THE ANTI-CANCER EFFECTS OF THE NOVEL CURCUMIN ANALOG HO-3867 ON C6 GLIOMA STEM CELLS

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ABSTRACT

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Cancer is the second leading cause of global mortality and requires continuous research into novel treatment methods. Chemotherapy, radiation treatments, and surgery are often not adequate in part because of cancer stem cells (CSCs) that persist and cause tumor recurrence. Hence, researchers are examining natural remedies as complementary treatments. The turmeric ingredient curcumin is used in traditional medicine and exhibits potent anti-CSC properties but also low bioavailability that limits use in patients. Consequently, curcumin analogs with enhanced bioavailability and pharmacokinetics have been synthesized to suppress cancers. This project examined efficacy of curcumin analog HO-3867 in preparation for testing whether it also targets CSCs.

Cells in gliomas, a type of brain cancer, contain circadian clocks that offer potential for suppressing tumor growth by manipulating the molecular mechanism that generates circadian rhythms. Here, we examined effects of HO-3867 on rat C6 glioma cells that are a model for human glioblastoma, the deadliest type of glioma. We tested whether the circadian clock regulates the anticancer effects of HO-3867. Our initial dose-response curves indicated the EC_{50} for HO-3867 in C6 cells is 26.3 μM. To test whether the circadian clock modulates HO-3867 anticancer effects, the circadian clocks within the cells were synchronized and one-day treatments of 20 μM HO-3867 were delivered at 6-hour intervals for two days. Efficacy was evaluated using an assay of cell viability and morphological measures of cell death. No circadian rhythm effect on HO-3867 efficacy was detected, indicating its delivery as a drug at any time of

day is not likely to be restricted by a circadian rhythm in pharmacodynamics, although circadian rhythms in the body might modulate pharmacokinetics.

The circadian rhythm in cancer cells might help them survive by evading immune surveillance that fluctuates in a circadian rhythm. Immunotherapy shows promise in cancer treatments by targeting immune checkpoint proteins such as CD47, which inhibits phagocytosis of cancer cells. We evaluated CD47 expression in C6 cells to determine whether it is more highly expressed in CSCs than non-stem cancer cells as predicted from human glioma studies. Our preliminary results confirmed this prediction. Combining immunotherapy with agents that eliminate CSCs holds significant therapeutic potential.

Key Terms: Cancer, Glioblastoma, Curcumin, HO-3867, Circadian Rhythm

I dedicate this thesis to all first-generation college students, encouraging you to never believe anyone who says your dreams are unreachable.

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INTRODUCTION

Background

Curcuma longa (turmeric) is a well-known plant in many cultures, but it has more benefits than just its flavorful taste (Sharifi-Rad et al., 2020). Within the rhizome of turmeric is a phytochemical known as curcumin. Curcumin has been used in traditional medicine for more than 2000 years (Hatcher et al., 2008). Because of the antioxidant and anti-inflammatory properties that curcumin possesses it is a wonderful remedy in eastern cultures (Hatcher et al., 2008). Curcumin has been used in clinical trials and lab studies, due to its ability to inhibit the STAT3 pathway (Liu et al., 2018). Because curcumin has low bioavailability it was determined that curcumin would not be an effective cancer fighting agent (Zoi et al., 2021). To work around the problem of poor bioavailability researchers are working with curcumin congeners, metabolites, and analogs.

Curcumin has hundreds of congeners, metabolites, and analogs. Each of these have some variation relative to the original form of curcumin. Some of these variations are found in other parts of the *Curcuma longa* plant, some are biosynthesized, and others are made when the body breaks down food that has curcumin in it. HO-3867 is a synthetic curcumin analog that appears to have the same benefits as curcumin but is not metabolized as quickly (Dayton et al. 2010). Recently, HO-3867 effects have been studied with various types of cancer (Bixel et al., 2017).

To make curcumin and its different phytochemicals more effective it can be beneficial to work with natural properties such as the circadian clock and rhythms in the unfolded protein response (UPR) in cancer cells, which might allow curcumin to be more effective depending on circadian rhythms in bioavailability or efficacy. It has been determined that curcumin anticancer effects do have a circadian rhythm, and these can be predicted according to the circadian rhythm

in expression of period circadian regulator 2 (PER2), a gene used in the core circadian timing mechanisms (Sarma et al., 2016). It has been shown that the circadian clock plays an important role in cancer proliferation, and cancer treatment. When the circadian rhythm is continuously interrupted or when key circadian genes such as clock circadian regulator (CLOCK) or basic helix-loop helix ARNT like 1 (BMAL1) are interrupted or downregulated it has been shown that cancer proliferation has increased (Lee, 2021). However, causing an upregulation of PER2 has been shown to suppress tumor proliferation (Lee, 2021).

The UPR is the cell's way of controlling the endoplasmic reticulum when the cell is under stress. This stress typically happens when cancer treatments such as chemotherapy are introduced, and it is often persistently active in untreated cancer cells (Bonsignore et al., 2023). When ER stress is activated certain UPR proteins such as CD47, which gives off a "don't eat me" signal, and calreticulin (CALR), which provides an "eat me" signal, are expressed on the cell surface (Cook & Soto-Pantoja, 2017; Kaur & Roberts, 2016). It has been shown that in cancer cells that are being treated with methods such as surgery and chemotherapy CD47 is upregulated and CALR is downregulated (Hao et al., 2023; Kasikova et al., 2019). Targeting UPR proteins could be an effective way to treat cancer, and bringing down the levels of CD47 and upregulating the levels of CALR could make the cancer cells more vulnerable to immune cells.

Cancer and Chemotherapy

Within a eukaryotic organism there are complex biological mechanisms, which all are a part of cellular and molecular processes. The cells within an organism are equipped with biochemical signaling pathways that allow cells to communicate with other cells throughout the organism, which can have different functions, or to communicate with nearby cells. When biological

processes within cells such as DNA repair are interrupted, it can lead to persistent mutations, and if the damaged cell is not removed by phagocytosis, these cells can turn into cancerous cells. With these cancer cells spreading, it can cause damage to surrounding healthy cells and eventually it can lead to a tumor. Due to the complex biochemical signaling pathways, a damaged cell can spread to a different part of the body, and this is known as metastasis.

The earliest documented case of cancer was in 1500 BC, in Egypt (Fugue, 2014). Back then, surgery would be performed to remove the tumors and eventually the tumors would reoccur. These tumors result from cancerous cells that remain in the tissue, including cancer stem cells.

The first chemotherapy drug was discovered in 1942 by Louis Goodman and Alfred Gilman. Goodman and Gilman were working with the United States Army on developing a more effective biological warfare agent. Accidentally they discovered that the compound known as nitrogen mustard softened tumors in lymph nodes of mice, and they concluded that nitrogen mustard works on lymphoma (Devita & Chu, 2008). Chemotherapy is a common secondary treatment along with surgery for various types of cancer, and it can be used initially as well. Chemotherapy, while effective, can be very harmful for the patient receiving it. Chemotherapy damages healthy cells along with cancer cells, which can prevent the healthy cells from repairing themselves. While natural products have been a part of Eastern medicine for centuries, between the 1950s and 1960s there was a push to start using natural products to treat cancers (Huang et al., 2020).

