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SENIOR THESIS APPROVAL

This Honors thesis entitled

"Using Spheroids and a Bioluminescent Mouse Model to Determine the Effects of Ajulemic Acid on Ewing's Sarcoma"

written by

Drake Hardy

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.

Lori Hensley, thesis director

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Dr. Barbara Pemberton, Honors Program director

December 6, 2013

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Using Spheroids and a Bioluminescent Mouse Model to Determine the Effects of Ajulemic Acid on Ewing's Sarcoma

By

Drake Hardy

Abstract

Ewing's Sarcoma is a pediatric bone cancer with a five-year survival rate of only 30%. New treatment options for this highly aggressive disease are desperately needed. Ajulemic acid (AJA), a synthetic cannabinoid, has been the focus of our research, and has demonstrated the ability to decrease tumor cell viability and inhibit endothelial cell migration and angiogenesis. Based on these results, AJA is a potential therapeutic agent for Ewing's Sarcoma and other solid pediatric cancers. In order to create a realistic environment *in vitro* in which to study these tumors, we created 3-dimensional spheroids with three cell types, each of which fluoresces a different color. These include: fibroblasts, which give the spheroids a connective tissue component, endothelial cells, which line and form blood vessels in the body, and cancer cells. These spheroids were then treated with different concentrations of AJA and compared to control groups in an effort to understand cellular proteins and pathways being modulated by the drug. Also, in order to test AJA in a more realistic model of human cancer, we developed a novel bioluminescent mouse model of Ewing's Sarcoma. We compared luminescent to physical measurements and assessed the ability of AJA to decrease tumor growth *in vivo.* Our findings show support for AJA as a potential new cancer treatment option.

Introduction

Ewing's Sarcoma is a highly aggressive malignant tumor of the bone and soft tissues found in children (5). These tumors require the development of a vascular network in order to grow. Previous studies have shown that vascular endothelial growth factor (VEGF) supports angiogenesis and is up-regulated in patients with Ewing's Sarcoma. Studies have also shown that inhibiting VEGF activity prevents tumors from growing (7). Likewise, matrix metalloproteinase-9 (MMP-9) has been shown to regulate angiogenesis as well as tumor growth and cell migration. When it is suppressed, previous studies have shown that metastasis of the tumor is inhibited (5).

New treatment options are needed in order to increase the survival rates of patients with this disease. Studies have shown that cannabinoids are capable of inhibiting cancer cell growth in many types of cancer (3). Thus, they may be an appropriate treatment option for Ewing's Sarcoma. Ajulemic acid (AJA), specifically, is a synthetic non-psychoactive cannabinoid which shows promise as a treatment option because of its lack of adverse side effects. It also has a low potential for abuse, which makes it a more likely candidate for cancer treatment (1). Although its structure is similar to that of tetrahydrocannabinol (THC), the active compound found in the *Cannibis* or marijuana plant, AJA does not produce a "high" like THC at doses appropriate for therapy (1). Previous studies have shown that AJA has anti-inflammatory effects and is capable of inhibiting tumor growth in several types of cancer cells. Also, AJA had a lesser effect on normal, healthy cells in these studies and once treatment was stopped, normal cell growth resumed (1). While many cannabinoids have psychoactive properties, none have been detected in studies with AJA. This drug is also analgesic, more potent than other cannabinoids, and nontoxic, which adds to its potential as a possible cancer treatment option (4).

Previous research in our lab has shown that AJA decreases cancer cell viability and inhibits endothelial cell migration and angiogenesis, as well as tumor cell invasion. This indicates that AJA may be interfering with proteins that regulate these processes, such as VEGF and MMP-9 respectively. Without a vascular system to nourish a tumor and carry away its waste, cancer cells would struggle to survive.

In previous studies, *in vivo* 3-dimensional models called spheroids have been used to study tumors. Until recently, spheroids were made up of two cell types, cancer cells and endothelial cells, suspended in hanging drops of medium (6). Our current research has incorporated a third cell type, fibroblasts, into the spheroid in order to create an even more realistic *in vitro* model. Fibroblasts give the spheroid a connective tissue component, which is more representative of the environment a tumor is exposed to in a living organism. Each of the three different cell types used in our spheroids were transfected to fluoresce a different color. This will enable us to keep track of the different cell types when the spheroids are treated with AJA. By treating the spheroids with AJA, we have also studied its effects on the expression of some of the proteins necessary for tumor survival and progression, such as VEGF and MMP-9.

