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Acute Promyelocytic Leukemia as a Possible Source for Ajulemic Acid Binding

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SENIOR THESIS APPROVAL

This Honors thesis entitled

**“Acute Promyelocytic Leukemia as a Possible Source for
Ajulemic Acid Binding”**

written by

Brian Christopher Monk

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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Acute Promyelocytic Leukemia as a Possible Source for Ajulemic Acid Binding

Brian Christopher Monk

Abstract

Acute promyelocytic leukemia (APL) is a cancer that affects bone marrow, the blood-forming tissue (of the body). This type of leukemia accounts for approximately 10% of acute myeloid leukemia (AML) cases. Normally this cancer is treated with a drug called all-trans retinoic acid (ATRA), but other variations of treatment do exist. The importance of this research is that APL cells express contains a receptor, retinoic acid receptor- α (RAR α), which ajulemic acid (AJA) may bind to in order to induce apoptosis in Ewing sarcoma (ES), a pediatric bone cancer. Therefore, this research will focus on the ability of AJA to induce the differentiation of APL cells, as ATRA does, thus demonstrating that AJA may be binding to RAR α .

Introduction

Acute Promyelocytic Leukemia

APL is a type of cancer that affects the bone marrow. Normally, bone marrow produces cells known as hematopoietic stem cells, which give rise to a variety of different cells within the body. Some of the cells produced include: red blood cells (erythrocytes), white blood cells (leukocytes), and cell fragments called platelets (thrombocytes) (Collins, 2002). However, in patients with APL, stem cells are not able to differentiate, or become specialized into mature functional white blood cells. Instead, immature white blood cells (WBC) accumulate within the bone marrow. This results in a shortage of a normal amount of erythrocytes, leukocytes, and

thrombocytes within the body. Figure 1 shows the pathway that a hematopoietic stem cell takes to reach one of its three outcomes: erythrocyte, leukocyte, or thrombocyte.

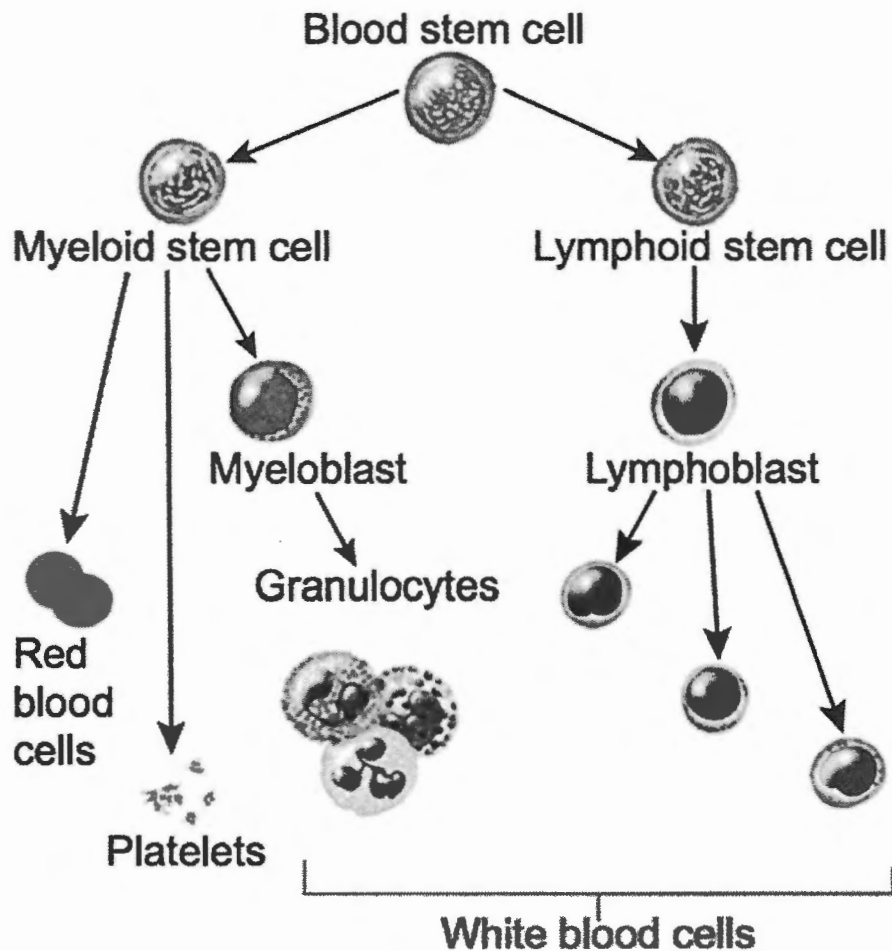


Figure 1: The image above shows the normal route of a hematopoietic stem cell takes to become a red blood cell (RBC), platelet, or WBC.

From: The National Cancer Institute "What You Need to Know About Leukemia."

In patients with APL, the problem arises in a cell called a promyelocyte. Promyelocytes are derived from myeloblasts and eventually give way to granulocytes. These then go on to produce neutrophils, eosinophils, basophils, and mast cells (Figure 2). In APL the promyelocytes are unable to

differentiate into these different types of WBCs and cause blockage within the marrow, leading to the overall effect of fewer RBC, WBC, and platelets.

