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**β -Cyclodextrin Inclusion Complex with Oxazine-4 Derivative for the Treatment of
Glioblastoma Multiforme**

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Abstract

Glioblastoma multiforme (GBM) is the most common type of high-grade glioma and accounts for as much as 50% of all primary brain tumors. With the current standard of care, survival of GBM patients remains poor at 15 months after diagnosis. In this study, the antiproliferative effects of a novel oxazine-4 derivative called 0108 were examined. Delivery of 0108 was accomplished via complexation with (2-hydroxypropyl)- β -cyclodextrin (HP- β -CD) to form 0108CD. The association constant, K_a , of 0108CD was determined and stability of 0108CD formulations made with 1, 2.5, 5, 7.5, and 10% HP- β -CD were assessed over 24, 48, and 72 hours. The *in vitro* toxicity of 0108 and 0108CD was quantified via IC_{50} in U87-MG, U118-MG, SF298, and SF268 cell lines. The effects of 0108 on cell phase were examined in U87-MG.

The 5% HP- β -CD formulation was established as the optimal 0108CD treatment based on maximum 0108 encapsulation without HP- β -CD oversaturation. Stability of the 5% HP- β -CD formulation decreased after 24 hours. Incorporating 0108 in a HP- β -CD vesicle did not change the potency of the drug in SF295-MG and U118-MG, and increased its potency in U87-MG and SF268-MG. 0108 treatment at the highest dose showed a time-independent increase in the number of cells in G0/G1 and S phases and a decrease in G2/M phase.

Key Words

Brain cancer; Cyclodextrin; Drug delivery; Inclusion complex; Glioblastoma multiforme

Introduction

Cancers of the central nervous system (CNS) are leading causes of cancer deaths in adults younger than 40 years with an estimated annual mortality of 128,000 out of the 176,000 diagnoses worldwide.¹ Glioblastoma multiforme (GBM), a type of high-grade glioma (HGG) or malignancy arising from the supportive tissue of the brain, is one of the most frequently occurring and aggressive intracranial tumors. As of 2017, patients with GBM had one-, five-, and ten-year survival rates of 39.7, 5.5, and 2.9%, respectively.¹

The current standard of care in patients with GBM is maximal safe resection of the tumor, followed by radiotherapy and adjuvant temozolomide (TMZ) chemotherapy. The median survival for GBM patients receiving maximal therapy remains poor at 15 months after diagnosis.¹ Systemic delivery of TMZ is said to only result in moderate benefits, attributed to its short serum half-life and numerous dose-limiting side effects, such as blood toxicity and cardiomyopathy, suppression of bone marrow activity, and oral ulcerations.^{2,3} Treatment of GBM and other CNS cancers is limited by the inability of chemotherapeutics to cross the blood-brain barrier (BBB) and further, the blood-brain tumor barrier (BBTB). In GBM specifically, the significant metabolic demands of the HGG induce a hypoxic environment with overexpression of vascular endothelial growth factor and angiogenesis, factors that disrupt the BBTB.⁴ The GBM core resembles this disrupted BBTB which is highly “leaky” in nature. However, the invasive nature of gliomas leaves the larger majority of the tumor with an otherwise intact BBTB with similar characteristics to the BBB, including tight junctions, electrical resistance, and a network of compound-inactivating enzymes.

Drug delivery systems offer an alternative to systemic delivery of chemotherapeutics. They possess appropriate sizes, surface properties, and ligands which allow them to penetrate the BBB and BBTB to offer targeted delivery of both hydrophilic and lipophilic drugs. In recent years, researchers have been using drug delivery systems to enhance TMZ treatment and reduce toxicity. One study, however, found that longer survival time and reduction in tumor volume exhibited by the TMZ liposomal formulation were statistically insignificant in comparison to free TMZ.⁵ The poor aqueous and organic solubility of TMZ has led to significant difficulties in its encapsulation in polymeric nanoparticles.⁶ Other modalities of TMZ delivery for treatment of GBM are being studied, most notably, penetration of the BBB for direct delivery to the site of the lesion, gold nanocarrier delivery systems, and nose-to-brain administration.

This work focuses on cyclodextrins (CDs), cyclic oligosaccharides that form non-covalent complexes with a range of hydrophobic drugs and increase drug bioavailability, stability, and solubility. CDs promote drug absorption across the brain, dermal, nasal, and intestinal barriers, a function attributed to their ability to extract cholesterol from

The association constant, K_a , of 0108CD was determined and stability of 0108CD formulations made with 1, 2.5, 5, 7.5, and 10% HP- β -CD were assessed over 72 hours. IC_{50} values of 0108 and 0108CD in U87-MG, U118-MG, SF298, and SF268 cell lines were determined. The effects of 0108 treatment on cell phase were examined in U87-MG.