Animal models are necessary in order to more accurately study the effects of AJA on Ewing's Sarcoma. Thus, we have developed a novel intratibial bioluminescent mouse model of Ewing's Sarcoma to serve as a more realistic version of the disease found in humans. Tumors can be grown in the bone, as they grow in children, and the growth and regression of tumors can be measured through the intensity of light produced by the tumor cells. The cancer cells created for this model express high levels of luciferase (2), the enzyme that catalyzes the reaction allowing fireflies to light up. Therefore, when the cells are exposed to luciferin, the substrate for the reaction, they luminesce. In these experiments, we implanted the tumor cells subcutaneously

and made both physical and luminescent measurements. We hypothesized that physical measurements would correlate with luminescent measurements, demonstrating that our bioluminescent intratibial model is a solid model in which to study Ewing's sarcoma. We also studied the ability of AJA to decrease tumor growth in the mice when used at varying concentrations.

Materials and Methods

MTT Assay on TC71-PM4-GR cells

TC71-PM4-GR cells (a generous gift from E.S.Kleinerman, University of Texas, M.D. Anderson Cancer Center), which are Ewing's sarcoma cells taken from secondary lung tumors, were plated in a 24-well plate and treated with 16μM, 32μM, and 64μM AJA. We used these concentrations because the LD50 of SK-ES cells, a primary Ewing's line in which all initial experiments were completed, when treated with AJA is 32μM. Some of the cells were also left untreated. 72 hours after treatment, growth media was aspirated. MTT was added as a 1:50 mixture in minimum essential medium with L-glutamine (Gibco Life Technologies). The plate was then incubated for 1 to 2 hours and the media was aspirated. Cells were solubilized in 0.5mL DMSO and plates were rocked for 15 minutes at room temperature. Absorbance was measured at 570nM.

MTS Assay on TC71-PM4-GR cells

TC71-PM4-GR cells were plated into 96-well plates and treated with 16μM, 32μM, and 64μM AJA. Some of the wells were also left untreated. 72 hours after treatment, an MTS assay was run according to the manufacturer's recommendations (Promega).

Spheroids

In order to provide a moist environment for the spheroids, 0.5mL of PBS were pipetted into each well of a 48-well plate. Cells were then counted. For spheroids containing 2 cell-lines, 1,500 cells were needed for each cell-line. For spheroids containing 3 cell-lines, 1,000 cells were needed for each cell line. All spheroids contain a total of 3000 cells in a final volume of 20µl. Spheroids containing 2 cell-lines were made up of TC71-PM4-GR cells and EAhy-RED cells. Spheroids containing 3 cell-lines were made up of TC71-PM4-GR cells, WI-38 cells, and EPC cells. The cell suspension was pipetted onto the middle of the circle on the lid of a 48-well plate, and the lid was carefully flipped and placed over the well plate containing PBS. The plate was then incubated at 37°C for 6 days. Spheroids were treated with 10µM, 20µM, and 32µM AJA. Control wells were untreated.

ELISA assay for VEGF and MMP-9

Media samples from spheroids were removed 18 hours post-treatment and ELISAs were run for VEGF and MMP-9 according to manufacturer's recommendations (R&D Systems).

Physical and bioluminescent measurement of tumors in mice

SK-ES cells stably transfected with a luciferase reporter were injected into *nu/nu* mice subcutaneously on the rear flank. Mice were then treated with a high dose (0.2mg/kg) or a low dose (0.01mg/kg) of AJA. Each experimental group included 8 mice. 7 mice were treated with vehicle (DMSO) only, serving as controls. Physical tumor growth was measured in all mice twice per week with a caliper. Cell luminescence was also measured twice per week in all animals using an IVIS imaging system. In order for the tumors to luminesce, luciferin (Xenolight RediJect, Caliper Life Sciences) at a concentration of 30mg/mL was injected into the mice at

5μL per gram of body weight 30 minutes before imaging. If the tumors became too large or the mice lost a significant amount of weight, they were euthanized according to UAMS IACUC standards.