Curcumin and HO-3867

Curcumin (Figure. 1), which is a polyphenol component found in the rhizome of the *Curcuma longa* plant was first used scientifically in research in 1815. Even though curcumin has been recently extracted and researched, it has been an effective medical tool in Eastern cultures

for centuries. Curcumin is known for having anti-inflammatory and antioxidant properties making it a wonderful candidate for cancer research. However, due to curcumin being hydrophobic and easily degraded it has very low bioavailability making it a poor cancer fighting agent. Due to the low bioavailability, and limited research in humans, the Food and Drug Administration (FDA) has not approved curcumin for use in cancer treatments (Hassanzadeh et al., 2020).

Curcumin interacts with multiple biochemical and molecular pathways (Figure 2) leading to it having effective anti-inflammatory, anti-microbial, and wound healing properties. Curcuminoids can inhibit the activity of transcription factors such as nuclear factor-κB (NF-κB) and the signal transducer and activator of transcription (STAT) family, as well as genes that regulate cell proliferation and apoptosis such as p53. The STAT inhibition is through suppression of the JAK2 pathway (Rath et al., 2014; Liu et al., 2018).

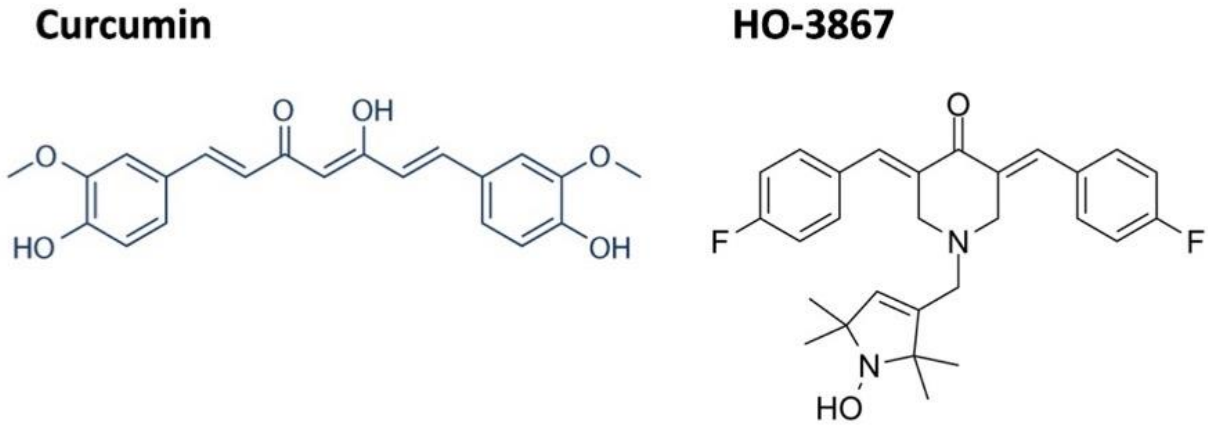

Figure 1: Comparing chemical structures. HO-3867 is a synthetic curcuminoid analog and a more specific STAT3 inhibitor than curcumin. From PubChem Compound (https://pubchem.ncbi.nlm.nih.gov/compound/46871899).

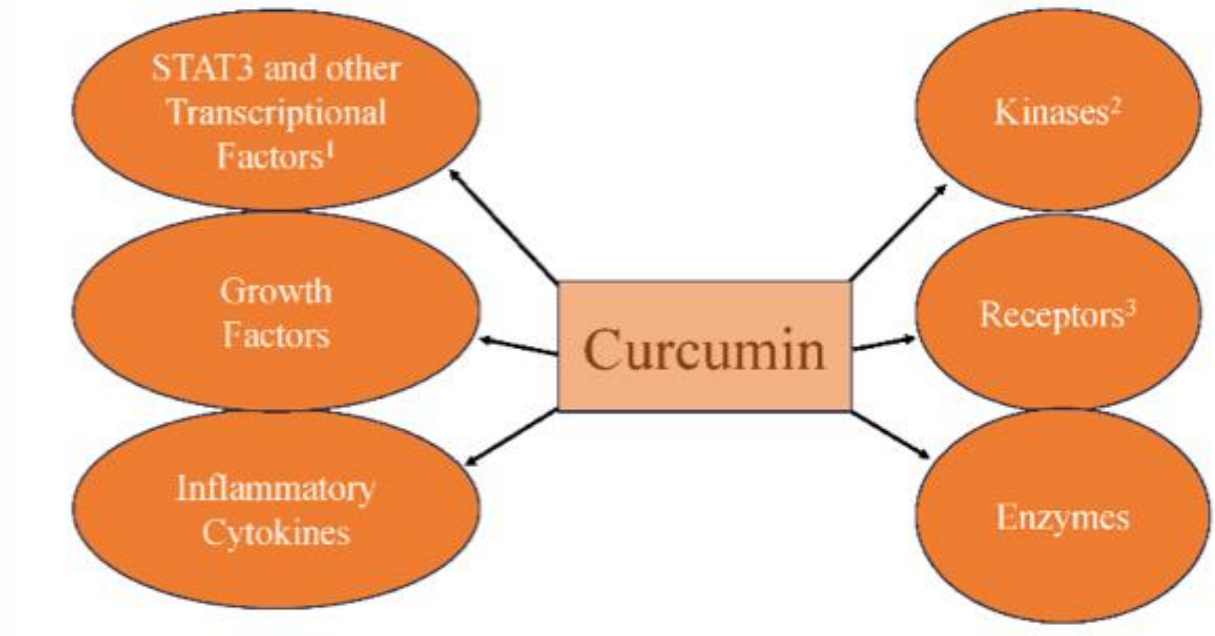

Figure 2: Molecular targets of curcumin. Multiple pathways are inhibited by curcumin. In contrast, curcumin upregulates (1) transcription factors ERE, Nrf-2, and PPAR-y; (2) JNK kinase pathway; (3) DR-5 receptor, p53, and DEF-40. Derived from: Noorafshan, A. et al., 2013 Current Pharmaceutical Design 19: 11.

HO-3867 (Figure 1), a biosynthesized version of curcumin also known as a curcumin analog. was patented in 2012 at The Ohio State University and was initially reported as having anticancer effects on ovarian cancer cells (Kuppusamy and Hideg, 2010). HO-3867 is known for having higher bioavailability than curcumin and for being a selective STAT3 inhibitor. On top of being a selective STAT3 inhibitor, HO-3867 binds to mutated p53 in cancer cells and causes it to act like wild-type p53. When HO-3867 restores the wildtype p53 this also changes the transcriptional activity and anticancer functions. Gaining the wildtype p53 has successfully caused a decline of cancer growth in mouse models (Madan et al., 2018). This restoration of p53 functioning helps to induce apoptosis within the cell (Chen et al., 2022). HO-3867 has been

tested on various forms of cancer including, lung, breast, pancreatic and many more but not gliomas. Throughout these studies it was noticed that HO-3867 targets cancerous cells but not noncancerous cells (Chen et al., 2022). Finding a treatment to target cancer cells is essential because, ideally, healthy cells should be minimally impacted when fighting cancer. HO-3867 has also been identified as having a longer bioavailability than curcumin (Dayton et al., 2010).

