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Ajulemic Acid Induces Cell Death in Retinoblastoma Cells

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SENIOR THESIS APPROVAL

This Honors thesis entitled

“Ajulemic Acid Induces Cell Death in Retinoblastoma Cells”

written by

Emily Evans

and submitted in partial fulfillment of the
requirements for completion of the
Carl Goodson Honors Program
meets the criteria for acceptance
and has been approved by the undersigned readers.

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Ajulemic Acid Induces Cell Death in Retinoblastoma Cells

Emily A. Evans

Abstract

Retinoblastoma is a cancer of the retina affecting one in every 15,000-20,000 births. It is normally diagnosed in infancy or early childhood and although it is treatable, enucleation is commonly required. Other treatments include radiation, photocoagulation, or cryotherapy and are often followed by chemotherapy. In an attempt to find an alternate method of treatment that lowers the frequency of enucleation and the serious side-effects resulting from current treatments, ajulemic acid (AJA), a synthetic derivative of *Cannabis*, has been used to treat retinoblastoma cells. Thus far, significant cell death has occurred among retinoblastoma cells that have been treated with AJA concentrations of as low as 50 μ M. We are also currently investigating how the AJA treatment of retinoblastoma cells compares with carboplatin treatment, one of the leading chemotherapy drugs currently used in treating retinoblastoma.

Introduction

Retinoblastoma

The purpose of our research was to investigate ajulemic acid's (AJA) ability to induce retinoblastoma cell death and to compare it with one current chemotherapy method of treatment, the drug carboplatin (CBP).

Retinoblastoma (Rb) is a pediatric cancer that develops in the retina of the eye and is the most frequent primary eye growth found in infants and children (Blachford, 2002). This disease occurs in nearly one out of every 15,000-20,000 live births world-

wide (Aerts, 2006). Rb usually occurs and is diagnosed before the age of 4 (Beers, 2003) However, it can be identified in patients at the ages of 5-7 years (Murphree and Clark) an even more rarely in adulthood (Aerts, 2006). A consistency of cases among all races has been observed and there is no significant difference in the number of cases between male or female or between incidences in the left or right eye (Kufe et al., 2003). In developed countries, there is a high survival rate for children with retinoblastoma; however children with the disease in underdeveloped parts of the world rarely survive (Murphree and Clark).

There are a variety of ways that retinoblastoma can present, but the most common signs are leukocoria, a white pupillary reflex, seen in 50% of patients and strabismus, crossed eyes, observed in 25% of patients (Blachford, 2002). Other symptoms may include red painful eye, poor vision, iris rubeosis (abnormal blood vessel growth of the iris), orbital cellulites (orbital tissue infection), hypopyon (pus in the eye), amblyopia ("lazy eye"), congenital glaucoma, exophthalmia (eyeball protrusion) and heterochromia (difference in iris coloration) (Aertes, 2006; Blachford, 2002).

Retinoblastoma develops in a few different ways. Endophytic growth, most commonly observed, refers to when retinoblastoma grows into the vitreous cavity outward from the retina toward the eye's center. Growth of the tumor into the choroid and away from the underside of the retina is referred to as exophytic growth. Exophytic growth is frequently associated with retinal detachment and the spread of the tumor into the choroid and optic nerve. A more uncommon growth pattern is known as diffuse infiltrating in which a mass is not formed, rather the tumor is dispersed throughout the retina (Murphree and Clark).

Retinoblastoma Etiology

Retinoblastoma can be inherited or can occur sporadically. About 45% of cases are the nonhereditary form, or non-germinal, and approximately 55% of the cases are inherited, or germinal, in which there is an immediate family member who has previously been diagnosed with the disease (Aertes, 2006). The nonhereditary form normally occurs unilaterally and is unifocal, a single tumor that affects one eye (Blachford, 2002). The hereditary form always presents either bilaterally (affecting both eyes) or unilaterally and multifocal (multiple tumors in one eye).

When the disease is inherited, a germline mutation has typically already occurred in one of the alleles of the retinoblastoma gene (RB-1) that codes for the protein pRB. If a person inherits one mutated allele and one normal allele, the person will not develop cancer as long as the normal allele does not become mutated. However, when a somatic mutation of the second allele occurs, the gene will code or miscode for the defective pRB and cell division will no longer be regulated. This lack of regulation results in cancer. Instead of inheriting mutations, people with sporadic retinoblastoma have two independent somatic mutations occurring in both alleles of the RB-1 gene. Again, the correct coding of pRB is prevented and cancer results. This idea that retinoblastoma is the result of two mutational events is known as Knudson's two hit hypothesis (Tobias and Black, 2002).

