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The Effects of Ajulemic Acid on the Metatastic Potential of Ewing's Sarcoma

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SENIOR THESIS APPROVAL

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"The Effects of Ajulemic Acid on the Metastatic Potential of Ewing's Sarcoma"

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Abstract

Ewing's Sarcoma belongs in the Ewing's Sarcoma Family of Tumors (ESFT) which represents approximately 2% of all cancers found in children and young adults (1). These tumors are generally treated by chemotherapy along with either surgery or radiation therapy which can themselves have debilitating effects on the patients (2-6). Dr. Lori Hensley's research has shown that ajulemic acid (AJA), a synthetic cannabinoid compound, exhibits extraordinary anti-tumor effects. In addition, AJA has not expressed any psychotropic side effects typically seen with other cannabinoids. Furthermore, ajulemic acid has been shown to eliminate the tumors by apoptosis, the preferred method of cell death. The purpose of this research is to observe if AJA can decrease the ability of cancerous cells to metastasize.

Introduction

Ewing's sarcoma

Ajulemic acid has displayed the ability to effectively eliminate various types of Ewing's sarcoma cells in a dose-dependent manner without harming non-cancerous cells. The purpose of this research was to investigate if ajulemic acid could also decrease the ability of Ewing's sarcoma cells to metastasize.

The Ewing's Sarcoma Family of Tumors is made up of Ewing's sarcoma, primitive neuroectodermal tumors (PNETs), and Askin tumors, which are PNETs of the chest wall (7). These cancers are predominately pediatric cancers which develop in the bone. There are over two hundred new diagnoses of Ewing's each year and only approximately 15% of these cases are found in adults.

Most of the ESFT affect a lower extremity (41%) or the pelvis (26%). These tumors are nine times more likely to appear in Caucasians than in African Americans (8). Also, these tumors are found in higher instances in males than in females at a ratio of nearly 3:2 (9).

One of the most detrimental aspects of Ewing's sarcoma is its incredible ability to metastasize. Metastasis is the spreading of a tumor throughout the body. The most common metastatic site for a Ewing's tumor is the lung although it can metastasize to other parts of the body, such as the brain. Tragically, many Ewing's tumors go undetected until they have metastasized: "approximately 25% of patients will have [a] metastatic disease at diagnosis (10)." With current treatment options, the prognosis is grim for those whose tumor has metastasized by the time of their diagnosis.

Ewing's Etiology

It is believed that Ewing's sarcoma is not an inherited disease. Instead, it randomly occurs after the birth of the afflicted child (11). Other cancerous syndromes do not seem to imply causation of the malady (1).

The "cellular origin" of Ewing's tumors has so far eluded scientists and the argument over how these cancerous cells arise has been "highly debated (12)." However, according to

recent research done by M.L. Suvà, all lines of the Ewing's Sarcoma Family of Tumors are believed to arise from a common mesenchymal stem cell originating in the bone marrow (13). The incidence of Ewing's sarcoma has proven to be extremely steady over the past thirty years (14).

Diagnosis

A given tumor from the Ewing's Sarcoma Family of Tumors is hard to diagnose because there are typically few signs and/or symptoms associated with the disease at its onset. Signs are outward expressions on or produced by the body that a physician can see, hear, or feel. Symptoms are a patient's complaints about a problem communicated to the physician. Symptoms are generally less telling, since they involve patients' feelings and/or emotions, both of which are highly personal and subjective. However, the "most common presenting symptom is pain (1)," which is itself subjective.

The issue of diagnosing Ewing's becomes even more complex because sufferers are usually pediatric patients. The child must communicate his or her symptoms to their parent or guardian who then must take the child to a physician. The physician must then attempt to glean information from both the parent and child to make a correct diagnosis. Another sign that presents itself in patients with Ewing's is the breaking of a bone "for no known reason (7)." One other is hypersarcoplasia, the rapid growth of a tumor mass which can cause "fever and weight loss (1)."