Cancer Stem Cells

Cancer is characterized as unregulated proliferation of abnormal cells and divergent recognition by the immune system (Yin et al., 2021). Within the cancer there is a small population of cells, known as cancer stem cells (CSCs) that have properties of embryonic and somatic stem cells. CSCs can renew themselves, migrate or metastasize, and differentiate into rapidly dividing cells that form the bulk of the tumor (Yu et al., 2012).

When cancer occurs in the brain it is usually because glial cells have become transformed and replicate uncontrollably creating a glioma. Some gliomas are known as ependymomas, and they are very deadly if not caught early. The glioblastoma is a deadly form of brain cancer created by transformed astrocytes. With glioblastomas being so deadly it is essential to find a way to treat this form of cancer. The most common treatments for gliomas include surgery, chemotherapy, radiation treatments, and immunotherapy. The major issue with these treatments is they typically leave behind glioma stem cells, as well as damaging the healthy cells surrounding the tumor. Leaving behind these specific glioma stem cells can be the cause of tumor and cancer recurrence (Piper, 2021).

Circadian Rhythms of Mammals

Circadian rhythms are generated by an endogenous timekeeper in the cells known as the circadian clock, which is also present in glioma cells (Fujioka et al., 2006). In mammals the

master circadian clock is in the suprachiasmatic nucleus (SCN) within the hypothalamus of the brain, and clocks are present in cells of nearly all tissues throughout the body. The circadian clock is best known for controlling the sleep/wake cycle of animals, but it does so much more than just that. The clock and its various genes rhythmically regulate hormones, cell growth, and even tumor progression (Lee, 2021). Because the circadian clock plays such an important role in cell functions it has been proposed that working with the molecular clock components or the rhythms generated by the circadian clock will improve treatment results. For example, insomnia, which can be caused by an interrupted circadian clock, is often treated with melatonin that naturally regulates sleep.

Along with cells of the suprachiasmatic nucleus that generate neural and hormonal circadian rhythms acting on the body, most individual cells within the body have a circadian clock that generates rhythmic signals (Chaix et al., 2016). Gliomas have an individual circadian rhythm in which the cells are more vulnerable to cancer treatment when the expression of BMAL1 or CLOCK, two core circadian clock genes, is altered (Wang & Chen, 2022). Identifying a circadian phase at which the cells are most vulnerable to treatment is important, because then treatments will be more effective and targeted towards these cells. The phase of a circadian cycle is often referred to as a specific hour of these near-24 hour cycles.

When C6 rat glioma cells were treated with 5 μM curcumin, it was observed that the treatment was more effective about six hours before the PER2-luciferase gene maximum was expressed in the cell (Sarma et al., 2016) (Figure 3). This study laid the groundwork for using curcumin and its analogs to treat cancers by working with the circadian rhythms to find the most effective time to administer the treatment.

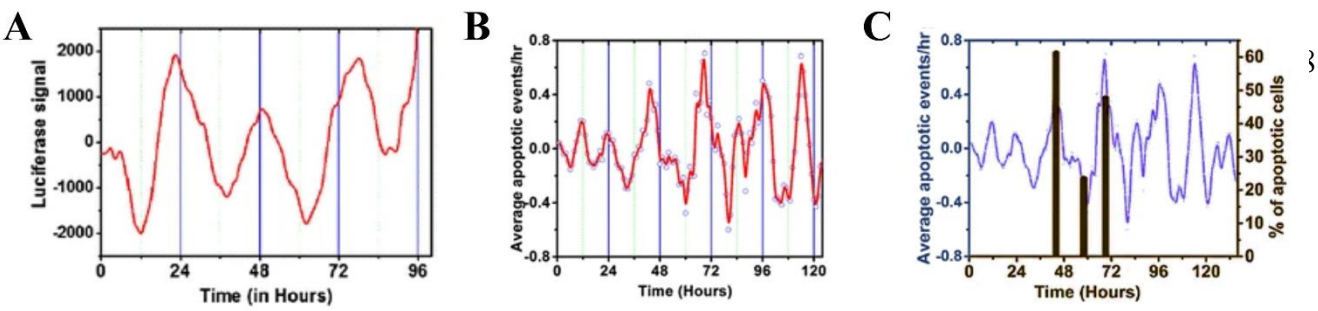

Figure 3: Circadian rhythms in C6 cells. A: Circadian rhythm in the expression of a reporter gene, *mPer2::mPer2:luc*, in a C6 rat glioma cell culture during exposure to a low concentration of curcumin (5 μM). Shown is the circadian rhythm in bioluminescence reflecting levels of the PER2-luciferase fusion protein. B: C6 cells given 5 μM curcumin at time zero show a circadian rhythm in cell death measured by apoptotic events (circles) observed through time-lapse imaging (line: 5-point running average). C: Intensity of immunofluorescence staining for apoptotic marker cleaved caspase-3 at three phases (bars) agrees with the rhythm in apoptotic rate shown in B. Shown are apoptotic events since treatment with forskolin to synchronize the circadian clocks in these cells. Derived from Sarma, A. et al., 2016 BMC Cancer 16: 759.

CD47

CD47 is expressed in cancer cells when the cell undergoes ER stress. CD47 is an essential protein for noncancerous cells because it protects the healthy cells from being phagocytosed by immune cells such as macrophages and microglia. When CD47 is expressed at the cell surface it provides a “don’t eat me” signal, suppressing the phagocytes. When this signal is expressed on cancer cells, it also inhibits their removal by phagocytosis. Typically, in cancer cells there is an over-expression of CD47 that protects the cells, and CD47 is reported to be further elevated in CSCs (Oliveira et al., 2024). When thinking about clinical treatments for cancer, it is very important to target CD47. Lowering expression of the protein or using immunotherapy with antibodies that block interaction of CD47 with its receptor, signal regulatory protein alpha (SIRPA), will allow for the immune cells to engulf the cancerous cells further preventing tumor growth (Jiang et al., 2021).

CALR

Like CD47, CALR is expressed on the cell surface during ER stress. CALR is an essential protein to allow macrophages and microglia to remove cells, particularly cells that are undergoing apoptosis following sustained ER Stress. CALR provides an "eat me" signal, inducing phagocytosis of the cell. In cancer cells, there is typically high cell-surface CD47 expression (Huang et al., 2020). When developing a cancer treatment, it may be beneficial to manipulate surface CALR and CD47 proteins simultaneously, which can be expressed at the same time on cancer cells. Upregulation of CD47 in cancer cells appears to be primarily at the transcriptional level (Sun et al., 2024), whereas increased surface CALR can result from changes in its translocation from the ER (Fucikova et al., 2021).

C6 Glioma Cell Line

The C6 rat astrocytoma cell line has been a research model to understand glioblastomas for decades. The C6 line originally was derived from a chemically induced tumor in a rat (*Rattus norvegicus*) (Benda et al., 1968). The C6 model has been tested and determined to have a circadian rhythm (Fujioka et al., 2006), and C6 cell cultures are inhibited by curcumin (Sarma et al., 2016).

Specific Aims

Aim 1: Test the anticancer effects of HO-3867 in C6 cell cultures.

HO-3867 is a curcumin analog reported to have better bioavailability than curcumin. These experiments tested whether it has anticancer effects on a glioma cell line and determined how its EC_{50} compares with that of curcumin. A dose-response curve was completed to find the EC_{50} for HO-3867 in C6 cell cultures. Two methods for quantifying cell survival were used, the

cell viability stain PrestoBlue and morphological changes in cell shape and size indicative of apoptosis.