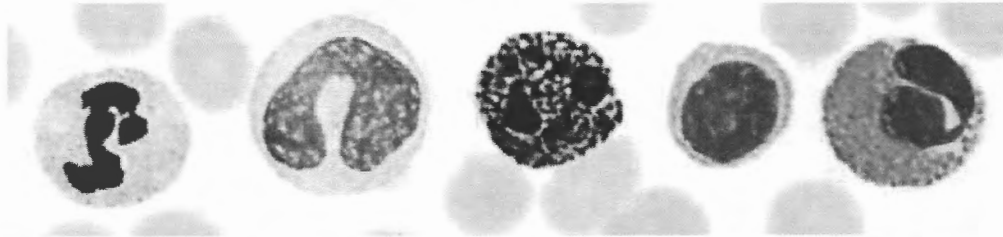


Figure 2: Promyelocytes that differentiate into different WBC. (Left to right: neutrophil, monocyte, basophil, lymphocyte, and eosinophil).

From: The University of California at San Francisco, Science & Health Education.

APL accounts for approximately 10% of all acute myeloid leukemia (AML), and occurs in 1 out of every 250,000 people in the United States (Yamamoto, et al., 2007). With that, it accounts for 21,700 deaths per year and has a higher incidence rate within the Hispanic community. The average APL patient is diagnosed around age 40, but can be diagnosed at any age.

APL is caused by a translocation of two chromosomes: 15 and 17 (Thé, 1991). Normally on chromosome 15, the PML gene, produces a protein that acts as a tumor suppressor (Salomoni, et al., 2002). This protein is able to prevent cells from growing and replicating too rapidly within the body. Chromosome 17, containing the RAR α gene, produces a protein that helps regulate gene transcription (the first step in the formation of protein production) (Thé, et al., 1991). This protein controls genes in WBC differentiation beyond the promyelocyte stage. With patients that have APL, 98% of the cases are due to the translocation, or fusion, of both chromosomes 15 and 17 (Zelent, et al., 2001). The fusion of these chromosomes produces a gene called PML-RAR α . This gene interferes with both the

normal processes of chromosomes 15 and 17. It does so by producing cells that are caught in the promyelocyte stage, leading to abnormal differentiation and accumulation. Ultimately this does not allow for the formation of mature and functional WBC. Interestingly enough, APL is not a genetic disease (Zelent, et al., 2001). It is acquired throughout one's lifetime and is only present in certain cells. This specific type of mutation is called a somatic mutation.

Diagnosis of this disease is done through observation of signs and symptoms, as well as diagnostic procedures. Normal symptoms that are observed in patients with APL are: fatigue, weakness, anemia (low RBC), bruising, bleeding, fever, and infection (Sanz, et al., 2010). In a proper diagnosis, a doctor would note all signs and symptoms of the patients, and order certain tests. These tests check the patient's complete blood count (CBC), electrolyte levels, prothrombin time (PT) (blood test to measure how long it requires blood to clot), and activated partial thromboplastin time (aPPT) (blood test that characterizes blood coagulation). In addition, a bone marrow biopsy would be done to test for the presence of abnormal WBC.

There are many different treatment options for patients with APL; the normal treatment being all-trans-retinoic acid (ATRA) in combination with a chemotherapeutic anthracycline drug (daunorubicin or idarubicin) (Attar, et al., 2013). ATRA helps promyelocytes differentiate into functioning neutrophils, reduces the total number of leukemic cells within the bone marrow, and aids in reducing the side effects of chemotherapy. For patients who cannot handle the use of anthracycline drugs or who relapse, ATRA with arsenic trioxide (AsO_3) is prescribed (Gore, et al., 2010). Overall, 80% of patients achieve short-term

remission when treated with ATRA alone and 70-80% achieve remission when treated with the combination of both ATRA and an anthracycline (The Leukemia and Lymphoma Society.). Those who achieve remission must undergo long-term follow-ups to ensure they are cured of the disease and determine if additional therapy is required. Therapy in order to maintain remission consists of treatment with 6-mercaptopurine (6-MP), methotrexate, and ATRA for at least two years (Gore, 2010).

Ewing's Sarcoma

Ewing's sarcoma (ES) is a cancer that occurs in either bones or soft tissues. There are several different types of ES, each affecting a different part of the body (Bailey, et al., 1994). Besides ES of the bone, there is extrasosseous ES, which are tumors that occur in the soft tissues that surround the bone (i.e. cartilage). Peripheral primitive neuroectodermal tumors (pPNET) occur within nerve tissues. Askin tumors, a subtype of pPNET, are located within the chest.

Out of all these different types of ES, 87% occur in the bones. Figure 3 shows the areas of the body that are affected (Sankar, et al., 2011). Common cases of ES occur in the femur (thigh), pelvis, or humerus (arm). Abnormal cases would consist of ES affecting the ribs, spine, etc. It is very common that ES will metastasize to another part of the body. Lungs, other bones, or marrow, are typical places to see the cancer spread.

ES affects approximately 3 out of 1 million children each year (Sankar, et al., 2011). In the United States, 250,000 are diagnosed with some form of ES each year, making it the second most common form of bone cancer. ES tends to affect children

and young adults who are between the ages of 10 and 20. Affecting more boys than girls, and rarely occurring in either African-Americans or Asian Americans, ES accounts for 1.5% of all pediatric cancers that are diagnosed.

Like APL, Ewing's sarcoma is not inherited genetically, making it a somatic mutation. ES arises from the translocation of two genes EWSR1 on chromosome 22 and FLI1 on chromosome 11 (May, 1993). When parts of these two genes fuse together, they create the EWSR1/FLI1 gene. This gene is what leads to the development of ES. Normally, the FLI1 protein helps regulate transcription as well as binding to DNA (May, 1993). The protein then helps control the overall development of certain cells by controlling the rate of transcription of certain genes. Regulating transcription is also the major function of the EWSR1 protein, under normal conditions (Mao, et al., 1994). However, when the two genes fuse together, the new protein turns transcription on or off on a variety of different genes, ultimately leading to abnormal differentiation and growth of cells. In patients diagnosed with ES, 85% of them have the ESWR1/FLI1 mutation.

