Lucanthone, a Potential PPT1 inhibitor, Perturbs Stemness, Reduces Tumor Microtube Formation and Slows the Growth of Temozolomide-Resistant Gliomas in Vivo

Daniel P Radin1,*, Sophie Shifman1,*, Ian R. Outhwaite1,*, Aryan Sharma1, Robert Bases, Markus A. Seeliger1, Stella E Tsirka1‡

1Department of Pharmacological Sciences, *Stony Brook Medical Scientist Training Program, Renaissance School of Medicine at Stony Brook University, Stony Brook, NY 11794-8651

†Correspondence should be addressed to:

Stella E. Tsirka, PhD, Basic Sciences Tower 8-192, Stony Brook University, Stony Brook, NY, 11794-8651; Phone: 631-444-3859; Email: Styliani-anna.Tsirka@stonybrook.edu

Conflicts of Interest: The authors have declared that no conflict of interest exists.

Manuscript Information: Pages: 30; Figures: 5; Tables: 0; Words in paper: 8981

Keywords: Lysosome, Autophagy, Cancer Stem Cell, Temozolomide, PPT1

Running Title: Lucanthone Targets TMZ-Resistant Gliomas

List of Abbreviations

APE1 – AP endonuclease I
BBB – Blood-brain barrier
CNS – Central Nervous System
CSF1R – Colony Stimulating Factor 1 Receptor
CTLA4 – Cytotoxic T Lymphocyte-associated protein 4
EGF – Epidermal Growth Factor
FGF1 – Fibroblast Growth Factor 1
GBM – Glioblastoma
GluC2 – luciferase+ GL261 cells
GSC – Glioma Cancer Stem Cell
IVIS – in vivo imaging system
LAMP1 - lysosomal associated membrane protein 1
NF1 – Neurofibromin 1
NtBuHA - N-tert butylhydroxylamine
PD-1 - Programmed Cell Death Protein 1
PFA – Paraformaldehyde
PPT1 – palmitoyl protein thioesterase I
TCGA – The Cancer Genome Atlas
TM – tumor microtube
TME – Tumor Microenvironment
TMZ – Temozolomide
Treg – Regulatory T Cell
TTF – Tumor-Treating Fields
UGCG – UDP-glucose ceramide glucosyltransferase
VEGF – Vascular Endothelial Growth Factor
Abstract

Glioblastoma (GBM) is the most frequently diagnosed primary CNS tumor in adults. Despite the standard of care therapy which includes surgical resection, temozolomide chemotherapy, radiation and the newly added tumor treating fields, median survival remains only ~20 months. Unfortunately, GBM has a ~100% recurrence rate, but after recurrence there are no FDA-approved therapies to limit tumor growth and enhance patient survival, as these tumors are resistant to TMZ. Recently, our laboratory reported that lucanthone slows GBM by inhibiting autophagic flux through lysosome targeting and decreases the number of Olig2+ glioma stem-like cells (GSC) in vitro and in vivo. We now additionally report that lucanthone efficiently abates stemness in patient-derived GSC and reduces tumor microtube formation in GSC, an emerging hallmark of treatment resistance in GBM. In glioma tumors derived from cells with acquired resistance to TMZ, lucanthone retains the ability to perturb tumor growth, inhibits autophagy by targeting lysosomes and reduces Olig2 positivity. We also find that lucanthone may act as an inhibitor of palmitoyl protein thioesterase 1 (PPT1). Our results suggest that lucanthone may function as a potential treatment option for GBM tumors that are not amenable to TMZ treatment.

Significance Statement

We report that the anti-schistosome agent lucanthone impedes tumor growth in a preclinical model of temozolomide-resistant glioblastoma, and reduces the numbers of stem-like glioma cells. In addition it acts as an autophagy inhibitor and its mechanism of action may be via inhibition of palmitoyl protein thioesterase 1. As there are no defined therapies approved for
recurrent, TMZ-resistant tumor, lucanthone could emerge as a treatment for glioblastoma tumors that may not be amenable to TMZ both in the newly diagnosed and recurrent settings.

Introduction

Gliomas are tumors that may arise from multiple cell types in the CNS. Glioblastoma (GBM), a stage IV glioma, is the most common and aggressive primary brain tumor diagnosed in adults. GBM carries a poor prognosis despite treatment with surgery, temozolomide (TMZ) chemotherapy, radiation and the newly added Tumor Treating Fields (TTF) therapy (Stupp et al., 2017). Median survival is still only 16-20 months (Stupp et al., 2017). When GBM recurs, there exists no effective treatment modality to significantly extend patient survival.

The non-surgical treatments used in the management of GBM all involve targeting rapidly dividing cells and the eventual induction of DNA-damage-dependent cell death. To that end, cellular mechanisms that confer resistance to TMZ by augmenting DNA repair may be induced and rapidly confer resistance to ionizing radiation and TTF therapy. Of the multiple pathways initiated by DNA damage in glioma cells, principle among them is the induction of cytoprotective autophagy (Kanzawa et al., 2004; Ito et al., 2005; Lomonaco et al., 2009; Lin et al., 2012; Knizhnik et al., 2013; Zou et al., 2014; Hori et al., 2015; Lee et al., 2015; Ye et al., 2016; Shteingauz et al., 2018). Autophagy has not only been implicated in resistance to TMZ, radiation and TTF, but also supports tumor cell survival in areas of hypoxia (Hu et al., 2012; Jawhari et al., 2016; Abdul Rahim et al., 2017) and dampens anti-tumor immune responses (Liang et al., 2012; DeVorkin et al., 2019; Valdor et al., 2019; Sharma et al., 2020; Kim et al., 2021). The latter may
partially explain the lack of efficacy of PD-1 inhibitors in GBM patients. Therefore, inhibiting autophagy in GBM may offer an effective method of managing this intractable neoplasia.