Hypothesis: HO-3867 will be an effective cancer fighting agent in C6 glioma cell lines.

Aim 2: Test whether HO-3867 is more effective when administered at a specific phase of the circadian clock.

C6 cells were selected for these experiments to determine the effects of the circadian clock on the anticancer effects of HO-3867. It has been shown that C6 cells have circadian rhythms and that they are an effective model for understanding glioblastomas. Pharmacokinetics and pharmacodynamics influence the efficacy of drug treatments, and both can be modulated by the circadian timing system at the level of the whole organism as well as at the cellular level. Examining the efficacy of HO-3867 in cancer cell cultures rather than intact animals allows us to predict the ability of the circadian clock within the cells to influence the compound's efficacy. Using the C6 cell model provides a view of the compound's effects without influences from the many other steps that would occur between administering the substance to a patient and when it reaches the cells.

If the C6 circadian clock alters effects of HO-3867, then this rhythm should be taken into consideration when evaluating the efficacy of this compound in vivo. On the other hand, if there is no significant effect of the cancer cell intrinsic clock in this experiment, then this part of the overall drug actions might be ignored when examining circadian influences on treatments based on HO-3867. Emphasis would then be placed on understanding how the circadian clock may alter its pharmacokinetics. Ultimately, it may be found in animal studies that HO-3867 effects are far less altered by the circadian system than other treatments and would then be useful when administered to patients at any circadian time.

Hypothesis: HO-3867 will cause greater cell death when applied 6-11 hours before the phase of maximal PER2 expression as observed previously when C6 cells were given curcumin that was allowed to remain in the cultures. Essentially, HO-3867 will show the circadian modulation reported for curcumin.

Aim 3: Determine whether the amount of cell-surface CD47 expression is different in stem-like C6 cells relative to non-stem C6 cells.

This aim was a preliminary experiment to use in eventually determining the effects of HO-3867 on the immune checkpoint protein CD47 in C6 CSCs through immunocytochemistry. Previous results in the lab confirmed that C6 cells express CD47 and CALR proteins on the cell membrane and their expression levels were correlated. Furthermore, CD47 was identified in CSCs in these cultures. This experiment provided an additional test of whether CD47 is expressed at higher or lower levels in CSCs relative to non-stem C6 cells.

Hypothesis: CSCs will have a higher expression of CD47 than non-stem C6 cells.

MATERIALS AND METHODS

Cell Cultures

The C6 cell line was obtained from ATCC (catalog number CCL-107). The cells were cultured at 37℃ and 100% humidity in Final Medium consisting of MEM (Modified Eagle Medium) without phenol red (Cellgro, Corning) with bicarbonate levels reduced to 4 mM, 10 mM Hepes (2-[4-(2-hydroxethyl) piperazin-1-yl] ethanesulfonic acid) buffer, and 10% fetal bovine serum (FBS). Penicillin, streptomycin and amphotericin B were added, and pH was adjusted to 7.2. This medium is designed for use in room air to prevent shifts in pH during manipulations outside a CO_2 incubator and was used previously for C6 cells (Sharma et al, 2014). For the PrestoBlue assay the C6 cells were grown in 96-well cell culture plates containing Final Medium. Brightfield images were captured with an Olympus CK 40 inverted microscope (S/N: 9J0671).

Synchronizing Circadian Clocks in C6 Cells

Bringing the circadian clocks within the cells to a common phase of the circadian cycle is essential when doing this circadian rhythm study of a cancer cell population. The resetting of the circadian clocks relied on a 2-hour 20 μM forskolin treatment that was used previously with C6 cells for this purpose (De et al., 2020). Placing all the cells on the same time schedule as each other makes it easier to determine the circadian phases in which the cells may be more vulnerable to the anticancer treatment.

PrestoBlue Absorbance Assay

PrestoBlue (ThermoFisher Scientific) is a cell viability reagent that requires a short incubation time after adding the reagent before data collection can begin. PrestoBlue was also selected because it allows cells to continue growing after adding PrestoBlue, thereby

strengthening the signal from the reaction product produced through reduction of PrestoBlue by the cells. To test the relative cell viability the absorption of PrestoBlue was measured at 540 nm and 620 nm with a Multiskan MCC plate reader. An average of three measurements was used to improve the signal-to-noise ratio. Each data set was processed using the subtraction analysis method provided by the PrestoBlue supplier (Life Technologies). Absorption at 620 nm (A_{620}) was subtracted from absorption at 540 nm (A_{540}), and to correct for cell density the average A_{540}-A_{620} from control wells was then subtracted.

Data were normalized to the control cells that were given only the DMSO vehicle and no HO-3867, for the maximum possible signal (100%), and to dishes without added cells, for the minimum. Averaged values from the normalized dose-response curves of two complete replicate experiments were then analyzed with GraphPad software to find the best fit of a four-point sigmoidal dose-response curve, which indicated the effective concentration producing a 50% loss of cell viability (EC_{50}).

Immunostaining and image analysis

C6 cells were grown in glass-bottom 35-mm plastic petri dishes (MatTek). Except where indicated, all remaining procedures were at room temperature and under dim lighting to avoid photobleaching the fluorophores. The medium was replaced with Final Medium without FBS, which was exchanged with Hoechst 33342 stock solution (ThermoFisher Scientific) diluted 1:1000 in Final Medium. After five minutes, the medium was removed, and the dish was rinsed twice with medium. The cells were fixed with 2 ml cold methanol for five minutes and then rinsed twice with 2 ml phosphate-buffered saline (PBS) three times for 5 minutes each. The dish was drained and given 2.5% normal horse serum (ReadyProbes, ThermoFisher Scientific) for blocking. After twenty minutes, the dishes were drained and given the primary antibody, mouse

monoclonal anti-CD47 (BioXCell, BE0283), diluted 1:100 in PBS. The plate was then wrapped in foil and stored at 5℃ overnight. After 19-hours the dishes were rinsed with 2 ml PBS three times for 5 minutes each. Secondary antibody was added, donkey anti-mouse IgG conjugated with Alexa Fluor 568 (Life Technologies), diluted 1:100 in PBS. After one hour the cells were drained and rinsed with 2 ml PBS three times for 5 minutes each. The PBS was replaced with 70% glycerol in PBS.

The cells were then imaged with a confocal microscope system consisting of a DMI3000B inverted microscope (Leica Microsystems, Buffalo Grove, IL, USA) equipped with a Spectra X LED light engine (Lumencore, Beaverton, OR USA), X-Light spinning-disk confocal unit (CrestOptics, Rome, Italy) and a Rolera Thunder cooled-CCD camera (Photometrics) with Metamorph software controlling image acquisition and data analysis (Molecular Devices, Sunnyvale, CA, USA).

Confocal images were collected in Z-series image stacks with 10x, 20x 40x objective lenses and with a 63x oil-immersion objective. Images were captured using standard DAPI and rhodamine filter sets. The signal intensity used for immunocytochemistry measurements was greater than the highest signal in control dishes of C6 cells in which the primary antibody was omitted but otherwise treated the same as the experimental dishes. When comparing immunofluorescence of Hoechst-positive and Hoechst-negative cells the maximum pixel intensity within an oval drawn around each cell was used as the signal intensity. Hoechst-negative cells were identified by eye.