Diagnosis of this cancer is done through both observations of signs and symptoms, as well as diagnostic procedures. Normal signs and symptoms that are apparent in patients with ES are: edema (swelling) or soreness around the area of the tumor, a low-grade fever, bone pain that specifically intensifies during exercise or while sleeping, and limping caused by the tumor being present on the bone (St. Jude). Using diagnostic procedures, a physician would use not only a physical exam, but also an MRI, CT, bone scan, bone marrow biopsy, or the use of PCR for the EWSR1/FLI1 gene, to help make a definitive diagnosis of ES. Typical treatment for

ES includes chemotherapy, surgery (possibly amputation to guarantee that all the cancer is removed) and radiation (St. Jude). The overall survival rate for children with ES is 70%, however that number drastically decreases to 30% for 5-year survival rate.

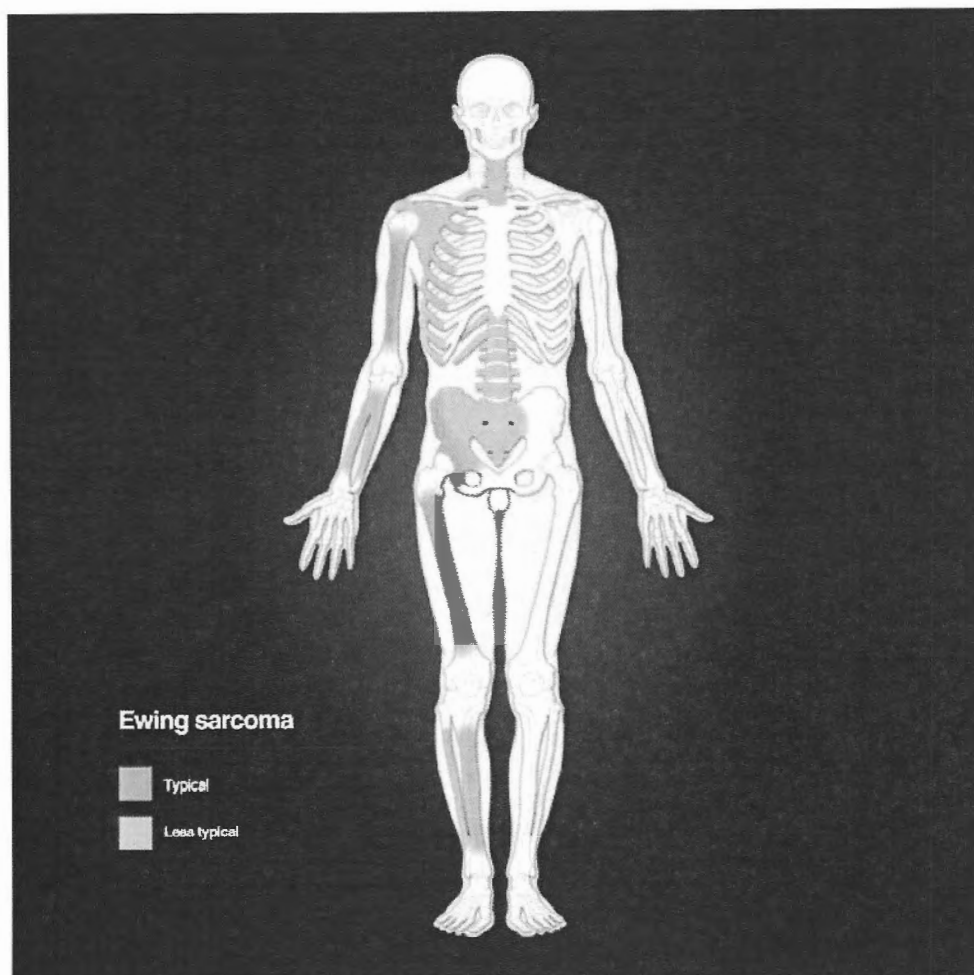


Figure 3: Image depicting occurrences of Ewing's Sarcoma (ES) within the body.

From: Dr. Frank Gaillard, et al.

Cannabinoids

Cannabinoids have been used for recreational purposes for sometime now. However, these recreational drugs are being studied for their potency on cancer.

Research defines a cannabinoid by its ability to bind to either CB₁ or CB₂ (Figure 4).

The CB₁ receptor is found throughout the body in places such as the brain, spleen, eye, testis, and uterus (Cridge, et al., 2013). CB₂ on the other hand, is found on cells that deal with the immune system as well as tumor cells. These receptors belong to a specific type of protein receptors called G-protein coupled receptors, whose purpose is to transduce signals from outside the cell. The CB₁ receptor mediates psychotropic effects of cannabinoids (Cridge, et al., 2013). On the opposite side, CB₂ functions in moderating the immune system's effect (Cridge, et al., 2013).