Lucanthone, a known anti-schistosome agent, has been since described as an inhibitor of topoisomerase I and II (Bases and Mendez, 1997; Dassonneville and Bailly, 1999) during replication and transcription, as well as of AP endonuclease (Ape1) (Mendez et al., 2002; Luo and Kelley, 2004; Naidu et al., 2011), a key enzyme in base excision repair. We and others have shown that lucanthone acts as an inhibitor of autophagy (Carew et al., 2011; Radin et al., 2022) and is efficacious in the treatment of some types of solid tumors when paired with ionizing radiation. Lucanthone crosses the blood-brain-barrier (Bases and Mendez, 1997) and induces regression of breast cancer metastases (Del Rowe et al., 1999). It is eliminated via the liver and kidney. Patients tolerate a total dose of 10 mg/kg per day (which achieves serum levels of ∼3-4 µg/ml or 8–12 μM) well, with no signs of nausea or other side effects (Del Rowe et al., 1999). Our published data revealed that lucanthone is effective against mouse and human GBM lines in culture and in vivo and preferentially targets glioma stem-like cells (GSC), normalizes tumor vasculature, reduces tumor hypoxia, and increases cytotoxic T cell infiltration into developing tumors (Radin et al., 2022). Lucanthone is structurally similar to chloroquine/hydroxychloroquine and may target palmitoyl protein thioesterase 1 (PPT1), whose pro-tumorigenic function has been evidenced in several types of peripheral tumors (Amaravadi and Winkler, 2012; McAfee et al., 2012; Rebecca et al., 2017; Nicastri et al., 2018; Rebecca et al., 2019; Sharma et al., 2020). Indeed newer generation PPT1 inhibitors exhibit oncolytic activity against tumors arising from cells that are resistant to radiation and
immunotherapy (Rebecca et al., 2017), suggesting that PPT1 inhibition may be useful for the treatment of cancer after recurrence.

In the current study we investigate in detail lucanthone’s mechanism of action in GBM and show that it is able to slow the growth of tumors derived from cells resistant to high concentrations of TMZ by inhibiting autophagy and reducing the number of Olig2+ GSC. We also find that lucanthone abates tumor microtube (TM) network formation in GSC. Importantly, We provide evidence that lucanthone may act to inhibit PPT1 in patient-derived glioma cell lines. Our data further support the need to investigate clinical applications of lucanthone in TMZ-resistant GBM.

Materials and Methods

Glioma cell lines

Glioma cell lines were described previously (Radin et al., 2022). Briefly, luciferase+ GL261 cells (GLUC2) were a kind gift from Dr. Michael Lim’s lab. They are derived from a chemically induced astrocytoma in C57BL/6 mice (Ausman et al., 1970). Luciferase+ KR158 cells were obtained from the labs of Drs. Tyler Jacks and Behnam Badie, and are derived from genetically engineered Nf1/Tp53 mutants (Reilly et al., 2000). Two primary patient-derived glioma cell lines were also utilized in these studies: GBM43 and GBM9, which carry Nf1 and Tp53 mutations and Kras and Tp53 mutations, respectively. The lines were obtained from the Mayo Clinic Brain Tumor Patient-Derived Xenograft National Resource. Cells were maintained in DMEM, 10% serum, 1% antibiotic, 1% sodium pyruvate and incubated at 37°C with 5% CO2. To enrich for glioma stem-like cells (GSC), serum was reduced step-wise over a week as described previously.
(Yi et al., 2013). GSC were cultured in serum-free DMEM medium containing F12 supplement along with pyruvate, antibiotics, N2 supplement, EGF, FGF and heparin (Yi et al., 2013).

**Phalloidin Texas-Red Stain**

Phalloidin Texas Red Stain was performed according to manufacturer’s instructions. Briefly, GBM9 and GBM43 GSC were plated onto glass coverslips that had been pre-coated with a 5% Matrigel solution for two hours and allowed to adhere overnight. Cultures were treated with medium or 3 µM lucanthone for 24 hours after which the medium was aspirated and cells were fixed with 4% PFA for 10 minutes. Plates were then washed 3x with 0.3% TX-100 in PBS and wells were blocked with 3% normal goat/donkey serum/0.3% TX-100 in PBS for 1 hour. Cultures were then stained with Phalloidin Texas Red diluted 1:40 in PBS according to manufacturer’s instructions for an hour. Cells were then washed 3x with PBS, counterstained with DAPI and imaged under confocal microscopy.

**Animals**

C57/Bl6 (WT) mice were bred under maximum isolation on a 12:12 hour light:dark cycle with food *ad libitum*.