Data analysis software

Images were analyzed with NIH ImageJ and Photoshop software. Data were analyzed and plotted using OriginLab, GraphPad, and Excel software. Data from HO-3867 cell viability

effects over time were analyzed with Vernier Graphical Analysis software to find the best fit of the sine wave function $y = a (\sin (bx + c)) + d$.

RESULTS

Aim 1: Test the anticancer effects of HO-3867 in C6 cell cultures

Determining EC_{50} for HO-3867

HO-3867 was tested at six concentrations: 0, 5, 10, 20, 40, and 80 μM to determine the EC_{50}. C6 cells were plated in 96-well dishes and maintained in culture for approximately three days. Brightfield images were captured before HO-3867 was added. On the third day, HO-3867 was added to eight wells at each of the concentrations listed above. The solvent for HO-3867, dimethyl sulfoxide (DMSO), was used at 0.2 or 0.8% v/v in Final Medium as a control. HO-3867 was administered for twenty-four hours at 37℃ and then replaced with Final Medium. 10% PrestoBlue in Final Medium was then added for 90 minutes at 37℃. The PrestoBlue was replaced with Final Medium, absorbance was measured, and relative cell viability was calculated. Brightfield images of the cells were then captured again. The absorbance was calculated for all the columns of the plate. From the fitted sigmoidal curve, it was determined that the EC_{50} for HO-3867 was 26.26 μM (Figure 4).

The brightfield images were analyzed using ImageJ, and cells were counted using the freehand draw feature. The cells displayed the typical shapes for C6 cultures, and there were also small, rounded cells previously identified as cells undergoing apoptosis (De et al., 2020). Roundness values were calculated using ImageJ (Shape Descriptors measurements). If the roundness was 0.60 or above, then the cells were considered potentially apoptotic. (Roundness ranges from zero to one, where one is perfectly round.) Roundness, as an indicator of cell death, increased significantly as HO-3867 concentration increased (Figure 4). These results provided a second measure of the treatment effects on C6 cells, after the PrestoBlue assay.

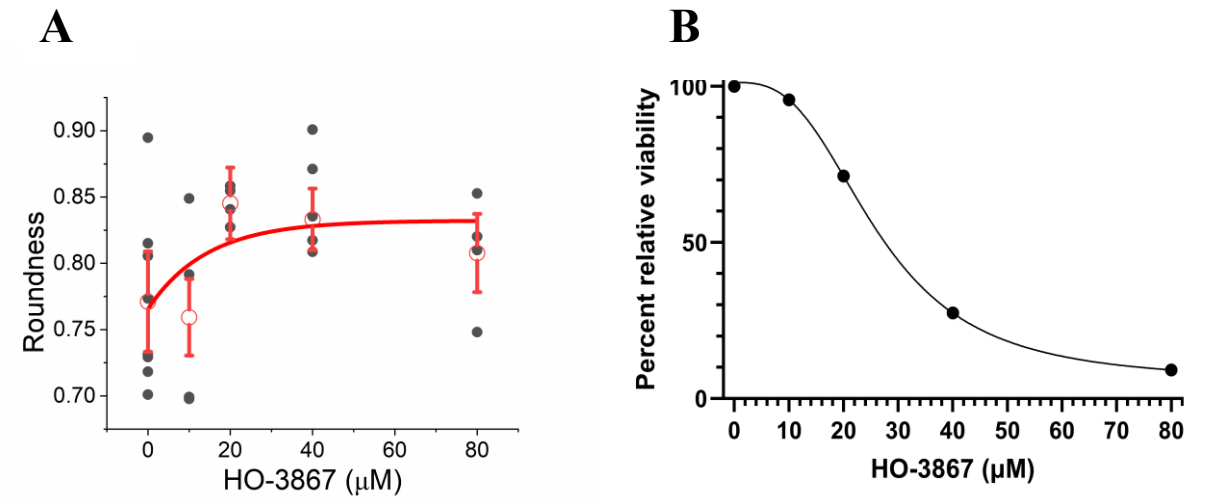

Figure 4: HO-3867 suppression of C6 cell viability. A: Average cell roundness as a measure of apoptosis increased with dosage of 24-hour HO-3867 treatments (Spearman's rank-order correlation, p=0.0395, n=25). B: Relative cell viability as measured with PrestoBlue decreased with increasing HO-3867 dosage.

Aim 2: Test whether HO-3867 is more effective when administered at a specific phase of the circadian clock.

Determining whether HO-3867 is more effective at a specific circadian phase

C6 cells were grown in multi-well dishes for three days. After the third day, the forskolin treatment was used to synchronize the circadian clocks in the cells. Forskolin was rinsed from the cells two hours later (Table 1). Two groups of 96-well plates were used so that the entire circadian cycle could be tested without performing experiments during the middle of the night. Group One (plates A-G) was treated with forskolin approximately 11 hours before Group Two (plates H-J) was treated (Table 1). Twenty-four hours later, 100 µL of Final Medium with 20 µM HO-3867 and DMSO was added to plate A. The other groups received this treatment according the schedule in Table 2. Twenty-four hours after the HO-3867 was administered, a rinse with medium was completed and then PrestoBlue was administered for 90 minutes (Table 3). The

images and absorbance data were analyzed. From these results it was determined that there was no circadian effect on cells treated with HO-3867 (Figure 5).

Measurements of cell roundness also provided evidence of cell death from HO-3867 delivered at multiple time points after forskolin treatment (Figure 6). Although average roundness varied over time, there was no evidence of the treatment effect being modulated by the circadian clock in these cultures (Figure 5). The cell roundness caused by 20 μM HO-3867 is shown in Figure 6. These observations indicate that the loss of viability in the cultures was from cell death caused by the treatment. HO-3867 could have also reduced cell proliferation, thereby providing an additional anticancer effect.

Table 1: The dates and times when forskolin was administered and then replaced with medium

Date	Plate	Time when forskolin was administered	Time when forskolin was replaced
10/20/23	A	8:41 AM	10:41 AM
10/20/23	B	8:45 AM	10:45 AM
10/20/23	C	8:50 AM	10:50AM
10/20/23	D	8:54 AM	10:54 AM
10/20/23	E	8:59 AM	10:59 AM
10/20/23	F	9:03 AM	11:03 AM
10/20/23	G	9:08 AM	11:08 AM
10/20/23	H	8:04 PM	10:04 PM
10/20/23	I	8:07 PM	10:07 PM
10/20/23	J	8:10 PM	10:10 PM

Table 2: The dates and times when HO-3867 was administered		
Date	Plate	Time
10/21/23	A	8:41 AM
10/21/23	B	2:45 PM
10/21/23	C	8:50 PM
10/22/23	D	8:15 AM
10/22/23	E	2:10 PM
10/22/23	F	8:01 PM
10/23/23	G	8:31 AM
10/23/23	H	2:14 PM
10/23/23	I	2:44 PM
10/23/23	J	7:56 PM

Table 3: The dates and times when PrestoBlue was administered after removing the HO-3867 treatment		
Date	Plate	Time
10/22/23	A	8:41 AM
10/22/23	B	2:45 PM
10/22/23	C	8:50 PM
10/23/23	D	8:15 AM
10/23/23	E	2:10 PM
10/23/23	F	8:01 PM
10/24/23	G	8:31 PM
10/24/23	H	2:14 PM
10/24/23	I	2:44 PM
10/24/23	J	7:56 PM