Due to pRB's involvement in other types of cancers as well as retinoblastoma, the patients that have a germinal mutation are at higher risks for developing nonocular neoplasms (Narins, 2000). Other cancers that are linked to a mutated RB-1 gene include cancer of the breast, brain, or prostate, osteosarcoma, soft tissue sarcoma, leukemia and

other sporadic cancers. Another risk for those with a germline mutation is “trilateral retinoblastoma” which involves a pediatric tumor in the midbrain (MacPherson, 2008).

The cause of retinoblastoma has been linked to loss of function of the tumor suppressor retinoblastoma gene (RB-1) at chromosome location 13q14 (Tobias and Black). There are 27 exons that encode the RB-1 gene ranging from 31 to 1,889 base pairs (Claudio, et al., 2002). RB-1 codes for the protein pRB, a nuclear phosphoprotein that is part of a pathway for cell division regulation, differentiation, apoptosis, and other important processes (MacPherson, 2008). If pRB does not function correctly due to its absence or gene mutation, uncontrolled cell proliferation is occurs leading to the development of cancer (Brigham, 2000). However, recent studies have suggested an additional mutation is needed for retinoblastoma to present.

There are three proteins that make up what are known as the “pocket proteins.” They include pRB, p107, and p130. Named for their pocket domain, they are important for binding E2F transcription factors and they form part of a signal-transduction pathway known as the RB pathway. These pocket proteins are similar in that all three consist of an amino-terminal domain, two conserved domains that make up the pocket region, a spacer region, and a carboxy-terminal domain. They differ in function and the specific E2F transcription factors that they bind to. Interestingly, high p130 levels have been found in retinomas (benign retinal tumors) and not in nearby retinoblastoma. This finding suggests the progression of retinoma to retinoblastoma may be linked to the lack of p130. The p130 might also be a tumor suppressor gene. Due to the discovery of consistent abnormalities in other chromosomes, the deletion of RB-1 may not be enough for retinoblastoma to occur (MacPherson, 2008).

Retinoblastoma Diagnosis & Treatment

Retinoblastoma is generally diagnosed using the results of a funduscopy exam and sometimes with the aid of ultrasound, magnetic resonance imaging (MRI) and/or computed tomography (CT). Ocular funduscopy is the process in which, under general anesthesia, the retina is observed using an indirect ophthalmoscopy. The lesion can be seen as a white tumor surrounded with irregularly arranged blood vessels. An ocular ultrasound expresses a mass made up of fine calcifications. When there is local extension of Rb, MRI is usually used to illustrate extensions of the tumor into the optic nerve, anterior chamber, and fat of the orbital. Intraocular tumors that are higher in density than the vitreous body are typically observed in using CT (Aertes, 2006).

Treatment is dependent upon the following types of retinoblastoma and stages of the disease; bilateral, unilateral, multifocal, unifocal, number of tumors, size of tumors, and disease extension. Two classification systems that are used to group retinoblastoma are the Reese Ellsworth classification and the ABC classification. After Rb staging has been determined, treatment is decided and may include a number of the following: enucleation, external beam radiation therapy, cryotherapy, transpupillary thermotherapy, laser photocoagulation, brachytherapy, chemotherapy (Aertes, 2006).

Enucleation (the complete removal of the eyeball) is recommended in certain situations that include large tumors, retinal detachment, and loss of functional vision. Although it can be traumatic, it is usually successful. Also successful is external beam radiation therapy; however, there are many adverse side effects that include secondary non-ocular malignancies, cataracts, radiation retinopathy (bleeding and exudates of the retina), impaired vision, temporal bone suppression and deformities (De Potter, 2002;

Abramson and Servodidio, 1997). In an attempt to avoid enucleation and external beam radiation therapy, first-line chemotherapy is often used followed by local treatment (De Potter, 2002).

Chemotherapy options include chemotherapy injections into the bloodstream (intravenous), subconjunctival chemotherapy (applied locally underneath the conjunctiva) and systemic chemotherapy (taken orally or injected into the muscles). Chemotherapy drugs commonly used for treating Rb include carboplatin, vincristine, and/or etoposide. After these tumors have been chemoreduced they can be treated with the more local treatments (De Potter, 2002).

Laser photocoagulation is the procedure of shining a beam of light through the pupil to permanently destroy the vessels feeding the tumor. Transpupillary thermotherapy uses an infrared laser to induce hyperthermia on the tumor. Adverse side effects can include optic disc and focal iris atrophy. Cryotherapy is a procedure that denatures smaller retinoblastoma tumors by freezing them. Brachytherapy is the suture of an iodine-125 (^{125}I) plaque to the tumor and subsequent removal to reduce tumor size. This procedure aims to reduce excess radiation and is often used in place of external beam radiation (De Potter, 2002; Abramson and Servodidio, 1997; Kufe, et al., 2003).