Early and accurate diagnosis of the malady is also difficult because normal lab studies do not directly point to a diagnosis of Ewing's sarcoma. An elevated "white blood cell (WBC)

count, erythrocyte sedimentation rate (ESR), and/or lactate dehydrogenase" (LDH) level can point to Ewing's sarcoma (1). The best way to firmly determine if the patient has Ewing's is to perform a biopsy, the removal of living cells for examination, and then perform a test on the cells to see if the CD99 or FL-1 proteins are present. Together, these "strongly suggest the diagnosis of ESFT (15)."

Prognosis and Treatment Options

An early and accurate diagnosis is critical for a patient's survival. For Ewing's sarcoma patients, the most important predictor of survival is whether they have a metastatic disease (16-18). Factors that do not appear to negatively affect a patient's prognosis are "pathologic fractures, (19)" "histopathology: the degree of neural differentiation, (15, 20)" or "EWS-FL1 translocation (21, 22)" of the gene.

The main treatment options are not preferred as they often produce debilitating results and decrease the patient's quality of life. Currently the only effective way to treat Ewing's sarcoma is by chemotherapy or surgical amputation, usually in conjunction with radiotherapy. After drastic steps have been taken, additional chemotherapy is normally used for local control (1).

Cannabinoids

In the past ten years, much research has been conducted in the field of cannabinoids. Cannabinoids are "the active components of marijuana and their derivatives. (23)" Many studies have documented valid medicinal benefits from this family of drugs. A specific drug, cannabidiol

(CBD), has been shown to possess "antitumor properties" (24) on human glioma cell lines. Another study suggested that CBD also inhibited the migration of this specific glioma (25). However, considerable controversies remain regarding the use of cannabinoids because they may exhibit unwanted—and in the opinion of many, unethical—psychotropic side effects along with their medicinal benefits.

Ajulemic Acid

The active component of marijuana is tetrahydrocannabinol (THC or Δ9-THC). It binds directly to CB1 receptors in the brain which mediate its psychotropic effects. After THC is metabolized, it becomes chiefly THC-11-oic acid, also called 11-COOH-THC. It is from this natural derivative on which the synthetic ajulemic acid is based.

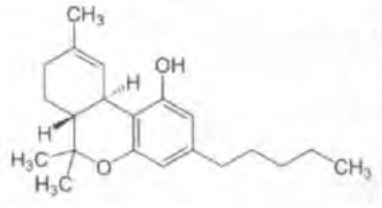
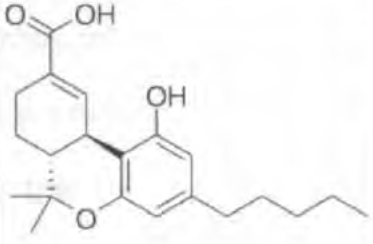
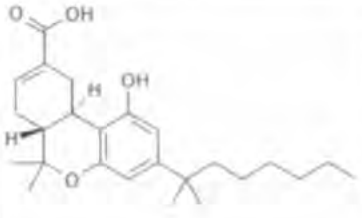
Tetrahydrocannabinol (THC)	THC-11-oic Acid	Ajulemic Acid
		

Figure 1. Structures of Cannabinoids

Ajulemic acid's organic structure is exceptionally similar to that of THC-11-oic acid, but a different location of the alkene present on the ring and the addition of the few carbons to the chain give ajulemic acid vastly different medicinal effects. Historically, Ewing's has been

difficult to eliminate, but ajulemic acid's ability to kill these hardy cells suggests that the drug could be a viable candidate for medicinal use. Further strengthening AJA's case is the fact that "there is no evidence that it produces psychotropic actions when given at therapeutic doses" (26) on the user, which gives ajulemic acid a sparkling outlook as a potential drug against these cancers.