**Murine glioma model**

Gliomas established in immunocompetent mice were established in 3-4 month old male and female mice as described previously (Miyauchi et al., 2016; Miyauchi et al., 2018; Caponegro et al., 2021). Parental and TMZ-resistant GLUC2 GSC were dissociated with accutase and counted.
Mice were anesthetized with 20mg/kg avertin, a midline incision was made in the scalp, the skin retracted and a small burr hole was drilled in the skull at the following stereotactic coordinates from bregma: -1mm anteroposterior and +2 mediolateral. $1 \times 10^5$ GLUC2 GSC resuspended in PBS were injected over a period of 4 minutes at a depth of 3mm. At the end of the injection, the needle was kept in the injection site for a further 3 minutes. After needle removal, the incision was sutured and mice were placed on a heating pad until they fully recovered from anesthesia. During the disease course if mice were found to have lost more than 15% of their initial body weight, they were euthanized. All animal procedures were approved by the Stony Brook University Institutional Animal Care and Use Committee.

**Lucanthone treatment in vivo**

Lucanthone was supplied by Dr. Robert Bases (Albert Einstein College of Medicine). Lucanthone was solubilized in 10% DMSO, 40% 2-Hydroxypropyl-β-cyclodextrin in PBS. After confirming the presence of gliomas on day 7 (using IVIS bioluminescence visualization), mice bearing GLUC2 GSC were randomly divided into control and experimental groups, and treated with either saline or 50mg/kg lucanthone i.p. daily (days 7 to 20). On day 21, tumors were visualized by bioluminescent imaging, as above. For studies utilizing tumors derived from TMZ-resistant GLUC2 spheroids, mice were treated starting on day 7 through day 26, and then imaged and euthanized on Day 27. 11 control mice (5 male, 6 female) were used in the experiments. For the lucanthone-treated mice, 9 were used (4 male, 5 female).
RNA extraction from tumors

To prepare RNA from the glioma tissue, tumor-bearing mice were deeply anesthetized, euthanized and perfused with 30 ml ice-cold PBS. Tumors were microdissected from brains and mechanically dissociated in Trizol (Caponegro et al., 2021). To obtain cDNA, one microgram of RNA was reverse transcribed on a Veriti thermocycler using the High Capacity cDNA Reverse Transcription Kit. Amplification was performed on a StepOnePlus real-time PCR machine using a SYBR green kit (Applied Biosystems).

Immunohistochemistry

Mice were anesthetized with 20 mg/kg avertin and transcardially perfused with 30ml PBS followed by 30ml 4% PFA in PBS. Brains were removed and post-fixed in 4% PFA in PBS overnight. They were dehydrated for 48 hours in 30% w/v sucrose in PBS.Brains were then embedded in optimal cutting temperature compound (OCT, Tissue-Tek) and 20μm coronal sections throughout the tumor were taken on a Leica cryostat (Nusslock, Germany) and collected on Superfrost plus microscope slides. For immunohistochemical analysis, slides were brought to room temperature, washed 3x with 0.3% TX-100 in PBS and then blocked with 1% BSA/0.3% TX-100 in PBS for 1 hour. Slides were incubated overnight with appropriate primary antibodies (Ki67, Olig2, P62, Cathepsin D, LAMP1). The primary antibody was removed and slides were washed 3x 0.3% TX-100 in PBS and incubated with appropriate secondary antibodies for 1 hour. Slides were washed 3x with PBS, and counterstained with DAPI. Immunoreactivity was visualized by confocal imaging using the Leica SP8-x system, with white light and argon lasers.
Western blot

Immunoblotting was done as described previously (Radin et al., 2022). Briefly, cells were lysed in 50mM Tris-HCl (pH 7.4) with 1% Nonidet P-40, 0.25% sodium deoxycholate, 150mM NaCl, 1% SDS and 1mM sodium orthovanadate. Proteins were denatured by boiling and treatment with β-mercaptoethanol. Proteins were run on SDS-page gels, and transferred onto PVDF membranes (Immobilon; Millipore). Membranes were washed with Tris-buffered saline with 0.1% Tween 20 (TBS-T) and blocked in a 5% BSA in TBS-T solution for 1 hour. Membranes were then incubated with LC3 (1:1000, CST 2775S), PPT1 (1:1000, Thermo Fisher PA5-102896), and β-Actin (1:2000; Sigma Aldrich) primary antibodies overnight at 4°C. Membranes were rinsed in TBS-T, probed with associated HRP-conjugated secondary antibodies and exposed to SuperSignal West Pico PLUS Chemiluminescent Substrate for 1 minute (Thermo Fisher Scientific), and subsequently developed using the iBright Imaging System.

Molecular docking

Receptors were prepared for docking using PDBs 1eh5 (Bellizzi et al., 2000) and 3gro (Dobrovetsky et al., 2009). Residues in bovine PPT1 (PDB 1eh5) were mutated to corresponding human residues. Unmodeled residues were built in 3gro. Selenomethionines in 3gro were replaced with methionines. Non-protein atoms were removed and hydrogen atoms were added to both structures. Charges were added with AMBER ff14sb (Maier et al., 2015) and structures were minimized. Charges were added to lucanthone using AMBER ff14sb. Lucanthone was docked using a standard DOCK6 flexible (FLX) docking protocol (Mukherjee et al., 2010) against
both receptors. Importantly, DOCK6 binding spheres were generated across each receptor in order to explore a variety of potential binding sites. Poses were ranked by DOCK6 gridscore and the highest-scoring pose was selected. Figure images were generated in UCSF Chimera (Pettersen et al., 2004).

Statistical analysis
Data comparing two population means with a normal distribution were analyzed using Student’s t-test. Data with non-normal distributions were analyzed using a Mann-Whitney test. Differences in cumulative distributions were assessed with the Kolomogorov-Smirnov test. Tumor cell circularity was calculated using the equation $\text{Circularity} = 4 \pi \frac{\text{area}}{\text{perimeter}^2}$).