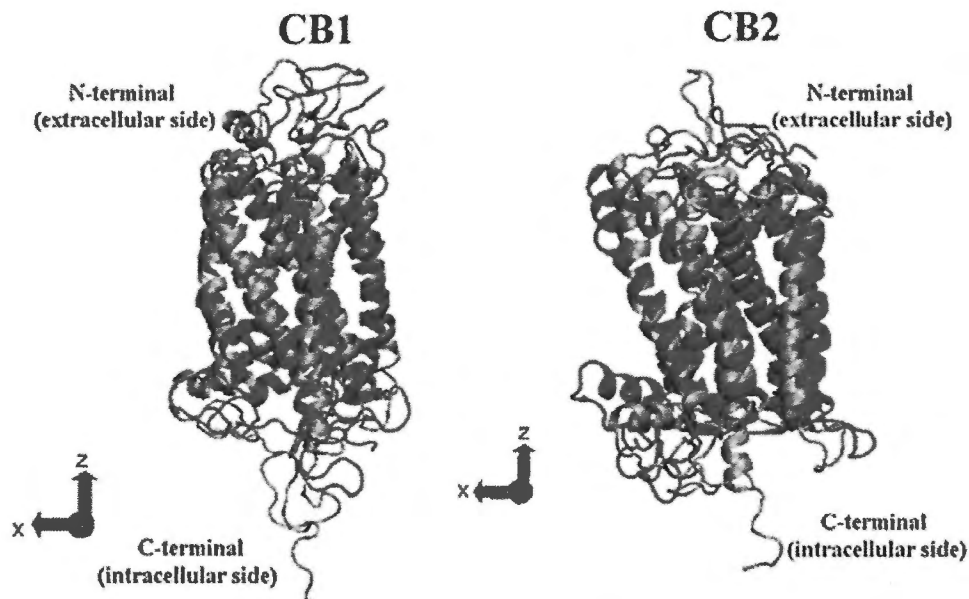


Figure 4: Image shows the two cannabinoid receptors (CB₁ and CB₂) and their physical shapes.

From: pubs.rsc.org

Research showing that cannabinoids do in fact inhibit tumor growth is extensive. A specific cannabinoid- Δ^8 -THC (tetrahydrocannabinol)-was used on mice with lung cancer. Researchers found that after twenty days of treatment, the drug inhibited growth of the tumor. Ceramides were found to be very prominent when either CB₁ or CB₂ were activated (Cridge, et al., 2013). Ceramides are very important in physiological signaling, controlling events such as apoptosis (cell

death), growth arrest (certain stage within the cell cycle), differentiation of cells, cell migration and adhesion. When either CB₁ or CB₂ were activated, researchers saw an increase in ceramide levels, which led to apoptosis or cell cycle arrest within tumor cells. Cannabinoids were found to prevent angiogenesis (the formation of new blood vessels). This is a critical necessity for tumors in order to develop and when undergoing treatment with cannabinoids, researchers found decreased angiogenic activity (Cridge, et al., 2013).

Although there are many derivatives of cannabinoids that are undergoing further studies to test for efficacy on cancer, our research focused on ajulemic acid (AJA). AJA (C₂₅H₃₆O₄), shown in Figure 5, is a crystalline solid at room temperature and has shown to inhibit psychotropic effects when administered (Burstein, 2004). This makes it a very good treatment for Ewing's sarcoma because children won't experience the high that is associated with normal cannabis plants. The drug has shown to bind effectively to both the cannabinoid receptors (Burstein, et al., 2013).

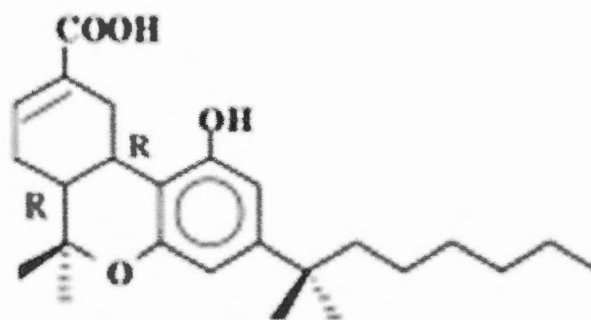


Figure 5: Ajulemic Acid (AJA) (C₂₅H₃₆O₄, MW= 400.55 g/mol) shows excellent capabilities in the treatment of cancers, especially Ewing's sarcoma. **From:** Sumner Burstein "Ajulemic Acid (IP-751): Synthesis, Proof of Principle, Toxicity Studies, and Clinical Trials", 2004

Relevance of Study

The purpose of this study is to test a possible receptor to which AJA may be binding. Previous research has indicated that AJA binds to a certain receptor called peroxisome proliferator-activated receptor gamma (PPAR- γ) (Liu, 2003). When ES cells were tested in the lab for the expression of this receptor via Western blot, PPAR- γ was not expressed. Therefore, it was concluded that AJA was binding to another receptor. Studies done from previous research found possible candidates for AJA's binding site. The RAR α and vitamin D receptors were among some of the strongest candidates. This particular research was to focus on the RAR α receptor. By using APL cells, which not only express the RAR α receptor, but ligand-binding to this receptor can be easily monitored by assessing the differentiation of APL cells. This experimental model was perfect to see if AJA binds to RAR α .

Several other drugs were tested alongside AJA. ATRA was used as a positive control in order to compare the efficacy of the two drugs. Other drugs, 9 cis-retinoic acid (cis-RA), cannabidiol (CBD), and hemp oil, were used to test the efficacy on APL. Hemp oil and CBD were used to test other derivatives from the cannabis plant.

Materials

Ajulemic Acid (AJA)

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

All-Trans Retinoic Acid (ATRA)

ATRA, was prepared in ethanol to a final stock concentration of 0.01 mM and 1 mM.

9 Cis-Retinoic Acid (cis-RA)

Cis-RA, was prepared in DMSO to a final stock concentration of 0.1 mM.

Hemp Oil

Hemp oil, was prepared in DMSO to a final stock concentration of 0.1 mM and 1 μ M.