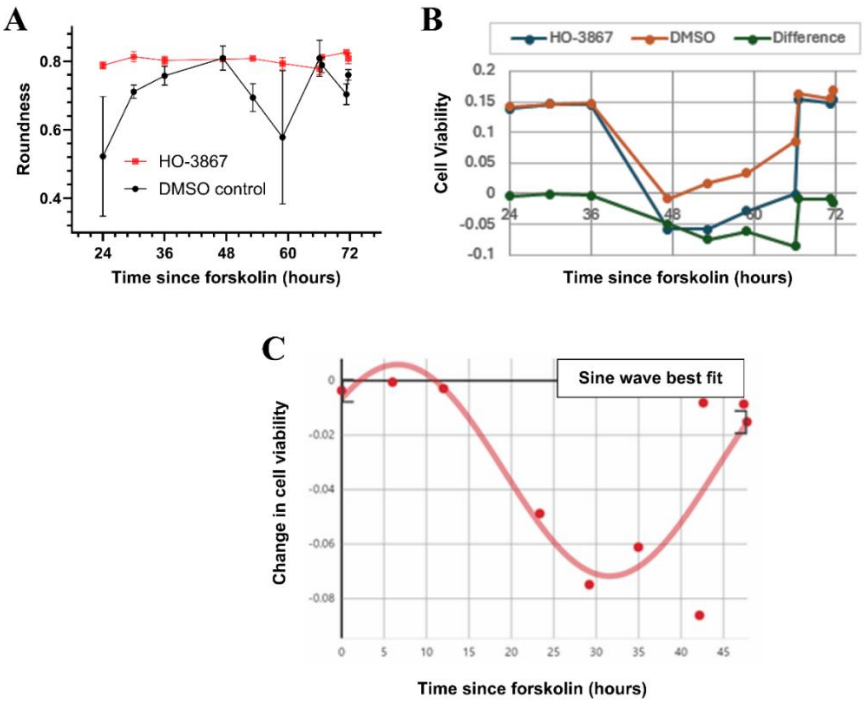

Figure 5: Changes in cell roundness and cell viability in response to 20 µM HO-3867 delivered at various time points. A: Average cell roundness increased significantly in cultures given HO-3867 relative to the vehicle 0.2% DMSO (Student's T-test, T=3.034, p=0.0141, n=10 time points). Error bars are SEM, about 4 measurements (wells) per group. Roundness was also significantly different between the HO-3867 and control groups when measurements at all time points were averaged and compared (T= -4.37, p<0.0001, n=38). B: Treatment with 20 µM HO-3867 caused a decrease in relative cell viability as measured with PrestoBlue in the same cell cultures as in A, but it did not show a circadian rhythm. C: Fitting a sine wave to the effect of HO-3867 (the difference between treated and control groups) indicates the best-fit period is 50 hours, well outside the circadian range.

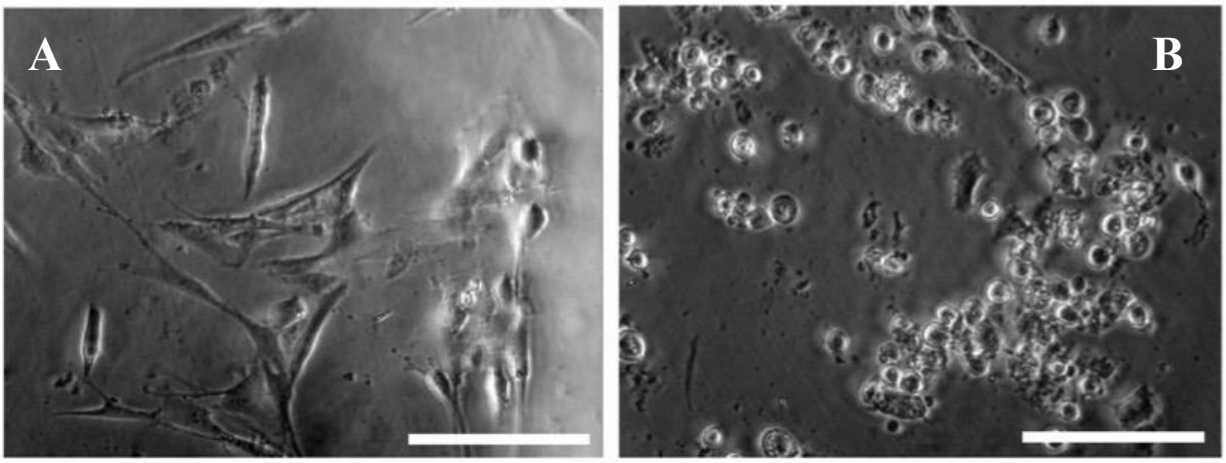

Figure 6: HO-3867 effects on C6 cell morphology. A: Control cells exposed to 0.2% DMSO (vehicle) for 24 hours. B: Cells treated with 20 μM HO-3867 and 0.2% DMSO for 24 hours. All cells were initially given a 2-hour pulse of 20 μM forskolin 24 hours earlier to synchronize circadian clocks in the cells (Table 1). Scale bar = 50 μm. (40x objective lens).

Aim 3: Determine whether the amount of cell-surface CD47 expression is different in stem-like C6 cells relative to non-stem C6 cells.

Immunocytochemistry

C6 cells were maintained in Final Medium in 35-mm sized glass bottom petri dishes for two days after plating. Two dishes were examined with cells at low density and one with cells at near confluence. Live cells were given nuclear stain Hoechst 33342, and then cells were fixed and CD47 was identified by immunocytochemistry. The cells were imaged using the brightfield, DAPI, and rhodamine channels, and Hoechst-negative cells were identified (Figure 7).

Of the three cultures tested, the one with the highest cell density had significantly higher CD47 expression in Hoechst-negative than Hoechst-positive cells (T-test, $p=1.78x10^{-06}$, n=25 cells each, Averages: 489.36, ±198.20 SD versus 270.4, ±35.01 SD). This result indicates elevated CD47 expression occurs in C6 Hoechst-negative CSCs. The number of these Hoechst-

negative cells was elevated in the densest cell culture relative to the other two dishes. Two Hoechst-negative cells were identified in each of the two low-density cultures, and their CD47 expression was not significantly different from that of Hoechst-positive cells.