Materials

0108 dye was obtained from the Summer Gibbs lab at Oregon Health and Science University. (2-hydroxypropyl)- β -cyclodextrin was purchased from Sigma-Aldrich (St. Louis, MI). Cell culture supplies including Dulbecco's Modification of Eagle's Medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), trypsin ethylene-diamine-tetra acetic acid (EDTA), penicillin/streptomycin (Pen/Strep) and Dulbecco's phosphate buffered saline (DPBS) were acquired from VWR (Radnor, PA). U87-MG, U118-MG, SF-295, and SF-268 cell lines were purchased from American Type Culture Collection (Manassas, VA). Vybrant® Dye Cycle™ Green stain was purchased from Life Technologies (Grand Island, NY).

Methods

Complexation and stability of 0108CD formulations

A series of 1, 2.5, 5, 7.5, and 10% HP- β -CD solutions were prepared for complexation with a suspension of 1.5 mg/mL of 0108. The complexes were dissolved in 95% EtOH, followed by removal of the solvent at 50 °C for 12 minutes using the rotary evaporator. Cyclodextrin thin films were hydrated at 50 °C and resulting formulations filtered using a 0.2 μ m Nylon membrane syringe filter (VWR, Radnor, PA). Stability of freshly prepared formulations were evaluated over 24, 48, and 72 hours by monitoring drug complexation. Drug complexation was directly proportional to absorbance at 610 nm. Measurements were done in triplicate, and data was presented as mean 0108 concentration (M) \pm SD. The 5% HP- β -CD solution was established as the 0108CD treatment in further studies.

In vitro toxicity of 0108 and 0108CD treatments

Cell viability was assessed for U87-MG, U118-MG, SF295, and SF268 at a density of 5,000 cells per well. U87-MG and U118-MG were cultured in DMEM media. SF295 and SF268 were cultured in RPMI. All media were supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were grown in a 96-well plate at 37 °C, in a humidified atmosphere, for 3 hours to allow for attachment. The cells were treated with 0.02, 0.2, 2, 20, 200, 2000, and 20000 nM of either 0108 or 0108CD. Control groups were treated with either DMSO (0108 control) or water (0108CD control). After 72 hours, cell viability was assessed using Resazurin solution at 10% of cell culture volume and fluorescence

intensity measured at excitation and emission of 560 and 590 nm, respectively, 2 hours later.

Half-maximal inhibitory concentration (IC_{50}) was determined with non-linear regression analysis using GraphPad Prism version 6.05 for Windows, GraphPad Software (La Jolla California, USA). Measurements were done in triplicate, and data presented as mean of IC_{50} (nM) \pm SD. A two-tailed unpaired t-test was performed at a p-value of 0.05 for comparison between 0108 and 0108CD treatment groups.

Effect of 0108 on U87-MG cell phase

The effect of 0108 was studied on the cell cycle of U87-MG cells using Vybrant® Dye Cycle™ Green stain. U87-MG cells were seeded at a density of 200,000 cells per well in a 6-well culture plate and allowed 3 hours for attachment. Cells were then treated with 30, 300, and 900 nM concentrations of 0108. The control groups were treated with DMSO. Plates were incubated at 37 °C and effects on cell cycle studied at 24, 48, and 72 hours post treatment. After the incubation period, media was aspirated, and cells were trypsinized and collected in 1.5 mL Eppendorf tubes. The cells were then centrifuged at 3500 rpm for 3.5 minutes, the supernatant aspirated, and cells reconstituted in new media. Centrifugation and reconstitution was repeated. Vybrant® Dye Cycle™ Green stain was added, as per the manufacturer's protocol, before analysis with BD C6 Accuri software. The results for three replicates were presented as mean percent of U87-MG cells \pm SD in G0/G1, S, and G2/M phases.

Results and Discussion

The 1:1 complexation of HP- β -CD with 0108 was studied as a function of increasing concentration of HP- β -CD (Figure 2) and the stability of each formulation was monitored over 72 hours (Figure 3).