Cannabidiol (CBD)

CBD, was prepared in ethanol to a final stock concentration of 0.1 mM and 1 mM.

Cell Culture

HL-60 cells (leukemic cells from an APL patient) were grown in suspension in IMDM media (20% fetal bovine serum (FBS) with penicillin and streptomycin) and cultured at 37°C with 5% CO₂.

Methods

Maintenance of Cell Line

Cells were maintained to produce enough viable cells in order to test the efficacy of different treatment options. One of the main indicators of cell line maintenance was a foggy, milky color being present. If present, this allowed us to properly split the HL-60 cells (place old cells into a new flask with fresh media and providing new media for the original flask as well). This process was repeated throughout the experiment. Another indicator we used was a microscope. When viewing the cells, if there were a lot of dead cells in the flask, this was an indicator that they too needed to be split.

NBT Assay

The efficacy of treatment of AJA on APL cells was done via NBT (nitro blue tetrazolium) assay composed of two tetrazole moieties (5-membered ring with 4 nitrogen's and 1 carbon). The NBT assay procedure was prepared by first spinning down cells in the centrifuge and vacuuming off media. Cells were suspended in 1 mL of NBT/PMA (composition of solution) that was warmed before submersion of cells. Cells were then placed into the incubator for 30 minutes at 37°C. After the 30 minutes, 83 μ L of HCl was added to the cells. The addition of HCl stops the reaction of the NBT/PMA with APL cells. Cells were then spun down and liquid was vacuumed off. Cells were then suspended in 0.5 mL of DMSO; which caused a purple color to form. The cells plus DMSO were moved to a well plate in order to be read by the TECAN Infinite Series at 560 nm. The machine measured absorbency by measuring the light that was absorbed. The effectiveness of treatment is indicated

by the shade of purple produced from the assay. Thus the darker the shade of color, the more effective and vice versa. Results were drafted and saved.

The NBT assay tests for oxidative free radicals produced by WBCs. In patients who do not suffer from APL, pathogens that are recognized are targeted with oxidative radicals produced from the WBCs. In patients with APL, this process does not occur. Thus, the darker shade of purple produced from the assay tells us that AJA caused the cells to differentiate into normal WBCs. The lighter the color, the less effective the drug was on APL cells.

The NBT assay was performed with all drugs used to test for the differentiation of APL cells.

Results

APL Treatment with ATRA

To establish a positive control, we first tested ATRA on APL cells. ATRA is the normal treatment for APL; therefore we would have a positive baseline for AJA when it underwent testing. HL-60 cells were grown and cultured, in order to get a sufficient amount to treat. Cells were either untreated (UT) or treated with ATRA (30 nM). The amount of cells needed for treatment was calculated and found to be 1×10^4 cells/mL. The average amount of cells in each flask (2 UT and 2 ATRA) were calculated via hemocytometer. The following results were obtained:

Untreated APL Cells	Average Number of Cell for UT (cells/mL)	ATRA APL Cells	Average Number of Cells for ATRA (cells/mL)
A	272×10^4	A	159×10^4
B	198.5×10^4	B	207.5×10^4

Following total cell count of the both UT and ATRA cells, NBT assay was performed on both groups. Figure 6 shows the results obtained from the experiment.

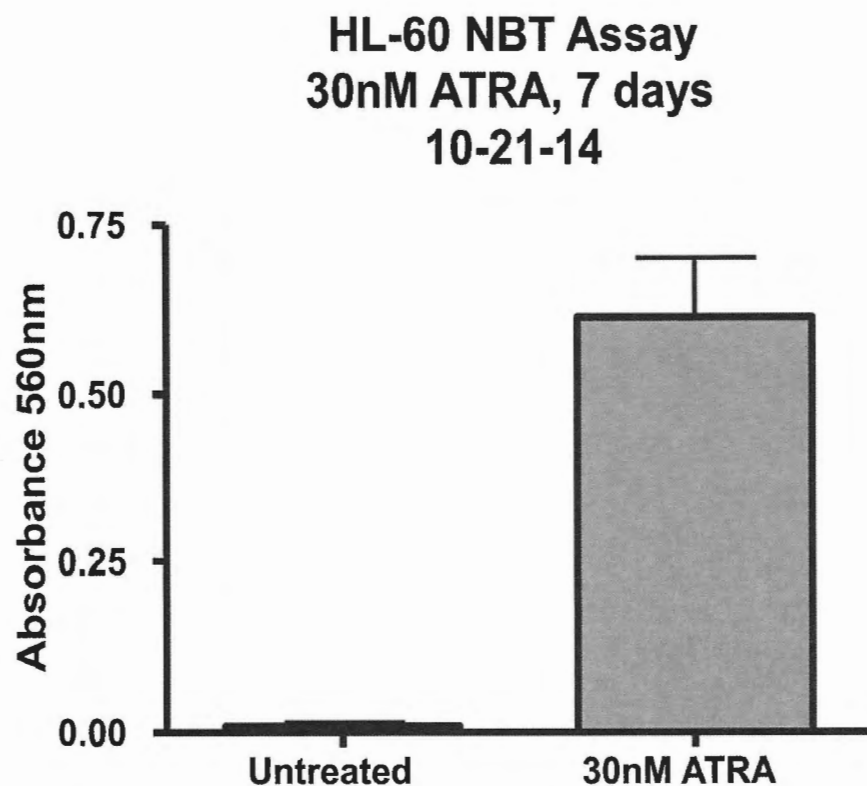


Figure 6: Results showing the absorbance of 560nm for UT APL cells and ATRA treated APL cells.