Cannabinoids

Several studies have examined the medicinal properties of *Cannabis*'s main active component THC (tetrahydrocannabinol). However, due to its psychoactive properties and the build-up of resistance, other cannabinoids have been investigated to find a more effective pharmaceutical. A group of cannabinoids that are THC metabolites actually

lack psychoactive effects and are called cannabinoid acids. These acids include all of the cannabinoids' carboxylic acid metabolites and their man-made analogs. The primary metabolite of the cannabinoid acids, derived after the oxidative metabolism of THC, is THC-11-oic acid. However, to make it more potent for pharmaceutical uses, the synthetic analog of THC-11-oic acid called ajulemic acid (dimethylheptyl-THC-11-oic acid) or AJA was developed (Structure shown in Figure 1) (Zurier). AJA has been found to relieve pain, reduce inflammation, and have anti-tumor effects without producing psychoactivity (Recht, 2001).

Ajulemic acid ($C_{25}H_{36}O_4$) has a molecular weight of 400.55 g/mol and is stable as a white crystalline solid. It is easily dissolved in many organic solvents and aqueous buffers with a pH above 8 (Burstein, 2005).

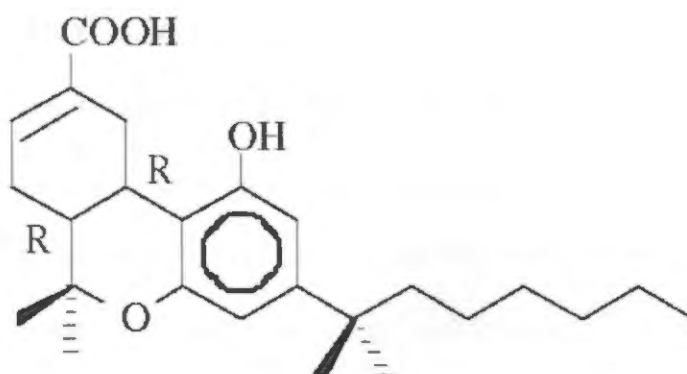


Figure 1. Structure of Ajulemic Acid

There has been much research conducted in an attempt to determine AJA's mode of action. Although there are still many unknowns, several propositions have been made. Firstly, it has been identified that AJA is highly stereospecific points to the idea that its mechanism is receptor mediated (Burstein, 2004). There is evidence that AJA moderately binds to CB_1 and CB_2 receptors; however, this is contradictory with AJA's non-psychoactivity (Burstein, 2005). CB_1 receptors are found throughout the peripheral

nervous system and the central nervous system. The areas in which the receptors are most heavily expressed (the hippocampus, cortex, basal ganglia, cerebellum, and spinal cord) account for the memory, cognitive, and movement effects of cannabinoids. The actual binding to the CB receptors activate G-proteins that affect many signal pathways by activation or inhibition (CNSforum).

When the cannabinoid receptors are activated by THC, the activated G-proteins inhibit calcium channels dependent upon voltage gradients, sodium channels, and adenylate cyclase. In addition, the G-proteins stimulate potassium channels and the MAPK signaling pathway. In all, the cell's cAMP concentration is decreased leading to neurotransmission inhibition. The sum of these affected pathways result in the euphoria effects involved in using *Cannabis* (CNSforum). Therefore, it has been suggested AJA may activate these receptors while antagonizing the pathway needed for the psychotropic effects (Brecht, 2005). In addition, there still may be other receptors with which AJA interacts, such as strychnine-sensitive α_1 - and $\alpha_1\beta$ -glycine receptors (Ahrens, et al., 2008) or the isotope γ of the peroxisome proliferator-activated receptor (PPAR γ) (Ambrosio, et al., 2007). Further research should be conducted to determine the receptors to which AJA binds both directly and indirectly.

Carboplatin

Carboplatin (CBP) is a chemotherapy drug that is currently being used in the treatment of several cancers including retinoblastoma. CBP (molecular weight 371.25 g/mol) is made up of a platinum-coordinated complex and is toxic to cells by alkylating DNA (Eljarrat-Binstock, et al., 2008).

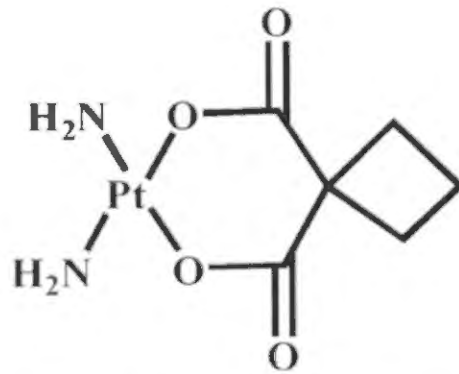


Figure 2. Structure of Carboplatin

Not only can carboplatin be used with other chemotherapy drugs intravenously, it has also been used singularly as a periocular injection (Figure 2) (Schmack, et al., 2006). However, a major side effect encountered with carboplatin periocular injections is ischemic optic neuropathy (Schmack, et al., 2006). Another accepted use is the subconjunctival injection of carboplatin. Side effects for this procedure include endophthalmus, atrophy of the optic nerve, and myelin sheath and nerve fiber loss (Kiratli, 2006).