ANOVA and Tukey test was used to analyze western blot data. Alpha value was set at 0.05 prior to starting experiments. Power analysis was used to determine the appropriate number of animals used in each experiment. Viability experiments were replicated with the two tumor lines. Statistical analysis was performed using Graphpad Prism (Graphpad Software Inc, La Jolla, CA).

Results
Lucanthone exhibits increased potency over TMZ in perturbing GSC growth, proliferation and sphere formation

TMZ is the FDA-approved chemotherapeutic agent for GBM shown to selectively kill non-stem glioma cells. The non-targeted GSCs subsequently drive tumor recurrence (Chen et al., 2012). Increasing concentrations of TMZ were used to treat the GSC lines and cell survival
was quantified. TMZ exhibited an IC50 of >200 μM in each cell line and an IC90 of >750 μM (Figure 1A). Given that TMZ accumulates in brain tumors at a concentration less than 20μM (Portnow et al., 2009), these results reemphasize that TMZ only poorly targets GSC.

We previously showed that lucanthone at 10 μM was effective in reducing the growth of patient-derived GSC (Radin et al., 2022). To gain a better understanding of the effective dose-response range of lucanthone against GSC, cultures were treated with up to 3 μM lucanthone. Lucanthone exhibited an IC50 of ~1.5 μM for each cell line with an IC90 of ~3 μM (Figure 1B).

We also assessed the ability of tumor cells to form spheres upon lucanthone treatment. GSC were dissociated with accutase and plated in 96-well plates overnight. GSC were then treated with 0, 1, 2 or 3 μM lucanthone for 5 days, after which survival was assessed. In both cell lines concentrations of 2 and 3 μM lucanthone strongly reduced GSC proliferation and sphere formation by 75% and 90%, respectively (Figure 1C). In a limiting dilution assay a small number of cells (100, 200, 400) were treated with 400 or 800 nM lucanthone (Mulkearns-Hubert et al., 2019). Cultures were allowed to grow for two weeks and then were scored for sphere formation using light microscopy. 400nM lucanthone markedly reduced GBM9 and GBM43 sphere formation ability by more than 60%. 800nM treatment nearly abolished self-renewal in these cultures (Figure 1D). As lucanthone can penetrate the BBB and was shown to accumulate in the serum of patients at 8-12 μM (Del Rowe et al., 1999), these data highlight lucanthone’s ability to limit GSC self-renewal at clinically relevant concentrations.

Lucanthone reduces tumor microtube formation in GSC
As reported in other types of tumors (Osswald et al., 2015; Weil et al., 2017; Venkataramani et al., 2019; Horne et al., 2021; Xie et al., 2021; Joseph et al., 2022), GSC cells in our cultures seemed to form connections with other cells, through structures that resemble tumor microtubes (TM). Cultures treated with lucanthone for 5 days had fewer connections. To interrogate this possibility quantitatively, GSCs were plated in Matrigel-covered glass cover slips and treated with vehicle or 3 µM lucanthone for 24 hours, after which cultures were stained with Texas Red Phalloidin to visualize actin-based networks. In GBM43 GSC, control-treated cultures exhibited extensive TM formation, with some TM extending beyond 100µm (Figure 2A). 24-hour lucanthone treatment resulted in a pronounced decrease in the number of these connections between GBM43 GSC as well as a modest decrease in mean TM length (Figure 2A, B). The morphology of dissociated GBM43 GSC treated with lucanthone correlated with an increase in cell circularity (Figure 2A, B). GBM9 GSC were also interconnected at baseline (Figure 2C, D), and lucanthone treatment reduced the number of TM per cell and the length of TM (Figure 2C, 3D). Similarly, lucanthone-treated GBM9 cells were more round and less spread out (Figure 2C, 3D). These findings suggest that lucanthone treatment disturbs TM formation in GSC.

Lucanthone slows the growth of TMZ-resistant gliomas

Our culture data have indicated that lucanthone may serve as a potent adjuvant to overcome cytoprotective autophagy induction after DNA damage elicited by TMZ (Radin et al., 2022). Unfortunately, the clinical utility of lysosomal autophagy inhibitors, such as chloroquine, is very limited because they augment the systemic toxicity of TMZ and limit the clinical utility of
this dual treatment regimen (Compter et al., 2021). To examine whether lucanthone would still
be able to maintain its efficacy in gliomas that exhibit resistance to TMZ, we utilized cells that
had been selected to grow in high TMZ concentrations. We have reported that GLUC2 cells that
grow in high concentrations of TMZ exhibit increased levels of expression of the stemness
marker CD133 (Radin et al., 2022) (Figure 3A), suggesting a dynamic response to this continued
TMZ treatment. GLUC2 spheres were subjected to 5 cycles of 500 μM repeated TMZ treatment.
The spheres were dissociated and implanted into the striatum of mice and allowed to form
tumors for 1 week (Figure 3B). Tumor engraftment was assessed by bioluminescent (IVIS)
imaging. Animals with similar tumor luminescent intensity were matched (Figure 3C), and then
treated either with vehicle-control or with 50 mg/kg/day lucanthone for approximately 3
weeks. Tumor burden was assessed by IVIS imaging over time. 27 days post-inoculation,
relative luminescence of lucanthone-treated mice was approximately 50% of that displayed by
control-treated mice (Figures 3D, 3E). To measure the tumor size more accurately, the animals
were euthanized and the brain sections subjected to H&E stain (Figure 3F). Quantification of
tumor volume revealed that lucanthone reduced tumor growth by ~50% (Figure 3G). Our
results demonstrate that even after the GBM cells adapt to persistent TMZ treatment in vivo,
they may still be sensitive to lucanthone, likely because it acts through a mechanism distinct
from TMZ.

