.K., L.H.O.,R.P.M., and P.A.F. analyzed the data, and G.S.K. wrote the manuscript.All authors critically revised the manuscript and approved the final version to be published. R.P.M. and P.A.F. supervised the project.

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**Conflict of interest**

The authors declare no conflict of interest.

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**Figures**

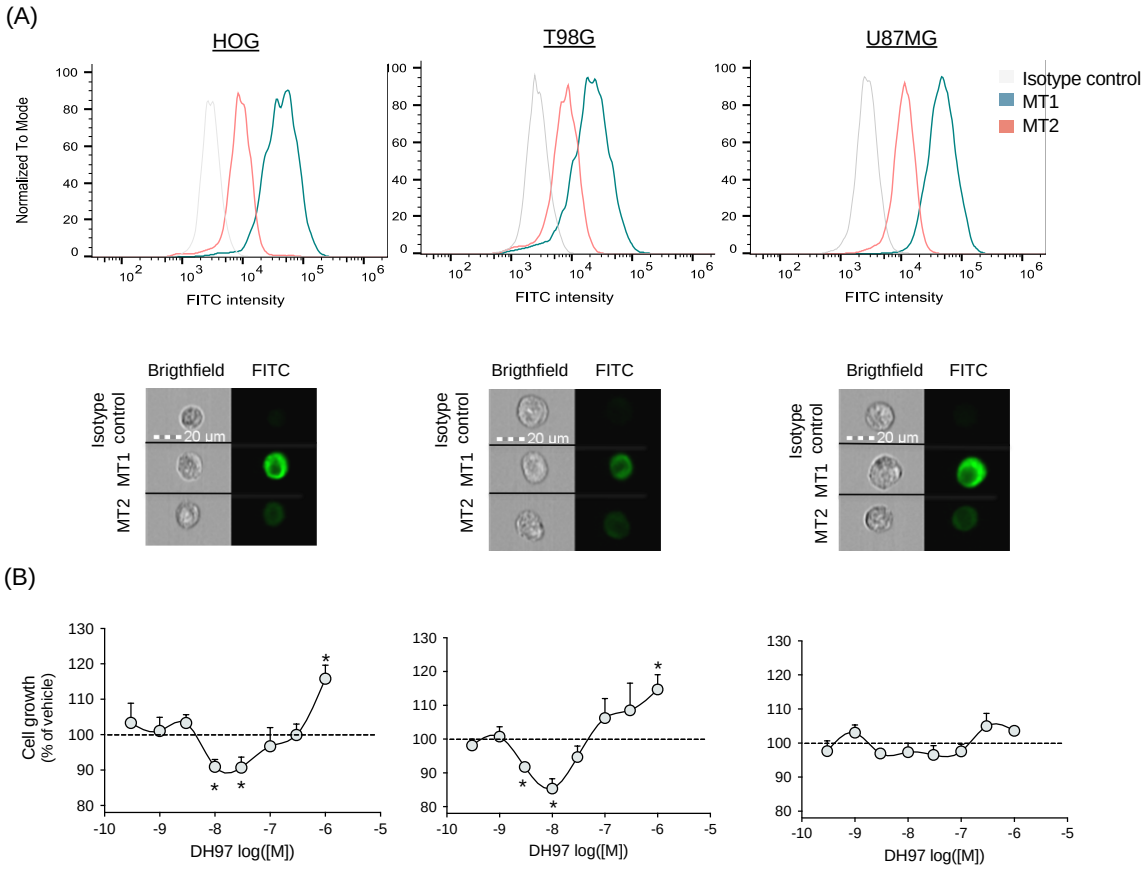


**Figure 1. The activation of MT1 and MT2 receptors play opposite roles in the control of glioma proliferation. (A)** Detection of melatonin membrane receptors by indirect immunofluorescence using imaging flow cytometry. Isotype control corresponds to cells stained with secondary antibody alone. **(B)** High- (HOG and T98G) and low-melatonin (U87MG) glioma cell lines were cultured for 48 h in the presence of DH97 ($3 \times 10^{-10}$ – $10^{-6}$ M), an MT2-selective antagonist (pKi = 8.03, 89-fold selectivity over MT1), or the respective vehicle ($7 \times 10^{-7}$ – $2 \times 10^{-3}$ % DMSO). Cell number was estimated by MTT assay and values were normalized by the mean absorbance detected in the respective vehicle group. Data are shown as mean ± SEM of four independent experiments in quadruplicates. * Significantly different from the respective vehicle group ($p < 0.05$) using the two-sided Student's t test.