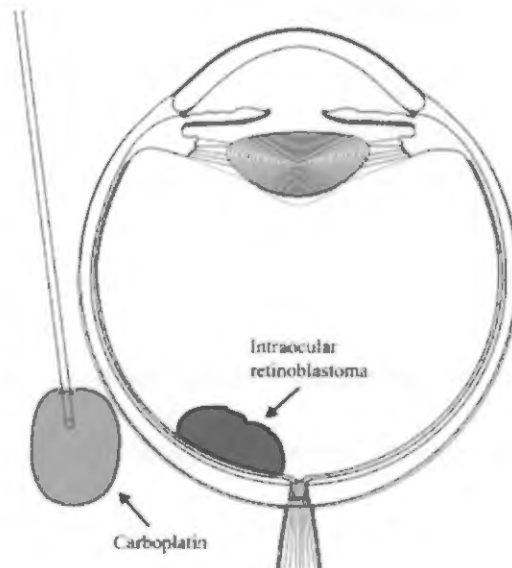


Figure 3. Periocular carboplatin injection.

From: Schmack: *The American Journal of Ophthalmology*, Volume 142(2).August 2006.311. © 2006 Elsevier Inc.

Materials

Ajulemic acid (AJA)

Ajulemic acid (AJA), dimethylheptyl-THC-11-oic acid was prepared in dimethyl sulfoxide (DMSO) to a final stock concentration of 10 mM.

Carboplatin (CBP)

Carboplatin (CBP) stock was obtained from Sigma-Aldrich and was prepared in sterile phosphate buffer saline (PBS) or ethanol (EtOH) to a stock concentration of 10 mM.

Cell Culture

Y-79 (human retinoblastoma) suspension cell line was obtained from ATCC and grown in RPMI-1640 medium containing a final concentration of 10% fetal bovine serum and was cultured at 37°C with 5.0% CO₂. Primary mice microglia and astrocytes were obtained from UAMS.

Methods

MTS Assay

Y-79 cell viability was determined by measuring mitochondrial activity using an MTS assay composed of [3-(4,5-dimethylthiazone-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] and an electron coupling reagent (phenazine methosulfate) (Promega). MTS solution was prepared by adding 42 mg of MTS Reagent Powder to 21 mL of DPBS, mixed on stir plate for 15min, and filter-sterilized through a

0.2 µm filter. MTS (40 µL) were added to each well of a 96-well plate, and the plate was incubated at 37°C for 1 hour. The plate was then read in a plate reader (TECAN infinite series) and absorbance was measured at wavelength of 490 nm.

MTT Assay

An MTT (3-(4,5-dimethylthiazone-2yl)-2,5-diphenyltetrazolium bromide) assay was used to assess cell viability of primary microglia and astrocytes. MTT solution was prepared by diluting MTT 1:50 with culture medium. Cell culture media was removed from cells and replaced with 500 µL MTT solution and incubated at 37°C for one hour. MTT solution was removed and cells were lysed by adding 500 µL DMSO/well. Plates were rocked for 30 min and read on a plate reader (TECAN infinite series) at 570 nm.

ELISA Assay

Caspase-3 levels were measured using an enzyme-linked immunosorbent assay according to manufacturer's recommendations (BD Bioscience).

Neuroprotection

Mouse cortical neurons were obtained from Lonza. They were plated in 36 wells of a 96-well plate and cultured according to manufacturer's recommendations. Fourteen days after plating, cells were treated with either AJA, INF-γ (interferon gamma), both or neither as indicated for 72 hr. MTS assays were then run as previously described to assess cell viability.

Results

Cell Density

In order to determine the ideal cell density at which Y-79 Rb cells grew the best and reacted most towards the treatment, multiple cell densities were investigated. The cells were plated in 5 various densities ranging from 4×10^4 cells/well to 4×10^5 cells/well in 6 wells each of the 96-well plate. After 24 hr, wells 1-3 of each density were left untreated and wells 3-6 of each density were treated with $50 \mu\text{M}$ ajulemic acid. An MTS assay was used to measure the cell viability by assessing the mitochondrial activity. The MTS reagent is a chemical that reacts with an enzyme present when the mitochondria are active. This reaction yields a soluble purple precipitate that can be measured in intensity on a plate reader at 490 nm. Cell viability was compared at each density with the untreated wells. Greatest cell viability loss due to AJA treatment was observed at the cell density 3.1×10^5 cells/well (Figure 4).