Lucanthone targets lysosomes, inhibits autophagy and reduces Olig2 levels in vivo in TMZ-
resistant gliomas
To examine whether the in vivo function of lucanthone was still to inhibit autophagy in the TMZ-resistant gliomas, tumor sections were stained for the autophagosome marker P62, the lysosomal protease Cathepsin D and the lysosomal membrane marker LAMP1. In control-treated tumors, P62 levels, as well as levels of Cathepsin D and LAMP1 were low (Figure 4A, B). In lucanthone-treated tumors, P62 was significantly increased as was Cathepsin D and LAMP1 (Figure 4A, B). These findings confirm that in vivo as well lucanthone exerted its oncolytic effects by inhibiting autophagy through targeting lysosomes.

We have previously shown that lucanthone’s effects on the glioma tumors results in reduced Olig2 levels (Radin et al., 2022). As shown in Figure 4C-D, control-treated tumors exhibited widespread Olig2 positivity, particularly near the edge of tumors. However, in lucanthone-treated tumors, Olig2 intensity as well as Olig2 area positivity were significantly decreased (Figure 4C, D), illustrating that lucanthone still retained its ability to reduce Olig2 levels in TMZ-resistant GLUC2 gliomas.

**Lucanthone may act as a PPT1 inhibitor**

Our previous results indicate that lucanthone targeted lysosomes and disrupted autophagic flux in glioma cells cultured in serum-rich medium and in culture conditions that enhanced the stem-like qualities of tumor cells (Radin et al., 2022). Drugs with autophagy inhibition activity, similar to lucanthone, have been shown to modulate the activity of palmitoyl protein thioesterase 1 (PPT1): the structurally-related autophagy inhibitor chloroquine was recently shown to act as an inhibitor of PPT1, which can enhance antitumor efficacy of anti-PD-1 treatment in melanoma (Sharma et al., 2020). We sought to examine whether PPT1 may be a
relevant marker in GBM, and whether lucanthone may act on PPT1. The prognostic value of PPT1 has been evaluated in peripheral cancers previously (Rebecca et al., 2019). To examine its potential prognostic utility in gliomas, we leveraged available TCGA data. Combining survival times of GBM and low-grade glioma, we found that patients with elevated PPT1 expression had a median survival of 33.7 months compared to 93.2 months for patients whose tumors exhibit low PPT1 expression (Figure s1A). We also found that PPT1 expression increases with tumor grade (Figure s1B), and is most elevated in the mesenchymal subtype of GBM (Figure s1C). Using the IVY GAP glioma atlas (Puchalski et al., 2018) we determined that PPT1 seems to be uniformly expressed among multiple different areas of the tumor (Figure s1D).

To investigate the interaction between PPT1 and lucanthone we modeled the preferred bound form of lucanthone. Bovine PPT1, which is 97.5% similar to human PPT1, assumes the same overall structure in the presence (PDB 1eh5) or absence (PDB 1ei9) of palmitate (Bellizzi et al., 2000). Only a single structure of human PPT1 is available (PDB 3gro) (Dobrovetsky et al., 2009) which has a Cα RMSD of 0.48 Å to 1eh5 excluding residues R151-A169 (PDB numbering); these residues hinge towards the palmitate-binding cleft (Figure 5A). The functional relevance of the 3gro conformation is unclear as it would not permit substrate binding (Figure 5B). Additionally, the 3gro conformation may be an artifact of crystal packing; R151-A169 are at the monomer interface and contribute to extensive intermolecular contacts.

We determined the most favorable pose of lucanthone using the flexible (FLX) DOCK6 protocol (Mukherjee et al., 2010). Prior to docking, bovine-specific residues in 1eh5 were converted to the analogous human residues. Importantly, we did not preselect any particular binding locations on the receptors and included all prepared DOCK6 binding spheres. The
The highest-scoring lucanthone pose across both PPT1 conformations was obtained with the PPT1 model generated from 1eh5. In this pose, lucanthone occupies a hydrophobic cavity distal to N-glycan modifications that is an extension of the palmitate-binding groove (Figure 5C). To our surprise, this cavity was previously identified as a predicted binding site for PPT1 inhibitors (Vergoten and Bailly, 2022). For example, PPT1 is a direct target of and potently inhibited by compound DC661 (Rebecca et al., 2019). The hydrophobic cavity occupied by lucanthone is also occupied by one headgroup of DC661 in the most energetically favorable pose of DC661 bound to PPT1 (Vergoten and Bailly, 2022). Together, the observations that the highest-scoring lucanthone pose occurs in a functionally associated PPT1 conformation, and that the predicted lucanthone binding cavity has also been described for known PPT1 inhibitors, support the hypothesis that PPT1 may be a target of lucanthone.

To gain additional insight into the molecular action of lucanthone, and whether its effects are mediated by PPT1, we used N-tert butylhydroxylamine (NtBuHA), a hydroxylamine derivative that has been shown to act as a chemical mimetic of PPT1 (Sarkar et al., 2013; Rebecca et al., 2017; Nicastri et al., 2018; Rebecca et al., 2019; Sharma et al., 2020; Brun et al., 2022). Previous studies have shown that NtBuHA reduces apoptosis of PPT1-deficient cells and alleviates motor and exploratory behavioral deficits exhibited by these mutant mice (Sarkar et al., 2013).