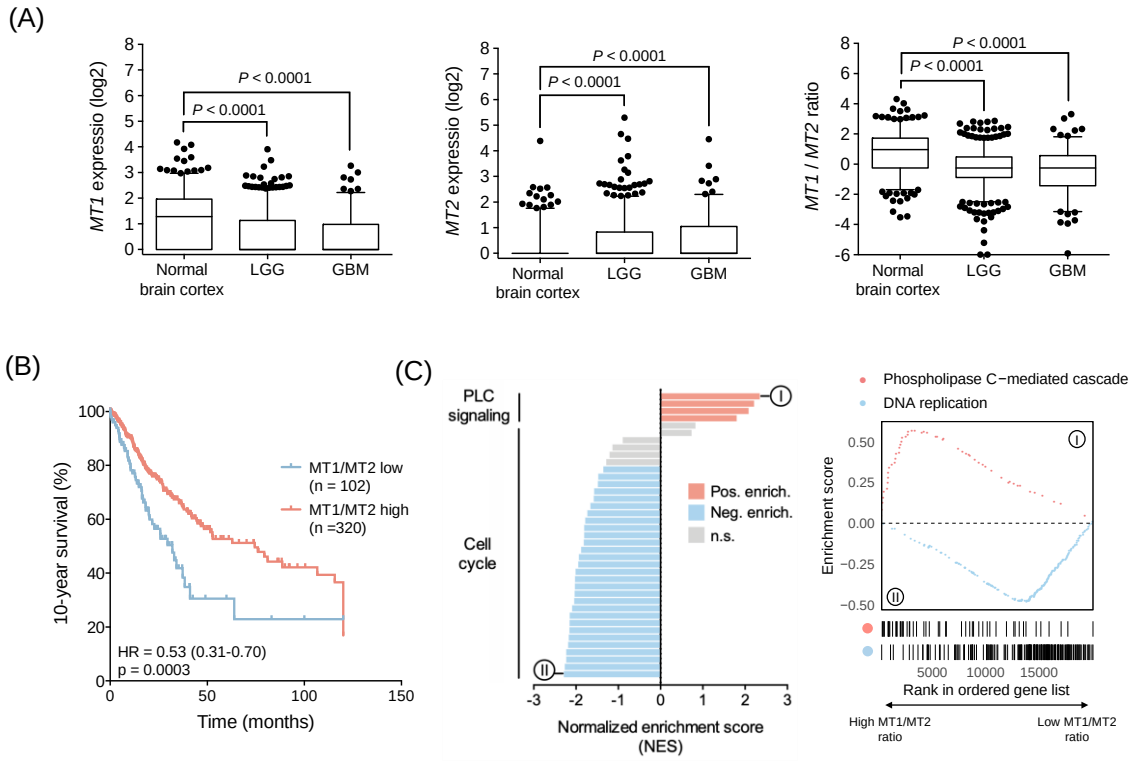


**Figure 2. Expression of *MT1* and *MT2* differentially impact glioma patient survival. (A)** Expression of *MT1* and *MT2* and the MT1/MT2 ratio in normal brain cortexes (n = 283) from the genotype-tissue expression (GTEx) and primary lower-grade gliomas (n = 509) and glioblastomas (n = 153) from The Cancer Genome Atlas (TCGA). Boxes extend from the 25th to the 75th percentile, the central bold line shows the median, and whiskers are drawn from the 5th to the 95th percentile. Comparisons were performed using the two-sided Student's t test. **(B)** Kaplan-Meier survival curves of gliomas patients with high vs. low MT1/MT2 expression ratios. Comparisons were performed using the log-rank test. **(C)** Gene set enrichment analysis testing the correlation between the MT1/MT2 expression ratio and the expression of genes related to the cell cycle and phospholipase C signaling in gliomas. Bar plots show normalized enrichment scores of the Reactome gene sets analyzed. FDR adjusted p values < 0.1 were considered statistically significant.