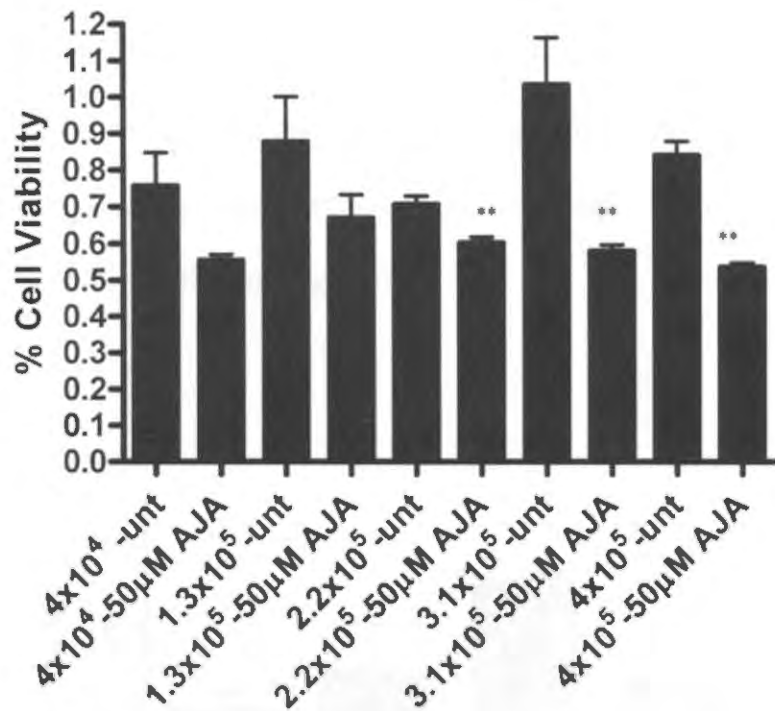


Figure 4. Cell Density.

Y-79 Rb cells were plated with indicated densities and treated in triplicate with 50 μ M AJA. Cell viability was measured using MTS assay 72 hr later. Values represent the average cell viability for triplicate cultures. Standard errors are indicated. Asterisks designate statistical significance of $p < 0.05$. Greatest difference in viability between untreated and treated wells was observed at the cell density 3.1×10^5 cells/well.

The Effects of DMSO on the viability of Y-79 Rb cells

In order to assess the effects of the solvent DMSO, in which AJA is dissolved, Y-79 cells were plated in a 96-well plate at a density 3.1×10^5 cell/well. Wells were treated in triplicate 24 hr later with the following increasing ajulemic acid concentration equivalents of dimethyl sulfoxide (DMSO): 50 eq, 75 eq, 100 eq. Cell viability was measured using an MTS assay. Our results indicate that DMSO does not significantly affect the viability of Y-79 RB cells (Figure 5).

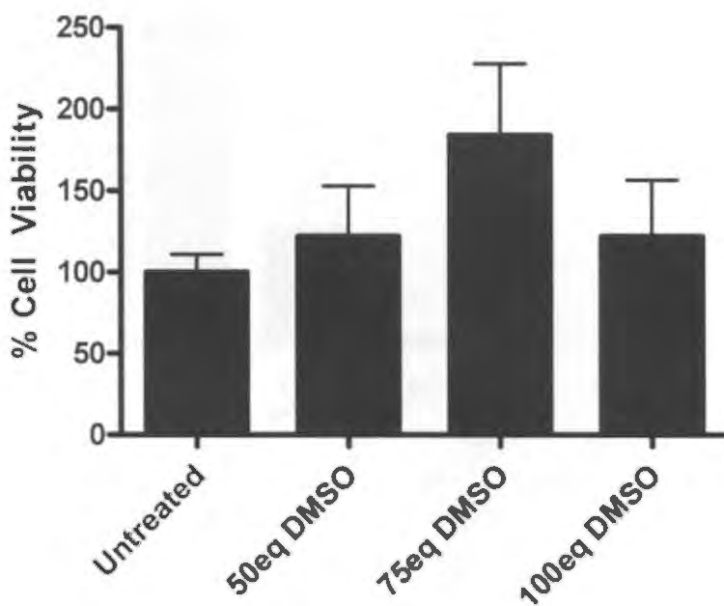


Figure 5. Y-79 Cultures with Dimethyl sulfoxide (DMSO) treatment.

Cells were treated 24 hr after plating with DMSO in equivalent volumes of AJA treatments as indicated to account for any cell death that was not caused by AJA. Cell viability was measured using MTS assay 72 hr later. Values represent the average cell viability for triplicate cultures. Standard errors are indicated.

Ajulemic Acid's Effect on Y-79 Rb cell viability

Y-79 cells were plated in a 96-well plate at a density 3.1×10^5 cells/well. Wells were treated in triplicate 24 hr later with the following increasing ajulemic acid (AJA) concentrations: 25 μ M, 50 μ M, 75 μ M, 100 μ M. After 72 hr, cell viability was measured using a MTS assay. Our data indicates significant cell death in Y-79 RB cells when treated with concentrations between 25 and 100 μ M AJA (Figure 6).