Metastasis

Ewing's sarcoma is a deadly disease, largely due to its ability to metastasize. Metastasis is the "process by which cancer cells spread to other parts of the body" (27) and has two main aspects: migration and invasion. The ability of cells to move themselves in the body is migration. Once Ewing's sarcoma cells develop in the bone they can navigate along the interior of the bone. This ability to migrate enlarges the tumor's mass and presence in the bone and allows the cancerous cells to travel to the blood and lymphatic vessels. To proliferate through the body, the cells must squeeze themselves through the basement membrane present in either of these vessels. This penetration of the cells through a basement membrane is defined as invasion. Once the cells have gained access to the blood or lymphatic vessels by means of invasion, they can spread to other parts of the body. Without the ability to migrate or invade, the malignant cells would not be able to effectively metastasize. The ability to halt or slow the metastatic potential of Ewing's sarcoma would greatly reduce the severity of the cancer and significantly improve the prognosis for patients diagnosed with the cancer.

Materials

Ajulemic acid (AJA)

Ajulemic acid was prepared at a 100mM concentration in DMSO by using a ratio of 40 mg/mL. This solution was diluted using DMSO to a working dilution of 10mM.

Cell Culture

Ewing's sarcoma SKES cells were cultured at 37° in 5% CO_2 in McCoy's 5A media with 15% FBS and penicillin / streptomycin. NIH 3T3 cells were cultured at 37° in 5% CO_2 in DMEM media with 10% FBS and penicillin / streptomycin. Ewing's sarcoma TC71 cells were cultured at 37° in 5% CO_2 in McCoy's 5A media with 15% FBS and penicillin / streptomycin.

Methods

Wound Migration Assay

SKES cells were plated at a concentration of 1 x 10^6 cells / well in the presence of wound inserts (Cell Biolabs, Inc.) at 500 µL per well in a 24-well collagen, laminin, and poly-D-lysine coated plate. Wound inserts were removed and wells were washed after mono layer formation and rehydrated with media plus indicated concentrations of AJA. Migration of cells across the wound field was observed at indicated time points.

Invasion Assay

Cell Biolabs, Inc. colorimetric basement membrane cell invasion assay was used according to manufacturer's recommendations with SKES cells.

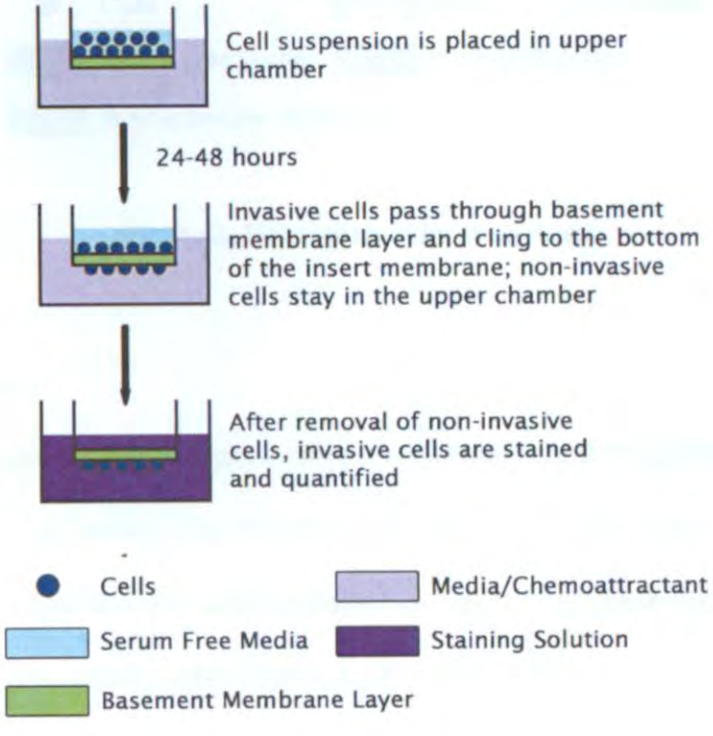


Figure 2. Invasion Assay

Haptotaxis Migration Assay

Cell Biolabs, Inc. colorimetric haptotaxis migration assay with fibronectin was used according to manufacturer's recommendations with SKES cells.