APL Treatment with ATRA & AJA

Having established a positive control for the experiment, comparing ATRA vs. AJA was next. Cell lines from the previous experiment were continuously grown and cultured. Cells were divided into three categories of treatment: UT, ATRA (30 nM), or AJA (30 nM). The amount of cells needed for treatment was calculated and

found to be 1×10^7 cells/mL. The average number of cells was calculated via hemocytometer. The following results were obtained.

Untreated APL Cells	Average Number of Cells for UT (cells/mL)	ATRA APL Cells	Average Number of Cells for ATRA (cells/mL)	AJA APL Cells	Average Number of Cells for AJA (cells/mL)
A	313.5×10^4	A	303×10^4	A	221.5×10^4
B	303×10^4	B	201×10^4	B	207×10^4

Following total cell count for UT, ATRA, and AJA, NBT assay was performed on all three trials. Figure 7 shows the results obtained from the exper

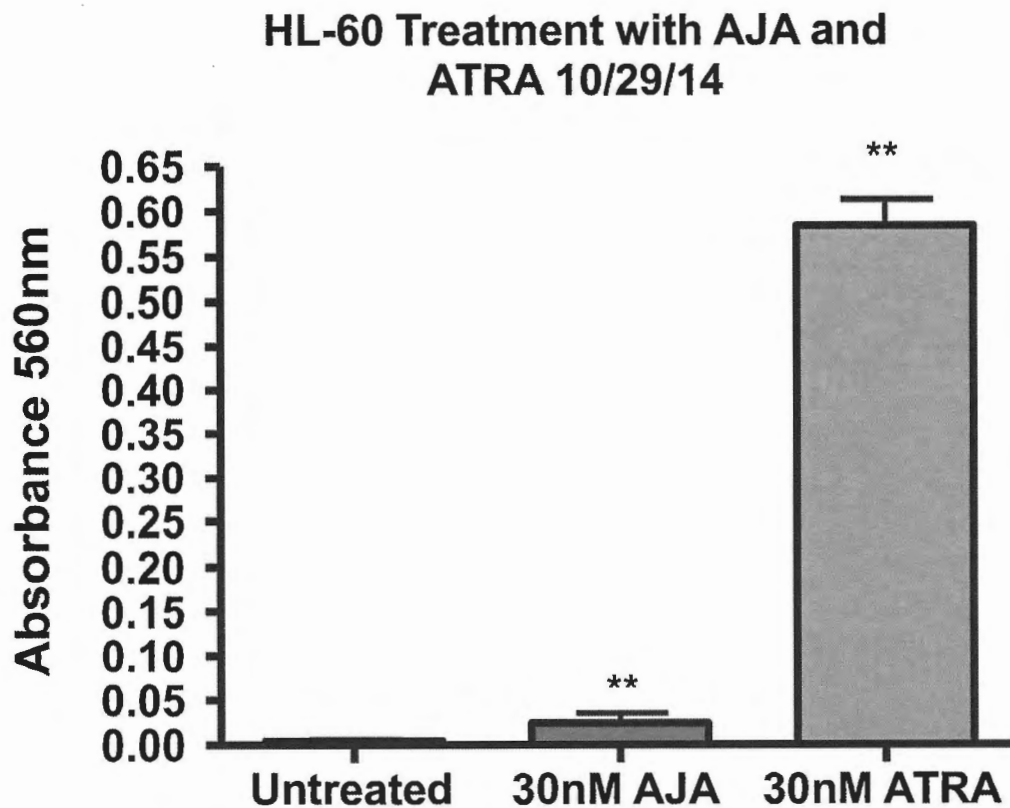


Figure 7: Results showing differentiation between APL UT, AJA, and ATRA (**p-value<.05).

APL Treatment with ATRA and Varying Concentrations of AJA

Evaluating the results shown in Figure 7, we decided to vary the concentration of AJA, to see if a higher molar concentration induced differentiation. Using the stock concentration of AJA we calculated the different molar concentrations ($M_1V_1=M_2V_2$). Cell lines were continuously grown and cultured. Upon treatment, cells were divided into five categories: UT, ATRA (30 nM), AJA (30 nM), AJA (300 nM), and AJA (3 μ M). The amount of cells needed for treatment was calculated and found to be 1×10^7 cells/mL. The average number of cells was calculated via hemocytometer. The following results were obtained.

Untreated APL Cells	Average Number of Cells for UT (cells/mL)	ATRA APL Cells	Average Number of Cells for ATRA (cells/mL)	AJA APL Cells (30 nM)	Average Number of Cells for APL (30 nM) (cells/mL)	AJA APL Cells (300 nM)	Average Number of Cells for APL (300 nM) (cells/mL)	AJA APL Cells (3 μ M)	Average Number of Cells for APL (3 μ M) (cells/mL)
A	237×10^4	A	233×10^4	A	212×10^4	A	244×10^4	A	237×10^4
B	196×10^4	B	214×10^4	B	207×10^4	B	241×10^4	B	213×10^4

Following total cell count for UT, ATRA, AJA (30 nM), AJA (300 nM), and AJA (3 μ M), NBT assay was performed on all five trials. Figure 8 shows the results obtained from the experiment.