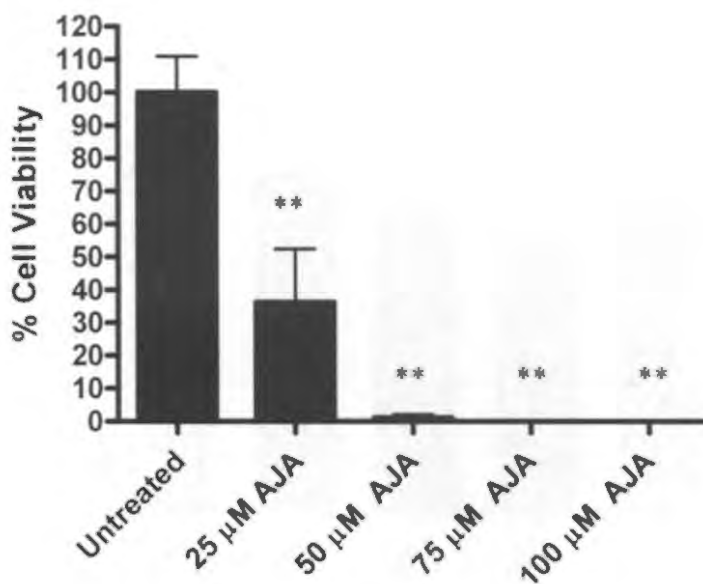


Figure 6. Significant cell death observed in Y-79 cultures with AJA Treatment.

Cells were treated in triplicate with the indicated AJA concentrations. Representative data are shown. MTS cell viability assay was run at 72 hr post-treatment. Values represent the average cell viability for triplicate cultures. Standard errors are indicated. Asterisks indicate statistical significance of $p < 0.05$.

Carboplatin's Effect on Y-79 Rb cell viability

Y-79 cells were plated in a 96-well plate at a density 3.1×10^5 cells/well. Wells were treated in triplicate 24 hr later with the following increasing chemotherapy drug carboplatin (CBP) concentrations: 25 μM , 50 μM , 75 μM , 100 μM . An MTS assay was used to measure the cell viability after 72 hr. Our results indicate that significant cell death occurs in Y-79 RB cells when treated with 50 μM concentration of CBP (Figure 7).

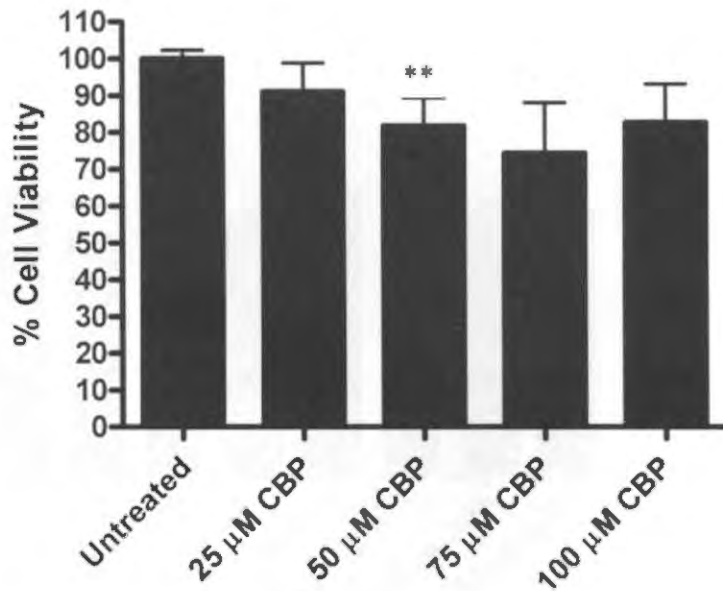


Figure 7. Y-79 cultures with CBP Treatment.

Cells were treated in triplicate with the indicated CBP concentrations. Representative data are shown. MTS cell viability assay was run at 72 hr post-treatment. Values represent the average cell viability for triplicate cultures. Standard errors are indicated. Asterisks indicate statistical significance of $p < 0.05$.

Effect of AJA on non-transformed neural-derived cells

Primary mouse astrocytes and microglia were plated and after 24 hr, treated in triplicate with increasing ajulemic acid concentrations of 50 μ M, 100 μ M, and 200 μ M. Cell viability was measured at 24 hr using an MTT (3-(4,5-dimethylthiazone-2-yl)-2,5-diphenyltetrazolium bromide) assay. The MTT reagent reacts with a mitochondrial enzyme that is active when the cell is alive and the chemical reaction yields an insoluble purple precipitate. Once the cell is lysed, the precipitate is released and the assay concentration can be measured on a plate reader at 570 nm. The results indicate that AJA concentrations between 100 and 200 μ M become toxic to astrocytes (Figure 8 – Chavis and Tull, 2007).