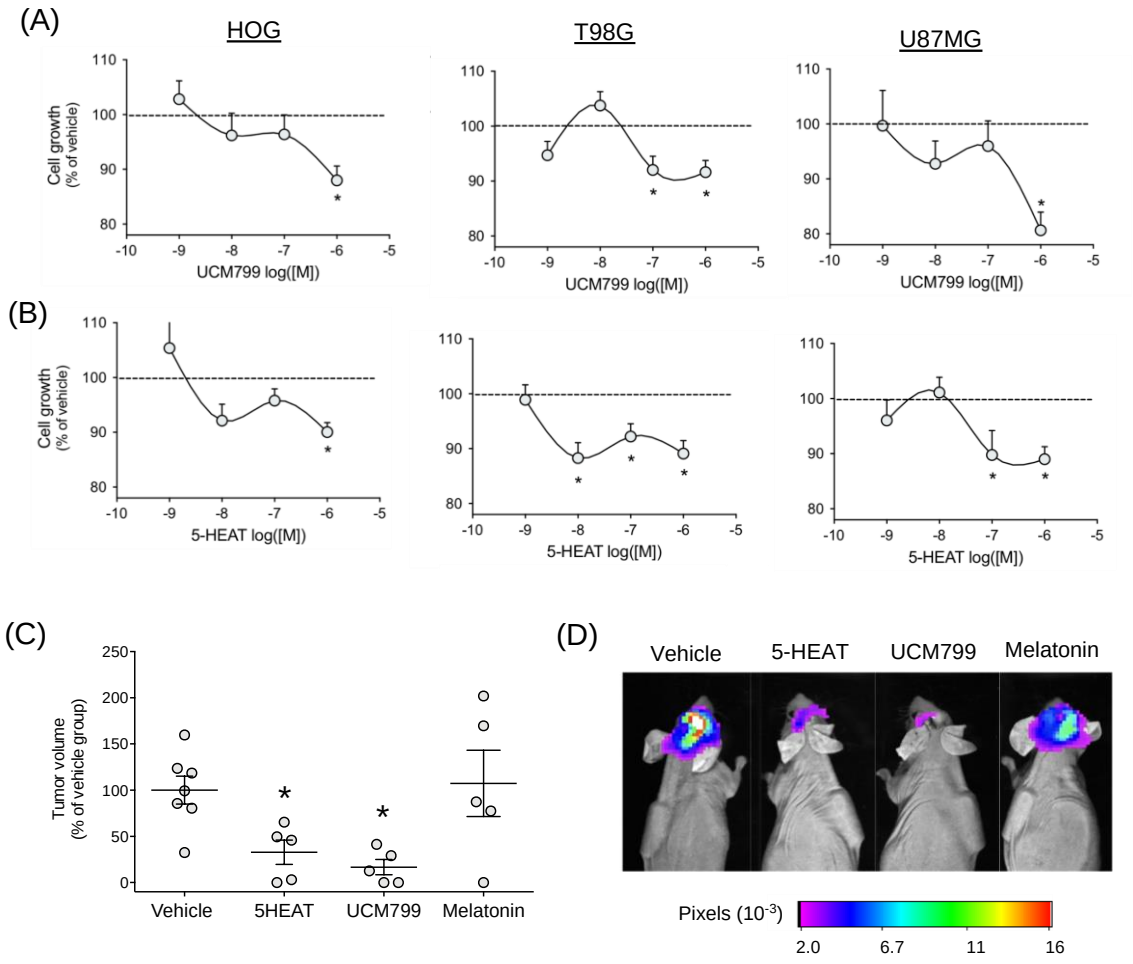


**Figure 3. The simultaneous activation of MT1 and inhibition of MT2 by fumctional selective melatonin receptor ligands impairs glioma growth in vitro and in vivo. (A)-(B)** High- (HOG and T98G) and low-melatonin (U87MG) glioma cell lines were cultured for 48 h with functionally selective drugs that simultaneously activate MT1 and inhibit MT2, 5-HEAT ($10^{-9}$ – $10^{-6}$ M, $pKi_{MT1}$= 7.77, $pKi_{MT2}$= 7.12) and UCM799 ($10^{-9}$ – $10^{-6}$ M , $pKi_{MT1}$= 7.3, $pKi_{MT2}$= 9.12), or the respective vehicle (2 x $10^{-6}$ – 2 x $10^{-3}$ % DMSO). Cell number was estimated by MTT assay and values were normalized by the mean absorbance detected in the respective vehicle group. Data are shown as mean ± SEM of four independent experiments in quadruplicates. **(C)** Mice with pre-stablished U87MG-luc orthotopic tumors received continuous brain infusions of vehicle (0.2% DMSO), $10^{-4}$ M 5-HEAT, $10^{-4}$ M UCM799 or $10^{-4}$ M melatonin. Mice were euthanized 14 days post treatment initiation for tumor volume evaluation. Values were normalized by the average tumor volume of the vehicle group. Data are shown as mean ± SEM of five independent

experiments.* Significantly different from the respective vehicle group ($p < 0.05$) using the two-sided Student's test . **(D)** In vivo bioluminescence imaging of tumor burden 14 days post treatment