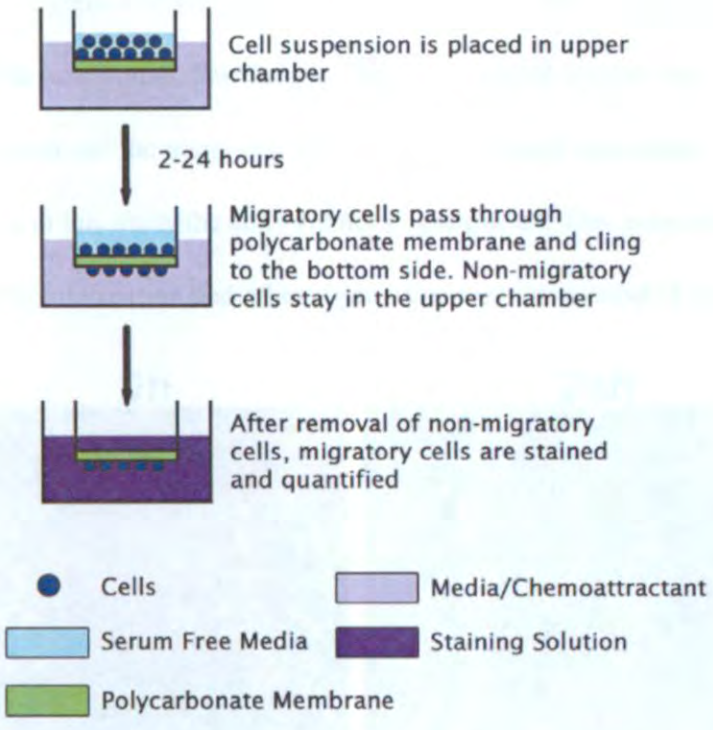


Figure 3. Haptotaxis Migration Assay

TC71 Migration Assay

Sigma Transwell plates and inserts were used according to the Haptotaxis Migration Assay instructions. Cell Biolabs Stain Solution and Extraction solution were used. The inserts were hydrated with Plain McCoy's medium before use and the cells were suspended in Plain McCoy's; both of these actions were to increase the migration ability of the Ewing's cells.

Results

Wound Migration Assay

A wound migration assay was used to determine if ajulemic acid could affect Ewing's sarcoma's ability to migrate. Cells were plated at 1 x 10^6 cells / mL in wound inserts. Pictures were taken at varying time points. The distance the cells traveled to move together could be quantitatively measured and the migratory ability between treated and untreated cells could be compared. However, in this assay the cells did not migrate at all. This assay was ineffective at conveying any usable information and other approaches were considered (Figure 4).