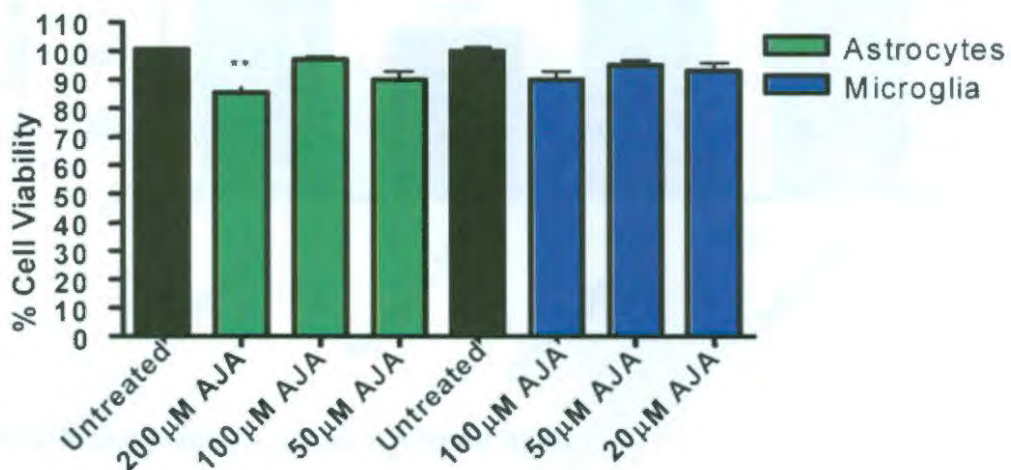


Figure 8. Ajulemic acid becomes toxic to non-transformed neural-derived cells at concentrations between 100 and 200 μ M. Cells were plated in triplicate and treated with the indicated concentrations of AJA. MTT cell viability assays were run at 24 hr post-treatment. Standard errors are indicated. Asterisks indicate statistical significance of $p < 0.05$ (Chavis and Tull, 2007).

Possible Neuroprotection with Ajulemic Acid

Primary mouse cortical neurons were plated in a 96-well plate. Neurons were treated with 500 units/mL interferon gamma (INF- γ). Significant cell death was observed. Mice neurons were pretreated with 40 μ M AJA and then treated with 500 units/mL INF- γ . Although not significant, less cell death was observed in the cells pretreated with AJA versus the control. Mice neurons were treated with 40 μ M of AJA and no significant cell death occurred (Figure 9 - Hurst, 2008).

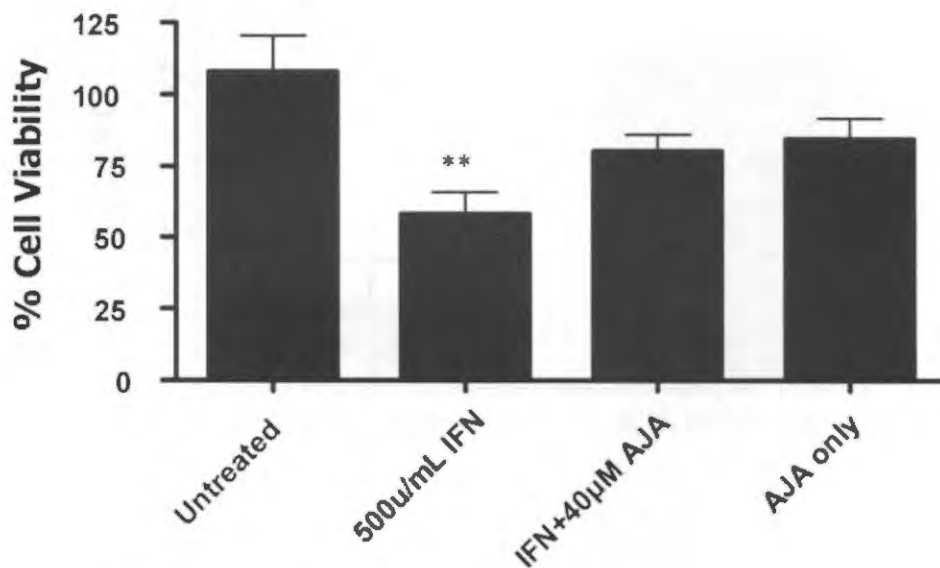


Figure 9. Neuroprotection with Ajulemic Acid.

Significant cell death occurred when interferon gamma (IFN- γ) was used to treat mouse cortical neurons. When treatment included both IFN- γ and AJA together, a lower amount of cell death was observed. There was also a lower amount of cell death observed when the AJA only treatment was used. MTS cell viability assay was run at 72 hr post-treatment. Values represent the average for triplicate cultures. Standard errors are indicated. Asterisks indicate statistical significance of $p < 0.05$ (Hurst, 2008).