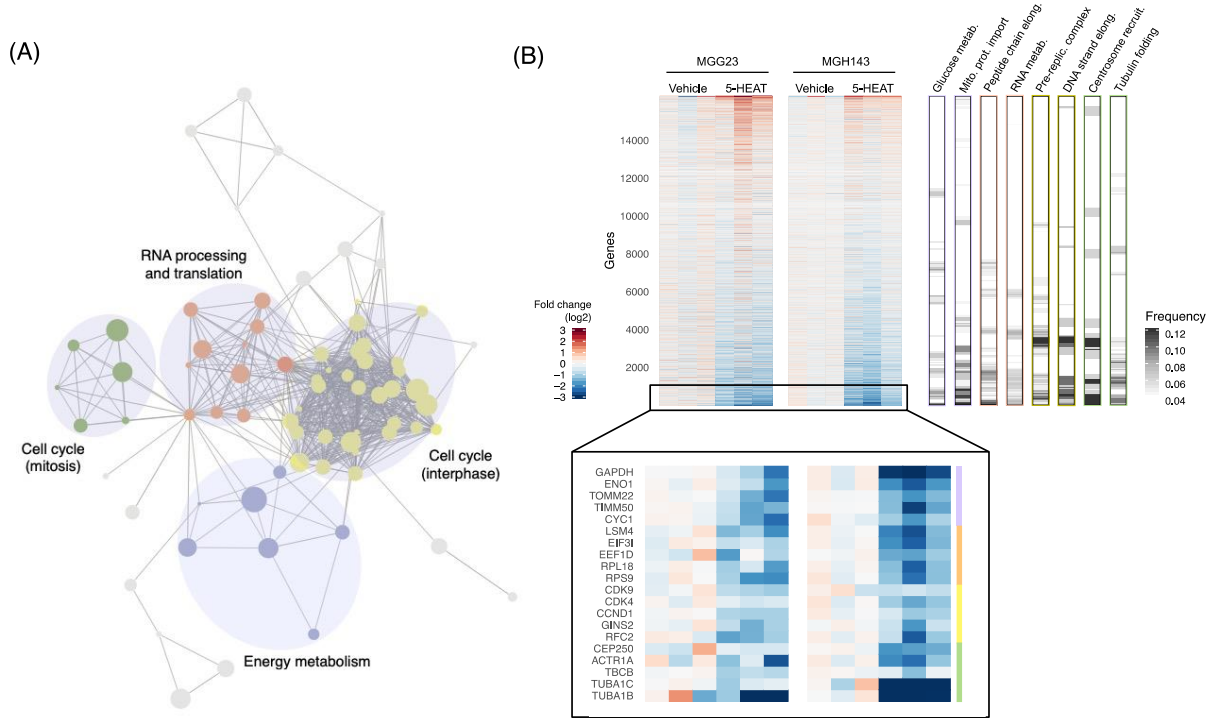


**Figure 4. Impact of 5-HEAT on the expression profile of glioma stem-like cells. (A)** MGG23 and MGH143 stem cell-enriched neurosphere cultures were incubated with $10^{-6}$ M 5-HEAT or vehicle ($2 \times 10^{-3}$ % DMSO) for 48 h and profiled by RNA-seq. Reactome gene sets differentially expressed in 5-HEAT treated cells compared to vehicle were identified using GSEA. Enrichment map shows negatively enriched gene sets (FDR adjusted $p < 0.1$) composing 4 main modules. Each gene set is a node and edges represent the similarity between gene sets. Node size shows enrichment significance (-log10(FDR-adjusted p)) and edge thickness is proportional to the overlap coefficient between gene sets. **(B)** Heatmap depicts the expression profile of 5-HEAT treated cells. Genes are ranked according to their average log2(expression fold change) in the 5-HEAT group compared to the vehicle. Bars on the right show the frequency of genes from selected gene sets within sliding windows of 500 genes. Relevant genes from each of the four gene set modules are highlighted in the bottom.

**Table 1. Multivariate Cox analysis of 10-year survival in gliomas.