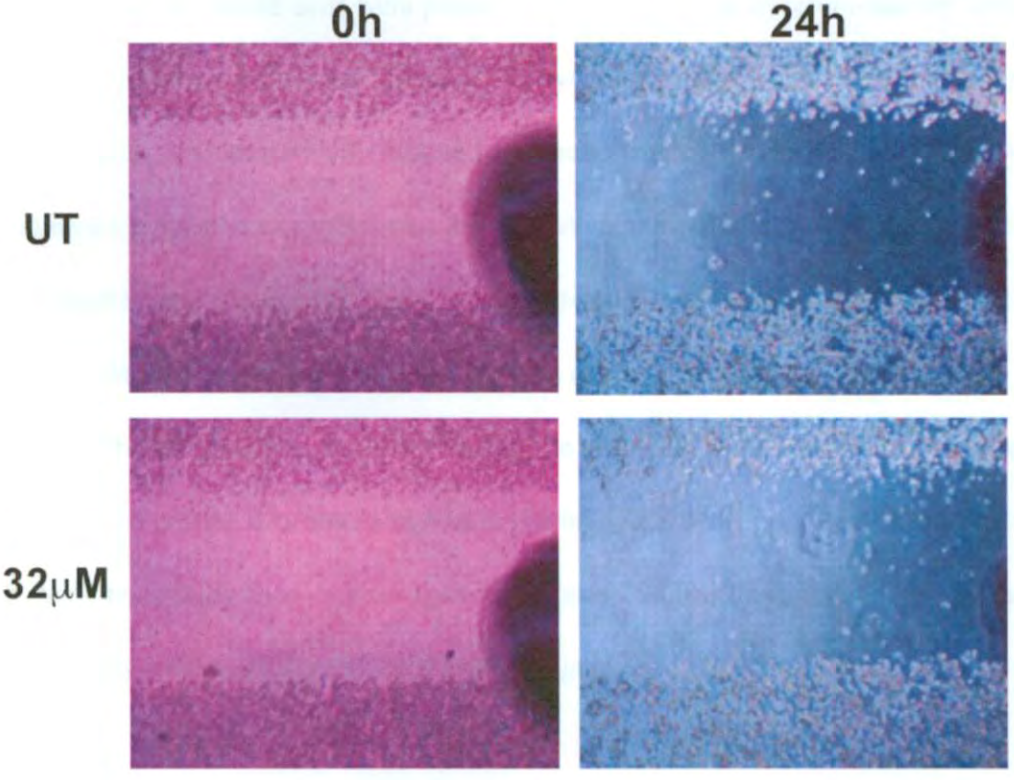


Figure 4. SKES wound migration assay is ineffective.

The cells were plated in triplicate and treated with the concentrations listed for the wound assay; the photographs were captured at the time points listed. Representative photos are shown. The

assay was run on plain tissue culture plates as well as on collagen-coated with laminin and poly-D-lysine. Cells were serum-starved at the time of plating and then treated in complete media with 10% FBS.

Haptotaxis Migration Assay

An alternate method by which to measure the migratory ability of SKES cells was to perform a haptotaxis migration assay since the wound migration assay failed to provide usable results. Since the Cell Biolabs invasion assay performed well, an extremely similar migration assay was chosen. SKES cells were plated at 1 x 10^6 cells / mL in serum-starved media in a 24 well plate in specialized inserts that had been hydrated with Plain McCoy's media. Three groups of SKES cells were treated with AJA and one group was an untreated control. The insert contained a polycarbonate membrane through which the cells could migrate towards the chemotactic agent fibronectin. These inserts were removed and the stain from the cells were extracted from the bottom of the insert using an extraction solution. Cell ratio values were quantified by using a plate reader to measure the absorbance of the different extractions for each of the treated groups. The ability of AJA to inhibit SKES cells' migration ability was compared. The haptotaxis assay showed the migratory ability of SKES cells but did not show that AJA significantly inhibited migration of the cells (Figure 5).