HL-60 Treatment With Varying Concentrations of AJA 11/11/14

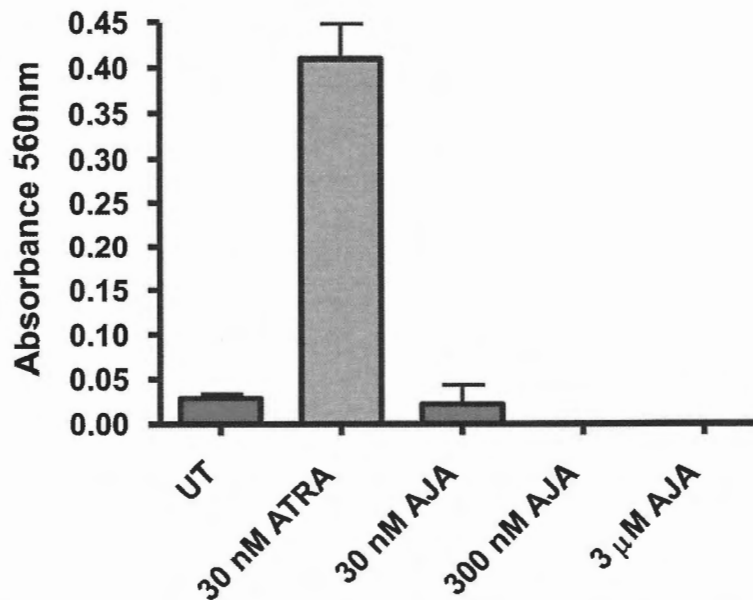


Figure 8: Results showing the differentiation between APL UT, ATRA, AJA (30 nM), AJA (300 nM), and AJA AJA (3 μM).

APL Treatment with ATRA, AJA, CBD, and Hemp Oil

The final experiment was to test different cannabinoids on the differentiation of APL cells. Cells were continuously grown and cultured before the experiment. Using the stock concentrations of the different drugs, we calculated the different molar concentrations ($M_1V_1=M_2V_2$). Upon treatment, cells were divided into nine categories: UT, ATRA (30 nM), cis-RA (30 nM), AJA (30 nM), AJA (3 μM), CBD (30 nM), CBD (3 μM), Hemp oil (30 nM), and Hemp oil (3 μM). The total number of cells needed for treatment was calculated and found to be 1×10^7 cells/mL. The average number of cells was calculated via hemocytometer. The following results were obtained.

Untreated APL Cells	Average Number of Cells for UT (cells/mL)	ATRA APL Cells	Average Number of Cells for ATRA (cells/mL)	Cis-RA APL Cells (30 nM)	Average Number of Cells for cis-RA (30 nM) (cells/mL)	AJA APL Cells (30 nM)	Average Number of Cells for AJA (30 nM) (cells/mL)
A	4x10 ⁷	A	4.5x10 ⁷	A	4.3x10 ⁷	A	3.2x10 ⁷
B	4.7x10 ⁷	B	4.5x10 ⁷	B	4.3x10 ⁷	B	3.6x10 ⁷

AJA APL Cells (3 μM)	Average Number of Cells for AJA (3 μM) (cells/mL)	CBD APL Cells (30 nM)	Average Number of Cells for CBD (30 nM) (cells/mL)	CBD APL Cells (3 μM)	Average Number of APL Cells for CBD (3 μM) (cells/mL)	Hemp Oil APL Cells (30 nM)	Average Number of Cells for Hemp Oil (30 nM) (cells/mL)	Hemp Oil APL Cells (3 μM)	Average Number of Cells for Hemp Oil (3 μM) (cells/mL)
A	3.2x10 ⁷	A	3.2x10 ⁷	A	4x10 ⁷	A	4.3x10 ⁷	A	3.7x10 ⁷
B	3.3x10 ⁷	B	3.2x10 ⁷	B	3.3x10 ⁷	B	3.5x10 ⁷	B	4.3x10 ⁷

Following total cell count for UT, ATRA, cis-RA, AJA (30 nM), AJA (3 μM), CBD (30 nM), CBD (3 μM), Hemp oil (30 nM), and Hemp oil (3 μM), NBT assays were performed on all nine trials. Figure 9 below shows the results obtained from the experiment