Potential Apoptosis Mechanism of Ajulemic Acid

Expression of caspase-3, an enzyme activated during the apoptotic cascade, was measured by ELISA. The results indicate that caspase-3 has been expressed in SK-ES Ewing's sarcoma cells when treated with 40 μ M ajulemic acid (Figure 10 - Daly, 2008)

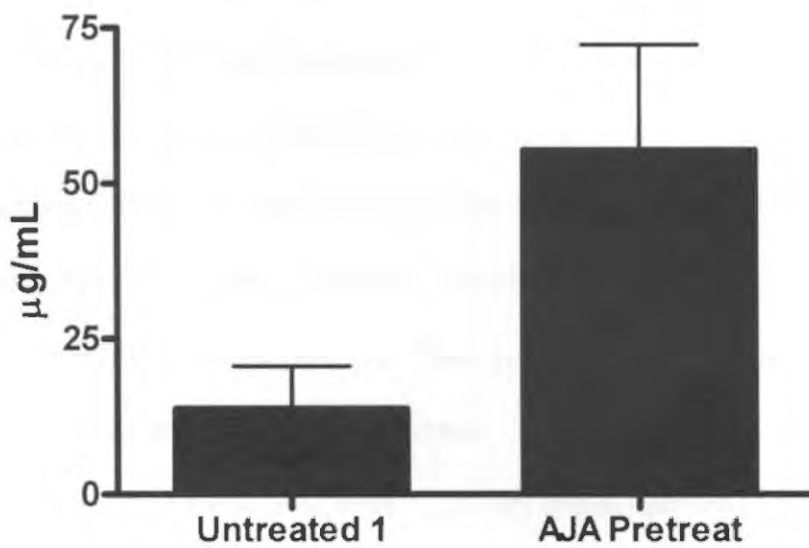


Figure 10. Caspase-3 ELISA.

SK-ES Ewing's sarcoma cells were pretreated with 40 μ M ajulemic acid and were measured for caspase-3 expression using ELISA assay. Indicated amounts of caspase-3 in μ g/mL were expressed in the untreated and AJA pretreated cells. Standard errors are indicated.

Discussion

The medicinal use of *Cannabis*' active ingredient THC (tetrahydrocannabinol) has attracted much attention. However, its psychotropic effects, although not usually considered addictive, keep it from being socially accepted. Therefore, there has been a large amount of recent research on its synthetic analog ajulemic acid (AJA) which does not cause psychoactive effects. This research has shown AJA's connection to pain reduction, anti-inflammatory properties, and tumor reduction without psychoactive effects. Up until now, there had been no prior research done on the effects of AJA on retinoblastoma, a neural derived cancerous tumor of the retina.

Cells from human retinoblastoma cell line Y-79 were treated with various concentrations from 25 to 100 μM of AJA. Complete cell death was observed after 72 hr using an MTS assay to measure viability. There is significant evidence that ajulemic acid is a potent retinoblastoma tumor cell suppressor.

Ajulemic acid was also used to treat primary mouse astrocytes and microglia. Only AJA concentrations above 100 μM induced significant cell death. Therefore, AJA has been shown to significantly kill the Rb tumor cells at concentrations below 100 μM without inducing cell death to the surrounding neuroglial tissue.

Preliminary data suggest ajulemic acid may also be neuroprotective. Significant cell death of primary mouse neurons occurred when treated with the cytokine interferon gamma ($\text{INF-}\gamma$) which causes cell apoptosis. However, when neurons were pretreated with 40 μM AJA and were then introduced to the interferon gamma, less cell death occurred. Although the data was not statistically significant, a greater number of trials

with larger sample sizes may result in a more concrete answer to AJA's ability to protect neurons from immune responses such as INF- γ .

Ajulemic acid's effects on RB cells may be induced through apoptotic mechanisms. The expression of caspase-3 indicates that apoptosis did occur in cells treated with AJA. Apoptosis is a preferable method of cell death over necrosis because cell rupture, as it occurs in necrosis, can cause leakage of lysosomal enzymes that may be deadly to nearby cells.

In our cell model, at equal concentrations ajulemic acid is more toxic to the Rb tumor cells than carboplatin, a current chemotherapy drug used for retinoblastoma treatment. Future research should investigate the effects of Rb tumor viability with lower ajulemic acid concentrations. Lower concentrations of AJA with the same loss of tumor cell viability could be a more beneficial treatment.

There is great potential for the movement of AJA in the direction of Rb treatment. Plans include continuing the research on AJA and its effectiveness as a tumor therapeutic in the hopes that one day, AJA may be a solution for retinoblastoma patients.

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