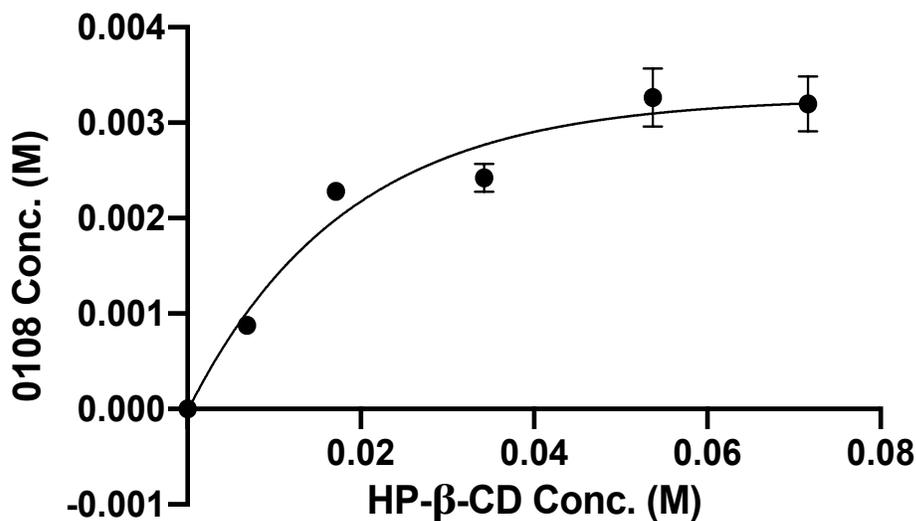


Figure 2: 0108 encapsulation as a function of HP- β -CD concentration. Data presented as mean \pm SD ($n=3$).

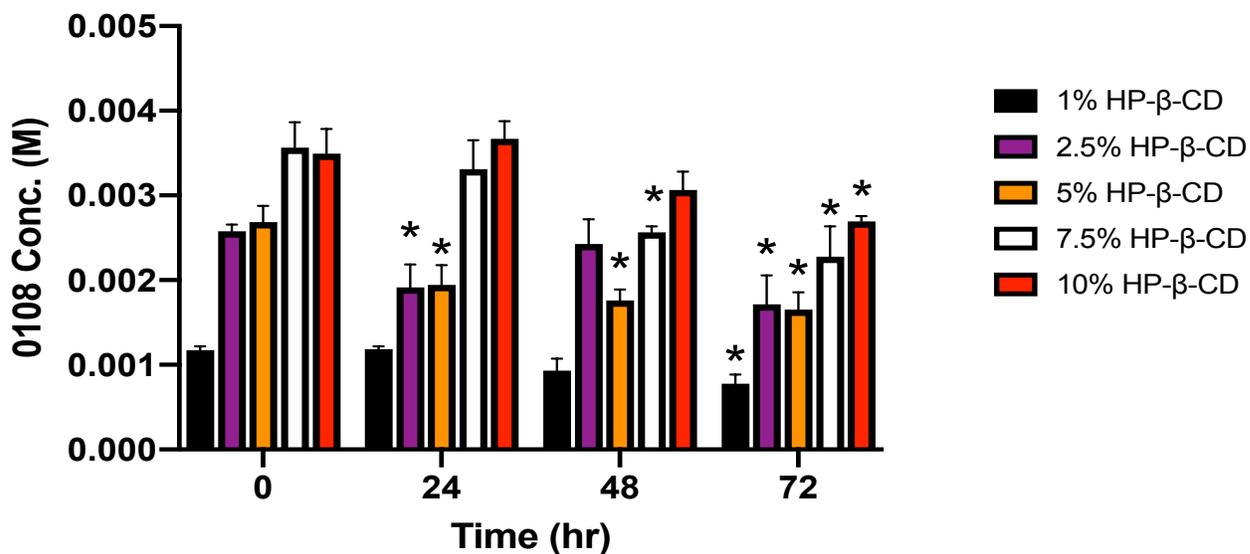


Figure 3: Stability of 0108 complexes with varying concentrations of HP- β -CD. Data presented as mean \pm SD ($n=3$). *indicates statistical significance as compared to 0108 conc. at 0 hr (p -value <0.05).

0108 with 1, 2.5, 5, 7.5, and 10% HP- β -CD was encapsulated at concentrations of 0.91 ± 0.039 , 2.36 ± 0.057 , 2.50 ± 0.121 , 3.38 ± 0.250 , and 3.31 ± 0.236 mM. K_a for 0108CD was determined by equation 2 to be 266.0 M^{-1} . Stability of the 2.5 and 5% HP- β -CD formulations decreased after 24 hours. That of the 7.5% HP- β -CD formulation decreased after 48 hours, and 1% and 10% HP- β -CD formulations became unstable after 72 hours. The intrinsic solubility of 0108 in water was 0.121 mg/mL.

The 5% HP- β -CD formulation was established as the optimal 0108CD treatment for further *in vitro* studies. This was based on maximum 0108 encapsulation without HP- β -CD oversaturation.