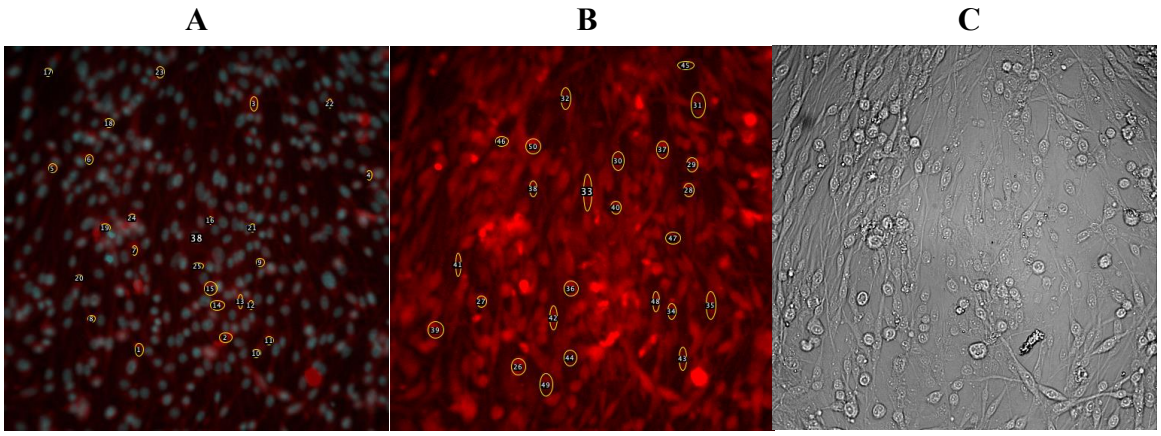

Figure 7. Comparing CD47 expression in CSCs and non-stem C6 cells. A: Regions-of-interest (ovals) showing Hoechst-negative cells measured in an image of CD47 immunostaining (red) with an overlay of Hoechst-stained nuclei (blue). B: Measured Hoechst-positive cells shown in the CD47 image alone. C: Brightfield image of the same cells in A and B. Imaged with 20X objective lens.

DISCUSSION

Aim 1

HO-3867 dose-response curve

In this study it was determined that HO-3867 had an EC_{50} of 26.3 μM for half maximal loss of viable C6 cells, which compares well with previous studies that reported an HO-3867 EC_{50} of 16 μM (Lu et al 2022). However, the lower EC_{50} could be because it was from a treatment for human osteosarcoma cells. To determine the EC_{50} we used the cell viability reagent PrestoBlue, and this could be another explanation why the EC_{50} is higher in this study. HO-3867 is an effective anticancer agent in C6 glioma cells. HO-3867 should be tested on mouse astrocytes to determine if HO-3867 has any negative effect on non-cancerous cells. Studies have shown that HO-3867 was an effective cancer fighting agent on cell lines other than C6. Some of these other cells lines include human breast, colon, liver, and human oral squamous cell carcinomas (Madan et al., 2018; Chen et al., 2022).

All treatment was done at 20 μM to prevent a ceiling killing effect. This EC_{50} was higher than previous studies. It is hypothesized that the EC_{50} is higher due to the C6 cell being used. Due to the different cell line and the difference in the organism. However, using a cell line that has been continuously grown could influence this higher EC_{50} as well.

Measuring viability

Measuring cell viability and apoptosis was performed two different ways, using PrestoBlue and measuring cell roundness. Two different methods were used to prevent apoptotic cells from not being counted. PrestoBlue measures the relative number of viable cells present, but it does not indicate how much of this measure results from cell death or suppression of cell

division during the 24-hour treatments. The roundness data helped by indicating how much cell death was occurring at the end of the HO-3867 treatment.

Difference in EC_{50} across studies

HO-3867 has been tested on various cell lines and EC_{50} ranged from 10-50 μM (Wu et al., 2023). The A549 human lung cancer cell showed an EC_{50} of 37.6 ±3.9 (Wu et al., 2023). Whereas C6 cells in this experiment showed an EC_{50} of 26.3. The higher reported EC_{50} values could be due to species-specific mechanisms found in human cells compared to other organisms tested.

Overall, this study does support the hypothesis of HO-3867 being an effective cancer fighting agent in C6 glioma cells because there was a lower EC_{50} than in most human cancer cell lines. Furthermore, other studies measured cell viability in different ways after HO-3867 was administered, which could affect EC_{50} determinations.

Aim 2

Circadian rhythm in C6 cells

Previous studies determined that C6 cells have circadian rhythms in gene expression, epithelial-mesenchymal transition, and cell death caused by low-curcumin dosage treatments (De et al., 2020). However, when testing whether HO-3867 anticancer effects are influenced by the circadian clock we determined there is no effect under the conditions tested here. This result could be for several reasons including the density of the cells, and how well the cells were growing during the two to three days after forskolin was applied. There was a decline after about 48 hours that might be caused by increased cell density or even uptake of HO-3867 into the cells. An additional study would be essential to determine whether a second dose should be applied to produce greater cell death.

A future study would be needed to determine if C6 cells treated with HO-3867 have a 50-hour oscillation. This is an important experiment because there could be an effect of HO-3867 outside the circadian range.

In this study HO-3867 did not appear to influence the circadian clock like curcumin. This leads to rejection of the hypothesis. A possible explanation for the lack of circadian influence could be due to this being a synthetic form of curcumin. However, it may show a 50-hour oscillation which is outside of the circadian range.

Aim 3

Overall, it seems that when there is a higher density of cells there will be a higher amount of Hoechst positive cells, but the Hoechst negative cells will be brighter. This was observed when three different densities were tested a low, medium, and high density. When looking at the low and medium densities, it seemed that even though there were cells present, there were very few Hoechst-negative cells. More confluent cultures have more Hoechst-negative cells, and individual cells express more CD47 than the non-dividing, astrocyte shaped Hoechst-positive cells that comprise most of the population. When there are more CSCs and more CD47 expression present in gliomas this could provide a greater suppression of phagocytes by the cancer cells than just elevated CD47 alone.

When the CD47 expression decreases the immune system will have limited inhibition, and this will allow the cancer cells to be engulfed by phagocytes. An experiment testing whether HO-3867 reduces the amount of cell surface CD47 would be valuable in determining whether this potential patient treatment acts though an additional anticancer mechanism along with its known actions. It would also be essential to determine if HO-3867 has any effect on CALR, and if so, whether it upregulates CALR to allow the immune system to engulf the cells.

This study supported the hypothesis of cancer stem cells having higher expression than non-stem cancer cell. Through immunostaining we were able to identify high amounts of CD47 on CSCs, and non-stem cancer cells consistently expressed lower levels.

REFRENCES

Askarizadeh, A., Barreto, G. E., Henney, N. C., Majeed, M., & Sahebkar, A. (2020). Neuroprotection by curcumin: A review on brain delivery strategies. *International Journal of Pharmaceutics, 585*, 119476. doi: 10.1016/j.ijpharm.2020.119476

Bixel, K., Saini, U., Kumar Bid, H., Fowler, J., Riley, M., Wanner, R., et al. (2017). Targeting STAT3 by HO3867 induces apoptosis in ovarian clear cell carcinoma. *International Journal of Cancer, 141*(9), 1856-1866. doi:10.1002/ijc.30847

Bonsignore, G., Martinotti, S., & Ranzato, E. (2023). Endoplasmic reticulum stress and cancer: Could unfolded protein response be a druggable target for cancer therapy? *International Journal of Molecular Sciences, 24*(2), 1566. doi:10.3390/ijms24021566

Chaix, A., Zarrinpar, A., & Panda, S. (2016). The circadian coordination of cell biology. *Journal of Cell Biology, 215*(1), 15-25. doi:10.1083/jcb.201603076

Chen, C., Hsieh, M., Ju, P., Hsieh, Y., Su, C., Chen, Y., et al. (2022). Curcumin analog HO-3867 triggers apoptotic pathways through activating JNK1/2 signalling in human oral squamous cell carcinoma cells. *Journal of Cellular and Molecular Medicine, 26*(8), 2273-2284. doi:10.1111/jcmm.17248

Cook, K. L., & Soto-Pantoja, D. R. (2017). “UPRegulation” of CD47 by the endoplasmic reticulum stress pathway controls anti-tumor immune responses. *Biomarker Research, 5*(1), 26. doi:10.1186/s40364-017-0105-8

Dayton, A., Selvendiran, K., Kuppusamy, M. L., Rivera, B. K., Meduru, S., Kálai, T., et al. (2010). Cellular uptake, retention and bioabsorption of HO-3867, a fluorinated curcumin

analog with potential antitumor properties. *Cancer Biology & Therapy, 10*(10), 1027-1032. doi:10.4161/cbt.10.10.13250

De, A., Beligala, D. H., Birkholz, T. M., & Geusz, M. E. (2019). *Anticancer properties of curcumin and interactions with the circadian timing system*. Los Angeles, CA: SAGE Publications.