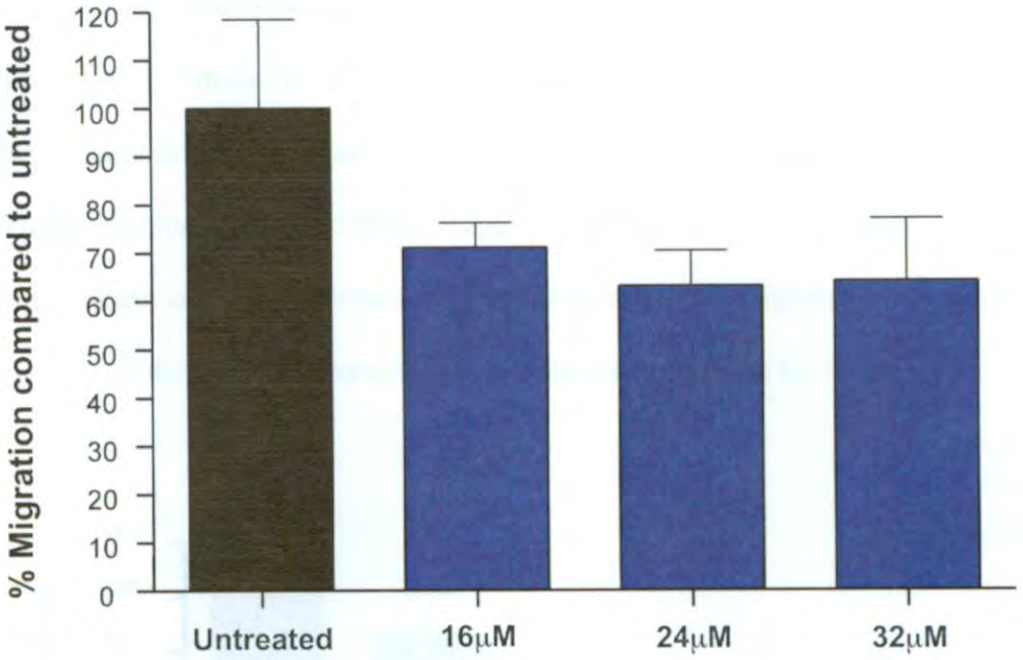


Figure 5. AJA has no significant effect on cell migration in haptotaxis migration assay. No significant migration or differences in migration between untreated and treated cells were observed in a haptotaxis assay with fibronectin as the chemotactic agent. Values represent the average for triplicate cultures.

Invasion Assay

The Invasion assay was selected from Cell Biolabs and used to assess the ability of AJA to affect the invasive ability of SKES cells through a basement membrane. SKES and NIH 3T3 cells were plated at 1 x 10^6 cells / mL in serum-starved media in a 24 well plate in specialized inserts that had been hydrated with Plain McCoy's media. Some groups of SKES cells were treated with AJA; NIH 3T3's are non-motile cells and were used as the negative control. The insert contained a basement membrane through which the cells could invade to reach serum-

containing media that had been plated on the bottom of the well. The cells were stained and the color was extracted from the invasive cells on the bottom of the insert by using an extraction solution. Cell ratio values were quantified by using a plate reader to measure the absorbance of the different extractions for each of the treated groups. The ability of AJA to inhibit SKES cells' invasive ability was compared. The invasion assay showed that AJA significantly decreased the invasive ability of the SKES cells in a dose-dependent manner (Figure 6).