Glioma cells (GBM9 and GBM43) were plated in a 96-well plate and allowed to adhere overnight. The following day, cultures were treated with 1 mM NtBuHA for 30 minutes followed by 10 µM (for GBM43) or 5 µM (for GBM9) lucanthone for 3 days. Cultures were then subjected to MTT assay (Radin et al., 2022). Lucanthone treatment alone reduced viability by 40-70% in
the glioma lines. While NtBuHA alone had no effect on cell viability, its combination with lucanthone resulted in significant reversal of lucanthone’s oncolytic activity (by 33-45%, Figure 5D). This rescue of cell viability provides evidence that lucanthone may act as an inhibitor of PPT1, and that lucanthone-treated cells are amenable to rescue by NtBuHA. Further mechanistic evidence was obtained by assessing levels of autophagy and PPT1 after treating GBM9 cells with lucanthone in the presence and absence of NtBuHA. As expected, lucanthone treatment increased the amount of LC3B-II in GBM9 tumor cells (Figure 5E, G), indicating a significant modulation of autophagy. We also found that lucanthone decreased the levels of PPT1 in GBM9 cells (Figure 5E, F). Treatment with NtBuHA alone subtly increased PPT1 and did not alter LC3B-II levels. However, NtBuHA restored PPT1 reductions induced by lucanthone to control-treated levels (Figure 5E, F). Additionally, when GBM9 tumor cells were treated with lucanthone and NtBuHA, we observed a reduction in the elevation of LC3B-II induced by lucanthone alone (Figure 5E, G). These data, in line with viability data, demonstrate that a PPT1 mimic is able to partially alleviate the oncolytic effects of lucanthone, further supporting its function as a PPT1 inhibitor.

To examine whether lucanthone was able to affect PPT1 levels in vivo, tumor sections from animals inoculated with TMZ-resistant glioma (as in Fig. 4) were stained for PPT1 and Cathepsin D. In control-treated tumors, PPT1 levels were high whereas levels of Cathepsin D were low (Figure S2). In lucanthone-treated tumors, PPT1 was significantly decreased while Cathepsin D levels increased. These findings suggest that treatment of glioma with lucanthone in vivo resulted in reduced PPT1 levels.
To interrogate whether PPT1 mRNA expression is altered in vivo in tumors treated with lucanthone, mice were inoculated with GLUC2 GSC. After determining using luminescent imaging that these mice had tumors (day 7), they were subjected to daily administration of vehicle or 50 mg/kg lucanthone for two weeks. At the end of the treatment period, tumors were harvested and PPT1 levels were evaluated. RT-qPCR data indicated that the mRNA expression of PPT1 was not significantly altered by lucanthone treatment (Figure S1E). However, lucanthone did reduce Olig2 mRNA expression in tumors (Figure S1E), in agreement with our previous in vitro and in vivo findings (Radin et al., 2022). This result indicates that lucanthone treatment may principally alter PPT1 protein levels without significantly modifying PPT1 mRNA levels.

Discussion

Our understanding of the cell-intrinsic and -extrinsic inputs underpinning the universal lethality of GBM has increased substantially over the past several years, yet they have not yet resulted in new therapies against GBM. The standard-of-care modalities are not sufficient to mitigate recurrence, which has very poor outcomes (Hegi et al., 2005; Stupp et al., 2005; Stupp et al., 2017). One possible explanation as to why novel, experimental treatments are not effective in recurrent gliomas is that they are only tested on treatment-naïve GBM cells/tumors, which have not been subjected to TMZ/radiation. When treatments that are shown to work in treatment-naïve pre-clinical settings are applied to the recurrent tumors, they often target glioma cells that have been selected for higher levels of, for example, drug efflux pumps. Augmented levels of drug efflux pumps may hinder the efficacy of novel drug
modalities even if those modalities operate in a manner distinct to that of TMZ (Veringa et al., 2013).