De, A., Beligala, D. H., Sharma, V. P., Burgos, C. A., Lee, A. M., & Geusz, M. E. (2020). Cancer stem cell generation during epithelial-mesenchymal transition is temporally gated by intrinsic circadian clocks. *Clinical & Experimental Metastasis, 37*(5), 617-635. doi:10.1007/s10585-020-10051-1

DeVita, V. T. J., & Chu, E. (2008). A history of cancer chemotherapy. *Cancer Research, 68*(21), 8643-8653. doi: 10.1158/0008-5472.CAN-07-6611

Faguet, G. B. (2014). A brief history of cancer: Age-old milestones underlying our current knowledge database. *International Journal of Cancer, 136*(9), 2022-2036. doi:10.1002/ijc.29134

Fujioka, A., Takashima, N., & Shigeyoshi, Y. (2006). Circadian rhythm generation in a glioma cell line. *Biochemical and Biophysical Research Communications, 346*(1), 169-174. doi: 10.1016/j.bbrc.2006.05.094

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell, 144*(5), 646-674. doi: 10.1016/j.cell.2011.02.013

Hao, Y., Zhou, X., Li, Y., Li, B., & Cheng, L. (2023). The CD47-SIRPα axis is a promising target for cancer immunotherapies. *International Immunopharmacology, 120*, 110255. doi: 10.1016/j.intimp.2023.110255

Hatcher, H., Planalp, R., Cho, J., Torti, F. M., & Torti, S. V. (2008). Curcumin: From ancient medicine to current clinical trials. *Cellular and Molecular Life Sciences: CMLS, 65*(11), 1631-1652. doi:10.1007/s00018-008-7452-4

Hu, J., Xiao, Q., Dong, M., Guo, D., Wu, X., & Wang, B. (2020). Glioblastoma immunotherapy targeting the innate immune checkpoint CD47-SIRPα axis. *Frontiers in Immunology, 11*, 593219. doi:10.3389/fimmu.2020.593219

Huang, C., Ye, Z., Huang, M., & Lu, J. (2020). Regulation of CD47 expression in cancer cells. *Translational Oncology, 13*(12), 100862. doi: 10.1016/j.tranon.2020.100862

Kalman H., & Kuppusamy, P. (2012). *Compositions and methods for inhibition of cancers* Retrieved from https://worldwide.espacenet.com/publicationDetails/biblio?FT=D&date=20121212&DB=EPODOC&locale=&CC=EP&NR=2451273A4

Kasikova, L., Hensler, M., Truxova, I., Skapa, P., Laco, J., Belicova, L., et al. (2019). Calreticulin exposure correlates with robust adaptive antitumor immunity and favorable prognosis in ovarian carcinoma patients. *Journal for Immunotherapy of Cancer, 7*(1), 312-z. doi:10.1186/s40425-019-0781-z

Kaur, S., & Roberts, D. D. (2016). Divergent modulation of normal and neoplastic stem cells by thrombospondin-1 and CD47 signaling. *The International Journal of Biochemistry & Cell Biology, 81*(Pt A), 184-194. doi: 10.1016/j.biocel.2016.05.005

Lee, Y. (2021). Roles of circadian clocks in cancer pathogenesis and treatment. *Experimental & Molecular Medicine, 53*(10), 1529-1538. doi:10.1038/s12276-021-00681-0

Liu, Y., Wang, X., Zeng, S., Zhang, X., Zhao, J., Zhang, X., et al. (2018). The natural polyphenol curcumin induces apoptosis by suppressing STAT3 signaling in esophageal squamous cell carcinoma. *Journal of Experimental & Clinical Cancer Research, 37*(1), 303. doi:10.1186/s13046-018-0959-0

Lu, P. W., Chou, C., Yang, J., Hsieh, Y., Tsai, M., Lu, K., et al. (2022). HO-3867 induces apoptosis via the JNK signaling pathway in human osteosarcoma cells. *Pharmaceutics, 14*(6), 1257. doi:10.3390/pharmaceutics14061257

Piper, K., DePledge, L., Karsy, M., & Cobbs, C. (2021). Glioma stem cells as immunotherapeutic targets: Advancements and challenges. *Frontiers in Oncology, 11*, 615704. doi:10.3389/fonc.2021.615704

PrestoBlue® cell viability reagent documentation

Rath, K. S., Naidu, S. K., Houghton, P., Hidge, K., Kuppusamy, P., Cohn, D. E., et al. (2014). HO-3867, a safe STAT3 inhibitor, is selectively cytotoxic to ovarian cancer. *Cancer Research (Chicago, Ill.), 74*(8), 2316-2327. doi: 10.1158/0008-5472.CAN-13-2433

Sarma, A., Sharma, V. P., Sarkar, A. B., Sekar, M. C., Samuel, K., & Geusz, M. E. (2016). The circadian clock modulates anti-cancer properties of curcumin. *BMC Cancer, 16*(1), 759. doi:10.1186/s12885-016-2789-9

Sharifi-Rad, J., Rayess, Y. E., Rizk, A. A., Sadaka, C., Zgheib, R., Zam, W., et al. (2020). Turmeric and its major compound curcumin on health: Bioactive effects and safety profiles for food, pharmaceutical, biotechnological and medicinal applications. *Frontiers in Pharmacology, 11*, 01021-01021. doi:10.3389/fphar.2020.01021

Sharma, V. P., Anderson, N. T., & Geusz, M. E. (2014). Circadian properties of cancer stem cells in glioma cell cultures and tumorspheres. *Cancer Letters, 345*(1), 65-74. doi: 10.1016/j.canlet.2013.11.009

Wang, Z., & Chen, G. (2022). Insights about circadian clock in glioma: From molecular pathways to therapeutic drugs. *CNS Neuroscience & Therapeutics, 28*(12), 1930-1941. doi:10.1111/cns.13966

Yin, W., Wang, J., Jiang, L., & James Kang, Y. (2021). *Cancer and stem cells*. London, England: SAGE Publications.

Yu, Z., Pestell, T. G., Lisanti, M. P., & Pestell, R. G. (2012). Cancer stem cells. *The International Journal of Biochemistry & Cell Biology, 44*(12), 2144-2151. doi: 10.1016/j.biocel.2012.08.022

Zoi, V., Galani, V., Lianos, G. D., Voulgaris, S., Kyritsis, A. P., & Alexiou, G. A. (2021). The role of curcumin in cancer treatment. *Biomedicines, 9*(9), 1086. doi:10.3390/biomedicines9091086