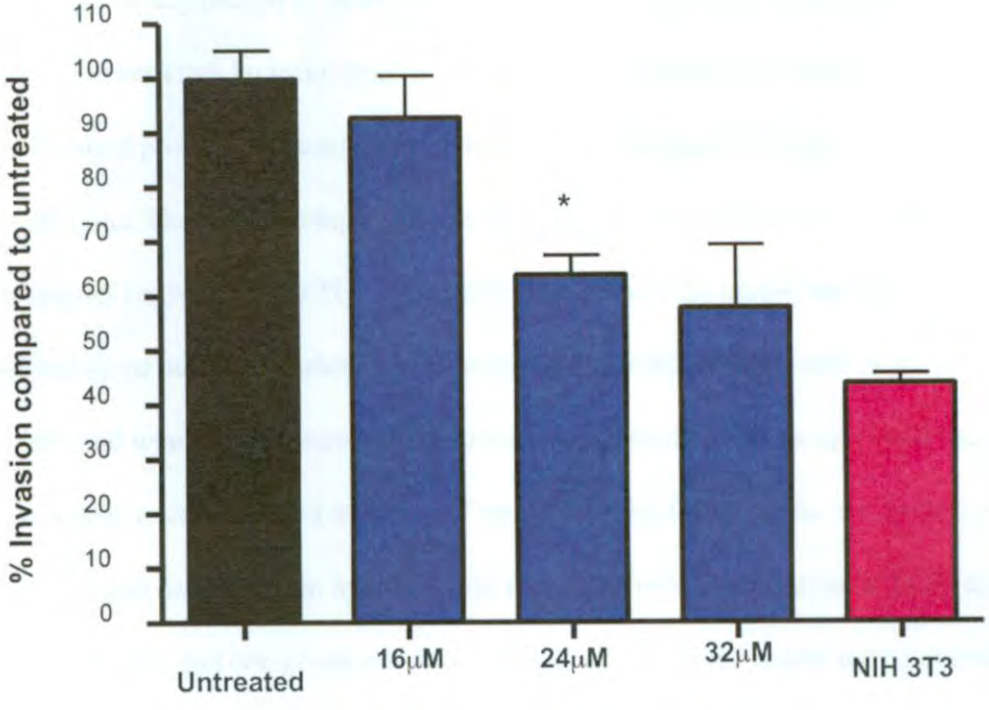


Figure 6. AJA inhibits invasion of SKES cells through a basement membrane.

Cells were plated in triplicate in serum-free media in the upper chamber of a transwell system containing a basement membrane with an 8μm pore size. Cells invaded the lower chamber in response to serum-containing media. Average values are shown with standard error indicated.

Values represent the average for duplicate cultures. Asterisks denote statistical significance of ($p < 0.05$).

TC71 Migration Assay

In the haptotaxis migration assay, the SKES cells did migrate through the membrane but not as much as was desired. Increased migration of the cancerous cells would provide a better gauge by which to test AJA's ability to inhibit such behavior. Also, purchasing migration kits directly from a company proved to be an expensive endeavor. Refining the haptotaxis migration assay served to lower costs by using generic inserts and well plates (Cell Stain Solution and Extraction Solution products were still used) and increase migration by using a different, more aggressive cell line. The TC71 Ewing's cell line was provided by Dr. Jeffrey A. Toretsky M.D. from Georgetown University. The TC71 line came from a recurrent tumor, one that had been treated but had come out of remission. It was anticipated that this tumor would be more expansionistic and would be more conducive to migration. No chemotactic agent was used.

These cells were plated at 1 x 10^6 cells / mL in Plain McCoy's media in a 24 well plate in transwell inserts that had also been hydrated with Plain McCoy's. Four groups of TC71 cells were treated with AJA and one group was an untreated control. These inserts were removed and the stain from the cells was extracted from the bottom of the insert using an extraction solution. Cell ratio values were quantified by using a plate reader to measure the absorbance of the different extractions for each of the treated groups. The ability of AJA to inhibit TC71 cells' migration ability was compared. The TC71 migration assay showed the increased migratory

ability of TC71 cells over the SKES cells and additionally showed that AJA significantly inhibited the migration of these cells (Figure 7).