HL-60 Treatment 11/24/14

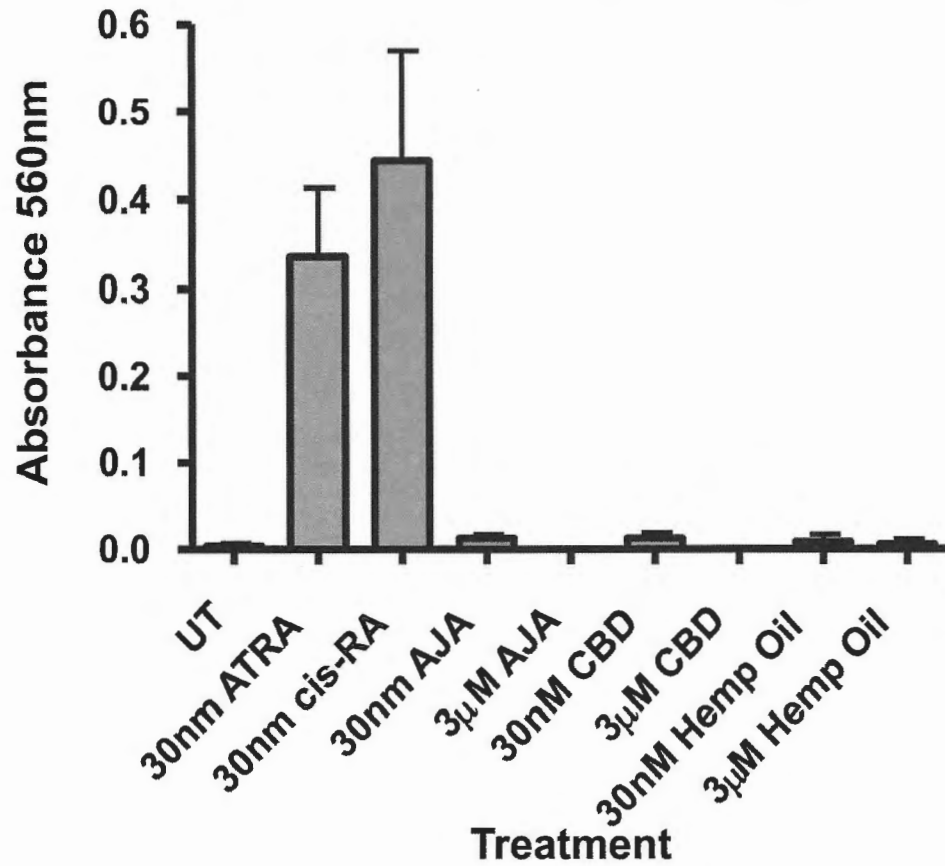


Figure 9: Results showing the differentiation between APL UT, ATRA, cis-RA, AJA (30 nM), AJA (3 µM), CBD (30 nM), CBD (3 µM), Hemp oil (30 nM), and Hemp oil (3 µM).

Discussion

The use of cannabinoids as a possible source for medical treatments has received a lot of attention. Large amounts of research have been focusing on how these drugs help treat diseases such as cancer. One of these cannabinoids, AJA, has been studied extensively because it elicits no psychotropic effects when compared tetrahydrocannabinol (a compound from the marijuana plant that causes psychotropic effects). This research focused on using APL cells to help determine the possible receptor that AJA may be binding to when treating Ewing's sarcoma cells.

APL cells (HL-60) underwent various forms of treatment throughout the experiment. Having the positive results from ATRA helped show the possible outcome if AJA did indeed bind to RAR- α . However, once the APL cells underwent treatment with AJA, there was little differentiation observed. Figure 10 shows the difference between normal WBC and abnormal WBC found in APL patients. The following figure is to show the desired affect of the drug, providing it did in fact bind to RAR- α .

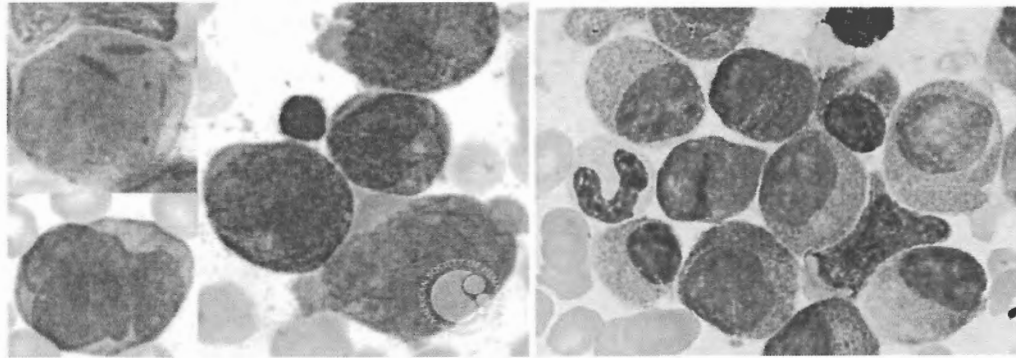


Figure 10: WBC observed in patients with APL (left) and WBC observed in healthy patients. The distinguishing difference between the two images in APL the nuclei of the cells are hard to distinguish (left) compared to those without APL (right). Therefore, if AJA did in fact bind to RAR- α , APL cells (left) would differentiate to normal WBC (right).

From: *American Society of Hematology Image Bank*, Luhan Swart, Ann van Eyssen, Margie Shuttleworth (left) and *Atlas Genetics Oncology* (right)

However, when APL cells underwent treatment with AJA, little or no differentiation was observed. Figure 11 shows the images taken from the lab with the various treatments (UT, ATRA (30 nM), and AJA (30 nM)). Even increasing the concentrations of AJA did not cause the APL cells to differentiate. Other sources of cannabinoids, such as CBD or Hemp oil, had the same results as AJA, little or no differentiation. Therefore, with these results, we believe that AJA does in fact bind to RAR α in APL cells, however at a very low affinity. This suggests that AJA may be binding to more than one receptor. Further research is needed to test the other possible receptors that AJA may be binding to.

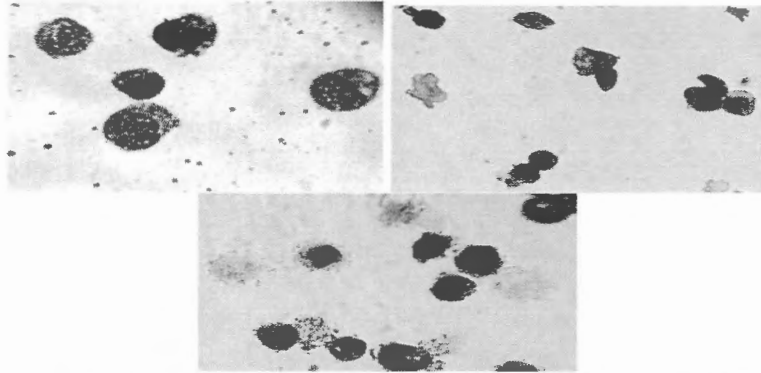


Figure 11: The top left image is APL cells UT, top right image is APL cells treated with 30 nM of ATRA, and the bottom left image is APL cells treated with 30 nM of AJA. There is clear evidence that AJA does induce some differentiation of APL cells, however a majority are still promyelocytes.

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