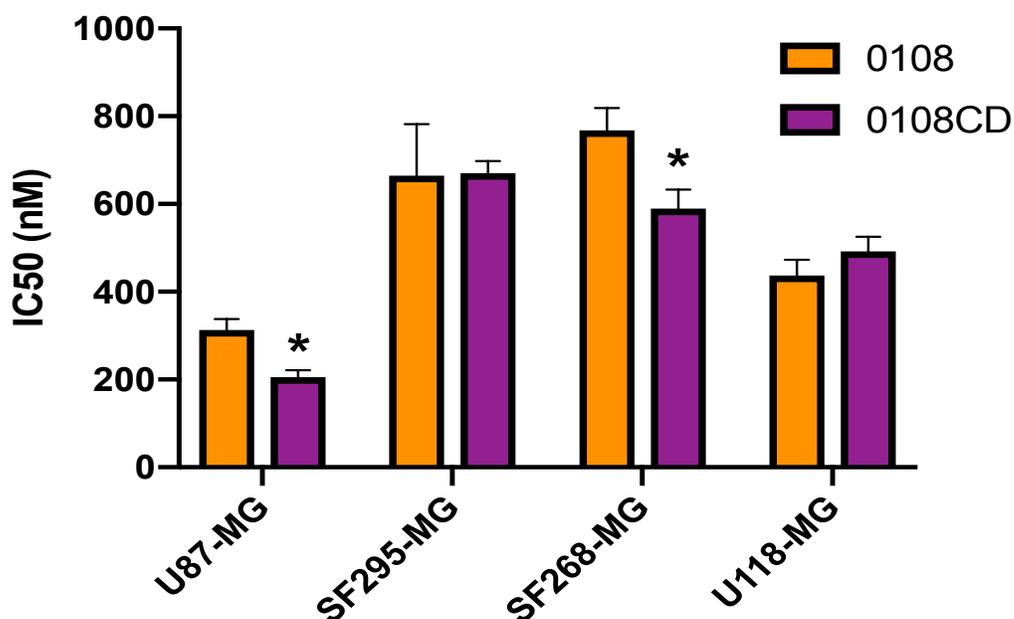


Figure 4: IC_{50} of 0108 and 0108CD in GBM cell lines. Data presented as mean \pm SD ($n=3$). *indicates statistical significance as compared to 0108 treated cells (p -value <0.05).

In vitro cell viability for both free and encapsulated 0108 were evaluated in four GBM cell lines to confirm that the CD complex did not modify the anti-proliferative effect of the drug (Figure 4). The IC_{50} of 0108 in U87-MG, U118-MG, SF295-MG, and SF268-MG was 312.5 ± 25.1 , 436.8 ± 35.8 , 661.3 ± 119.7 , 768.2 ± 50.2 nM, respectively. The IC_{50} of 0108CD in U87-MG, U118-MG, SF298, and SF268 was 205.6 ± 15.4 , 433.2 ± 131.8 , 670.3 ± 27.5 , and 589.5 ± 43.3 nM, respectively. The two treatments were statistically indistinguishable at a p -value of 0.05 in SF295-MG and U118-MG. The IC_{50} of 0108CD was lower than 0108 in U87-MG and SF268-MG. Incorporating 0108 in a cyclodextrin vesicle did not change the potency of the drug in SF295-MG and U118-MG, and increased its potency in U87-MG and SF268-MG.