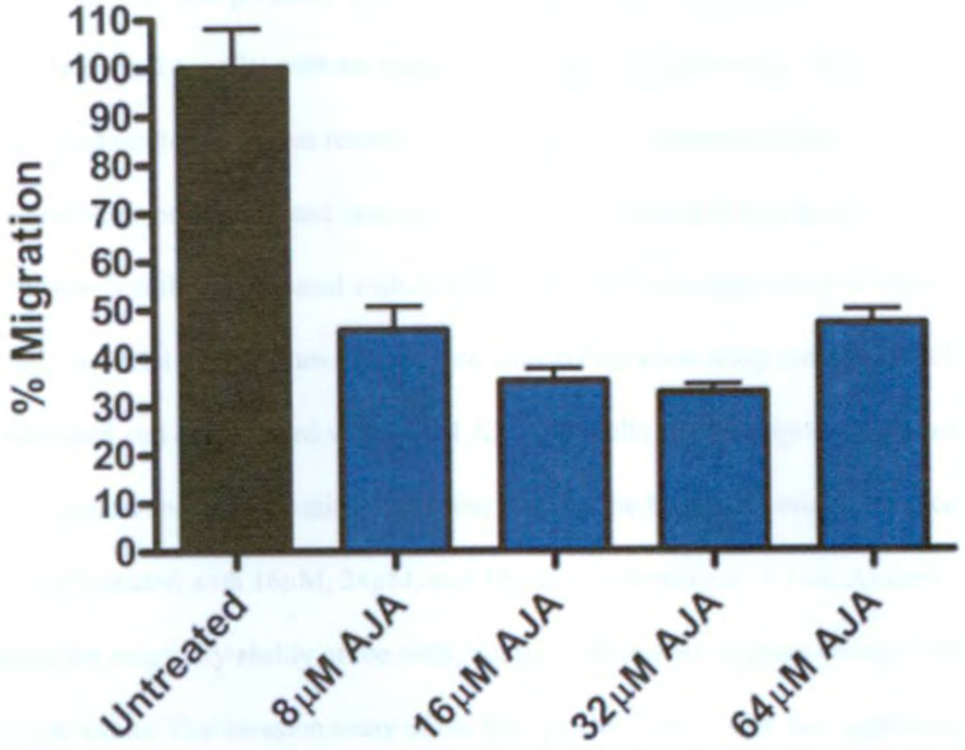


Figure 7. AJA inhibits the migration of TC71 cells.

Average values are shown. Values represent the average for triplicate cultures.

Discussion

The use of cannabinoids for medicinal purposes has been highly controversial because of the psychotropic effects generally seen in patients. However, ajulemic acid has exhibited potential medicinal benefits without causing the negative psychotropic effects seen with the use of other cannabinoids. Previous research has shown AJA to mediate tumor cell death without harming non-cancerous cells and to cause the preferred method of cell death, apoptosis.

Ewing's cells were treated with AJA to determine the drug's ability to inhibit the metastatic potential of the tumor. The scratch wound migration assay compared SKES cells that were untreated and cells treated with 32μM AJA. The cells did not migrate in the untreated or treated group, so another migration assay was used. In the haptotaxis migration assay, three sets of cells were treated with 16μM, 24μM, and 32μM concentrations of AJA. Ajulemic acid hampered the migratory ability of the cells, but the cells did not migrate enough to produce significant results. The invasion assay of the SKES cells showed that AJA significantly reduced the cells' ability to travel through a simulated basement membrane at a concentration of 24μM. A more aggressive cell line, the TC71 line, migrated further in another migration assay. Ajulemic acid reduced the migratory ability of these tumors in 8μM, 16μM, 32μM, and 64μM concentrations.

My assays showed that there is significant evidence to suggest that ajulemic acid reduces the metastatic potential of Ewing's sarcoma. The ability of the SKES cells to metastasize was curtailed by the addition of various concentrations of AJA as was the metastatic ability of the more aggressive TC71 cell line. These exciting results call for continued research.

Dr. Hensley has performed a mouse model study in conjunction with the University of Arkansas Medical Sciences in Little Rock, Arkansas. In the study, genetically engineered SKES cells were injected into the tibia of mice. These cells would luminesce if exposed to a specific substrate so the tumors could be visualized. Mice that developed tumors were separated into control and treated groups. The treated groups received injections of AJA into the bloodstream. The results of this study appear promising: a few of the treated mice experienced complete remission of the tumors. However, an untreated mouse also experienced complete remission of the tumor. These results indicate the need for further research and currently another model is planned.

Dr. Hensley is also working in conjunction with Dr. Perry and undergraduate students at Ouachita Baptist University to find the receptor to which AJA binds. The study uses software to model known potential receptors and AJA's structure. The program analyzes the docking ability of AJA at a given receptor and produces a quantitative value describing AJA's ability to precisely bind to that receptor. This research offers promising insight into how ajulemic acid mediates its medicinal abilities.

Ajulemic acid is an incredible drug that has previously been shown to effectively kill various type of Ewing's sarcoma cells. My research shows that AJA also can slow the metastasis of those tumor cells. Ajulemic acid has great potential for future use as an anticancer drug for sufferers of Ewing's sarcoma.

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