**

| | 10-year survival | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Low melatonin gliomas* | | | | High melatonin gliomas* | | | |
| | Univariate | | Multivariate | | Univariate | | Multivariate | |
| **Variable** | **HR (95% CI)** | **P value** | **HR (95% CI)** | **P value** | **HR (95% CI)** | **P value** | **HR (95% CI)** | **P value** |
| **Gender (male vs. female)** | 0.79 (0.52-1.22) | 0.297 | 1 (0.64-1.56) | 0.984 | 1.75 (1.06-2.9) | 0.03 | 1.46 (0.87-2.45) | 0.156 |
| **Age** | 1.07 (1.05-1.09) | <0.001 | 1.05 (1.03-1.06) | <0.001 | 1.07 (1.05-1.09) | <0.001 | 1.04 (1.02-1.06) | <0.001 |
| **IDH mutation (yes vs. no)** | 0.12 (0.07-0.2) | <0.001 | 0.16 (0.08-0.32) | <0.001 | 0.15 (0.09-0.25) | <0.001 | 0.32 (0.16-0.64) | 0.001 |
| **1p/19q codel (yes vs. no)** | 0.29 (0.14-0.6) | 0.001 | 0.62 (0.26-1.49) | 0.285 | 0.21 (0.09-0.49) | <0.001 | 0.38 (0.15-0.97) | 0.043 |
| **MT1/MT2 ratio (high vs. low)** | 0.61 (0.38-0.97) | 0.035 | 1.03 (0.63-1.69) | 0.897 | 0.48 (0.27-0.86) | 0.013 | 0.48 (0.27-0.87) | 0.015 |

HR: hazard ratio. CI: confidence interval

*As predicted by the ASMY:CYP1B1 index