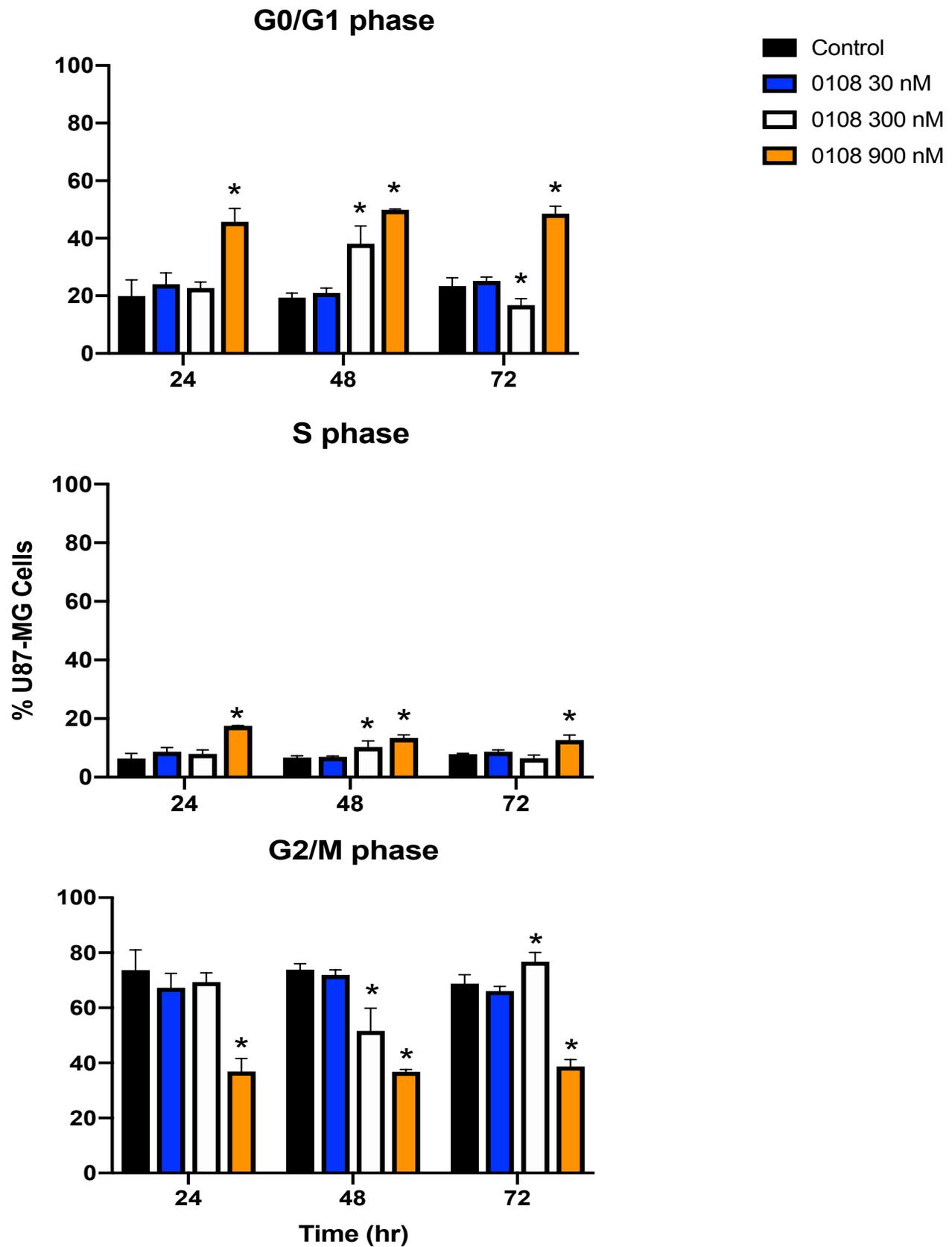


Figure 5: Effect of 0108 treatment below, around, and above IC_{50} in U87-MG cell cycle over 72 hours. Data presented as mean \pm SD ($n=3$). *indicates statistical significance as compared to control at each time point (p -value <0.05).

Effect of the dose of 0108 on different phases of the cell cycle was studied in U87-MG cells as a function of time (Figure 5). Cells were treated below, around, and above the IC_{50} of 0108 and percent of cells in G0/G1, S, and G2/M phases evaluated at 24, 48, and 72 hours. The percent of untreated cells in G0/G1 phase was 19.9, 19.4, and 23.3 at 24, 48, and 72 hours, respectively. The percent of cells in G0/G1 phase when treated with 900 nM 0108 was 45.7, 49.9, and 48.6 at the aforementioned times, respectively. The percent of untreated cells in S phase was 6.4, 6.7, and 7.9 while those with 900 nM 0108 treatment were 17.5, 13.4, and 12.7, respectively. The percent of untreated cells in G2/M phase were 73.7, 73.9, and 68.8 and those treated with 900 nM 0108 were 36.8, 36.8, and 38.7, respectively. 0108 treatment at the highest dose showed a time-independent increase in the number of cells in G0/G1 and S phases and decrease in G2/M phase.

Conclusion

0108 and 0108CD were shown to have antiproliferative effects against GBM *in vitro*. The efficacy of the CD formulation was similar to the free drug and even more potent in U87-MG and SF268-MG cell lines. Stability of the 0108CD formulation decreased by 24 hours, but freeze-drying storage techniques may improve stability over longer periods of time. Maximal treatment of 0108 shifted GBM cells from G2/M phase to G0/G1 and S phases, highlighting the potential mechanism of the drug. Real-time cellular proliferation and apoptosis studies of 0108 and 0108CD, and a comparison with well-known chemotherapeutics including doxorubicin, may further establish the mechanism and efficacy of this novel drug as a treatment for GBM.

Acknowledgements

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