In this study, murine glioma GLUC2 cells were treated with exceedingly high doses of TMZ to select for cells with resistance to this chemotherapeutic, and then inoculated mice with the surviving cells. Since TMZ preferentially targets rapidly proliferating cells, it is not surprising that tumors arising from these cells grew at a slower rate: as a way of comparison, tumors arising from these TMZ-resistant cells at Day 27 were 40% smaller than tumors arising from parental GLUC2 GSC at 21 days post inoculation (Radin et al., 2022), suggesting that these tumors were affected by high-dose TMZ well after discontinuing the exposure to TMZ. Though these tumors grew at a slower rate, they were still susceptible to lucanthone treatment (Figure 3F, 3G). Prior work has illustrated that dimeric quinacrine, mechanistically similar to lucanthone, which were shown to be potent PPT1 inhibitors, are effective as monotherapies against pancreatic tumors that are derived from tumors refractory to radiation and anti-CTLA4 therapy (Rebecca et al., 2017). Here we provide evidence that lucanthone may also act as an inhibitor of PPT1. Our molecular docking studies indicate that lucanthone binds PPT1 in a manner similar to that of dimeric quinacrine (Figure 5C). Pre-treatment of cultures with the PPT1 chemical mimetic, NtBuHA, attenuates the oncolytic effects of lucanthone (Figure 5D) and reverses lucanthone’s autophagy inhibition (Figure 5E-G), supporting the idea that PPT1 protein may be a molecular target of lucanthone. We will conduct appropriate experiments to biochemically validate the inhibitory effect of lucanthone on PPT1, using either cellular thermal shift assays or Surface Plasmon Resonance.
To our knowledge, we are the first group to look at changes of PPT1 levels at the protein and mRNA level after treatment with a putative PPT1 inhibitor. We found that while acute treatment with lucanthone reduces PPT1 levels in culture (Figure 5F), PPT1 mRNA levels in tumors remain relatively unchanged after 2 weeks of treatment. While PPT1 protein levels may decrease, there is evidence to suggest that there are PPT1-independent mechanisms that may confer resistance to PPT1 inhibitors. For example, recent evidence underpins the role of UDP-glucose ceramide glucosyltransferase (UGCG) in mediating resistance to oncolytic agents that exert their effects by targeting lysosomes (Jain et al., 2022). Treatment of tumor cells with PPT1 inhibitors resulted in increased glycosphingolipids, cholesterol and sphingolipids in lysosomes and plasma membranes, while UGCG and PPT1 inhibition synergistically repressed tumor growth in vivo (Jain et al., 2022). This resistance mechanism may also be active in glioma and warrants further examination. A cell-extrinsic mechanism of lucanthone resistance may be due to pro-tumorigenic modulation of the TME.

Another result that has become evident after lucanthone administration is on tumor microtubes (TM). TM and the extraordinary extent to which glioma cells are connected in vitro and in vivo are a relatively newly described process in the glioma literature. With the hypothesis that TM may convey resistance to radiation, efforts are ongoing to target them using gap junction inhibitors such as meclofenamate (Osswald et al., 2015; Weil et al., 2017; Venkataramani et al., 2019; Horne et al., 2021; Xie et al., 2021; Becker and Eisenmann, 2022; Becker et al., 2022; Joseph et al., 2022; Zeyen et al., 2022). The relationship between lysosomes/autophagy and TM has yet to be investigated, though there is literature to suggest that lysosomal targeting may affect the genesis of TM. In human retinal pigmented epithelial
cells, chloroquine inhibits microtubule nucleation by reducing p150\(^{\text{glued}}\), the largest subunit of dynactin (Osswald et al., 2015). This finding suggests that it might be possible for lysosomal targeting agents to prevent TM formation, which may subsequently sensitize GSC to radiation. Another possible explanation of lucanthone’s effects on TM formation includes downstream cellular consequences of lysosomal autophagy inhibition. In tumor cells, lysosome membrane permeabilization can cause zinc to spill out into the cytoplasm resulting in mitochondrial dysfunction (Du et al., 2021) and the release of reactive oxygen species (Vera-Ramirez et al., 2018; Chen et al., 2021). The release of reactive oxygen species from damaged mitochondria has a wide array of cytoplasmic targets (Joseph et al., 2022), including the collapse of actin polymers into aggregates (Varland et al., 2019), which may explain lucanthone’s effects on TM formation in GSC.

The finding that lucanthone retains efficacy in TMZ-resistant gliomas is particularly encouraging considering there are no established therapeutic protocols that significantly extend survival of GBM patients who are resistant to TMZ. Lucanthone still retains the ability to slow tumor growth in animals inoculated with TMZ-resistant glioma cells, inhibiting lysosomal autophagy (evident by elevated p62, Cathepsin D and LAMP1). Further, lucanthone-mediated reduction in the numbers and distribution of Olig2-expressing cells is promising, and warrants pairing lucanthone and radiation in pre-clinical models of recurrent or TMZ-resistant GBM, as it may be a safe and effective additional therapeutic. Further studies with mouse models harboring different driver mutations are necessary to generalize the results. These experiments set the basis to examine in the future if lucanthone-mediated autophagy inhibition may
enhance the effectiveness of immune therapies including those that modulate the immunosuppressive tumor microenvironment.

Acknowledgements:

The authors would like to thank members of the Tsirka lab and Drs John Haley, Kenneth Shroyer and Yusuf Hannun for thoughtful discussions. This work was partially supported by an NIH F30CA257677 (DPR), NIH T32GM008444 (DPR, SS, IRO), SBU URECA (AS), an NIH R35GM119437 (MAS), an NIH T32GM136572 (IRO) and a Stony Brook University OVPR Seed Grant (SET).

Data Availability Statement:

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.

Author contributions:

DPR, SS, IRO, SET: Participated in research design
DPR, SS, AS, IRO: Conducted experiments
RB: Contributed new reagents
DPR, SS, IRO, MAS, SET: Performed data analysis
DPR, SS, AS, IRO, MAS, SET: Wrote or contributed to the writing of the manuscript
References:


Nicastro MC, Rebecca VW, Amaravadi RK and Winkler JD (2018) Dimeric quinacrine as chemical tools to identify PPT1, a new regulator of autophagy in cancer cells. Mol Cell Oncol 5:e1395504.


Valdor R, Garcia-Bernal D, Riquelme D, Martinez CM, Moraleda JM, Cuervo AM, Macian F and Martinez S (2019) Glioblastoma ablates pericytes antitumor immune function through


Figure Legends

**Figure 1. Lucanthone exhibits increased potency over TMZ in perturbing GSC growth, proliferation and sphere formation.** GBM43 and GBM GSC were treated with TMZ (A) or lucanthone (B) for 5 days, after which an MTT assay was performed. Bars are mean +/- SEM. N=3-4 independent experiments ANOVA p<0.01 for each drug treatment. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, Dunnett’s multiple comparison test to control-treated cells. (C) Calcein-AM stained cells after treatment with lucanthone for 5 days, illustrating reductions in cell number, sphere formation and sphere size. (D) Limiting dilution assay. GBM43 and GBM9 were plated in 96-well plates at low densities (100, 200, 400 cells per well) and treated with indicated concentrations of lucanthone for 2 weeks, after which sphere formation was assessed and quantified. Line indicates median sphere formation. N= 6-7 independent experiments ANOVA p<0.0001 for each cell line. ****p<0.0001, Dunnett’s multiple comparison test compared to control-treated cultures.

**Figure 2. Lucanthone reduces TM formation in GSC in vitro.** GBM43 (A) and GBM9 (C) cultures treated with medium or 3 μM lucanthone for 24 hours visualized by phalloidin Texas Red stain. Quantification of TM length, TM per cell and cell circularity of GBM43 (B) and GBM9 (D) cultures. **p<0.01, ****p<0.0001, Kolmogorov-Smirnov test comparing distributions to control-treated cultures.

**Figure 3. Lucanthone slows the growth of TMZ-resistant gliomas in vivo.** (A) GLUC2 cells selected for TMZ resistance express the stemness marker CD133. (B) Treatment scheme used
for the study. (C) Mice were segregated into treatment groups with statistically similar baseline tumor luminescence. (D) Fold increase in luminescence of each mouse at Day 27 versus Day 7. (E) Tumor luminescence of 6 mice per treatment group. (F) Representative examples of tumors of control- and lucanthone-treated mice. (G) Tumor volume quantifications. Bars are mean +/- SEM. N=9 or more mice per group. *p<0.05, ***p<0.001, t-test.

**Figure 4. Lucanthone inhibits autophagy and reduces Olig2 in TMZ-resistant gliomas.** (A) Representative immunohistochemical images of P62, Cathepsin D and LAMP1 in tumors of control- and lucanthone-treated mice. (B) Quantification of P62, Cathepsin D and LAMP1 intensity in control- and lucanthone treated tumors. Bars are mean +/- SEM, N=5 animals per group. **p<0.01, ***p<0.001, t-test. (C) Representative immunohistochemical images of Olig2 in tumors of control- and lucanthone-treated mice. (D) Quantification of Olig2 intensity and Olig2⁺ area in control- and lucanthone treated tumors. Bars are mean +/- SEM, N= 6 animals per group. **p<0.01, Mann-Whitney test, ***p<0.001, t-test.

**Figure 5. Lucanthone is a potential PPT1 inhibitor.** (A) 1eh5 (grey, R151-A169 dark green ribbon) accommodates palmitate (light green surface) while 3gro (light blue, R151-A169 dark blue) occludes the palmitate binding site. (B) Views of R151-A169 highlight local conformational divergence and incompatibility between structure 3gro and the location of palmitate in 1eh5. Three views are presented: without palmitate, with palmitate in the same orientation, and with palmitate in a second orientation. (C) Lucanthone preferentially occupies a hydrophobic pocket in PPT1 distal to N-glycan modifications in the palmitate-binding groove. N-glycan modifications
are highlighted in teal and palmitate in green. Top insert: hydrophobic residues are colored yellow, acidic residues are colored red, and basic residues are colored blue. Bottom insert: residues within 5Å of lucanthone are highlighted with numbering according to 1eh5. For clarity, hydrogen atoms on lucanthone are not illustrated. (D) GBM43 and GB9 cells were pretreated with 1 mM NtBuHA or media for 30 minutes prior to treatment with 10 or 5 μM lucanthone for 72 hours, respectively, after which an MTT assay was performed. Bars are mean +/- SEM. N=7 independent experiments **p<0.01, t-test. (E) Representative western blot of 3 biological replicates showing relative PPT1, LC3B-I, LC3B-II, and β-actin levels in GBM9 cells. From left to right treatment conditions: DMSO, lucanthone (5 μM), NtBuHA (1 mM), and the combination; treatment time: 72 hours. (F) Quantification of PPT1 and (G) of LC3-II protein levels of 3 separate experiments described in (E), normalized to loading control (β-actin) and normalized to control treatment condition. One-way ANOVA and Tukey multiple comparisons tests were performed. Bars are mean +/- SEM. N=3 independent experiments, *p<0.05, **p<0.01, ***p<0.001.
Figure 3

A

Parental

CD133 KI67

CD133 KI67

TMZ-Resistant

CD133 KI67

B

Temozolomide

Recovery

Repeat 4 months Surviving 500 µM Temozolomide

Dissociate Temozolomide-Resistant Spheroids

Implantation

1 week incubation

Control or Lucanthone Tx 3 weeks

C

Day 7

Luminescence before treatment stratification

p = 0.98

Control Lucanthone

D

Fold increase in luminescence (Day 7 / Day 7)

E

Control

Day 27

Lucanthone

F

Control

Lucanthone

G

Tumor volume (mm³)

Control Lucanthone

***
Figure 4

Panel A shows immunofluorescent staining of P62, CATD, and LAMP1 in control and lucanthone-treated samples. Panel B displays quantitative analysis of P62 and LAMP1 intensity. Panel C depicts Olig2 staining in control and lucanthone-treated groups. Panel D illustrates Olig2 intensity and area as a percentage of the field of view.

**Figure 4**