Results

When treated with AJA, cell viability of TC71-PM4-GR cells was found to decrease in a dose-dependent manner. These results were obtained from MTT and MTS assays (FIG 1). Originally, MTT was the only assay being run. However, the results of this assay may not have been as accurate as possible since some of the cells (which grow both adherently and in suspension) could be vacuumed off of the plate. Thus, the MTS assay was also performed. Media does not have to be aspirated when this assay is performed. Both assays showed the same general trend. As the concentration of AJA increased, more and more cells were killed. The LD50, based on MTS data, was calculated to be 16.9μM.

In the past, spheroids were created using 2 cell-lines only. One of these cell lines was an endothelial cell line and the other was a cancer line. Over the summer, this lab created spheroids using fibroblasts, a third type of cell, giving the spheroids a connective tissue component. Since actual tumors incorporate, are surrounded by, and must invade through connective tissue in a living organism, this additional component should make our spheroids a more realistic model of human disease. By incorporating a different color of fluorescent protein into each of the cell types, it will be easier to observe what is actually happening to each of the cell types when they are treated with AJA (FIG 2A).

Certain proteins are required for tumor growth and invasion. Among these are vascular endothelial growth factor (VEGF) and matrix-metalloproteinase-9 (MMP-9). VEGF promotes

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the formation of blood vessels to the tumor. Tumors require blood vessels in order to receive the nutrients they need to grow and survive. MMP-9 promotes tumor invasion by allowing the tumor to break down surrounding connective tissue fibers (5). Previous studies in our lab (unpublished data) have shown that AJA inhibits angiogenesis in an aortic ring assay as well as tumor invasion *in vitro* using modified Boyden chamber assays. When spheroids were treated with AJA and compared to controls, it was found that AJA decreased both of these proteins (FIG 3). However, initial data suggested it did not do so in a dose-dependent manner.

The data of this study indicate that AJA inhibits tumor growth in a dose-dependent manner. The results for both physical tumor growth and tumor luminescence support this. However, the results of the data obtained are slightly skewed. While the data show an obvious inhibition of tumor growth in the low-dose group as compared to controls (FIG 5A&B), the data for the high-dose group reflect drug toxicity and have helped us to establish the upper limits of drug treatment concentrations. High-dose treatment was terminated at day 8 because weight loss in this group was deemed unacceptable (FIG 5D), and physically the mice were lethargic and did not look well. Though low-dose treatment caused a slight decrease in the weight of the mice, this decrease was not significant enough to consider the concentration toxic or discontinue treatment. When the mice being treated with a higher dose of AJA stopped receiving treatment, rapid tumor growth was observed.

Importantly, the results of this study indicate that there is a close correlation between the physical size of the tumor and tumor luminescence until the tumor becomes very large (FIG 5C). There are multiple reasons why the correlation may not be as good once the tumor becomes large. We favor the hypothesis that once the tumor becomes large enough, the tumor cells physically have no more space for mitosis and therefore become quiescent. Our data support this

hypothesis because once the tumor cells have metastasized, we again observe luminescence, this time in the secondary tumors. Alternatively, it is possible that once the tumor becomes too large, some number of cells in the tumor become necrotic because of low oxygen and nutrient conditions within the tumor. Dying cells would cease protein production, including luciferase production. It is also possible that the plasmid causing the cancer cells to produce luciferase is rejected over time, as there is no selective pressure in the animal for the cells to maintain it. If no luciferase is produced by the cancer cells, no luminescence will occur.

Conclusions

This study supports AJA as a possible candidate for the treatment of pediatric tumors in the future. It has shown that this compound decreases the viability of Ewing's sarcoma cells in a dose-dependent manner. It has also brought light to some of the mechanisms by which AJA may be inhibiting angiogenesis and tumor invasion. Furthermore, the data produced by this study have given us critical information regarding the toxicity limits of the drug. Most importantly for our future work demonstrating the efficacy of this compound *in vivo*, the subcutaneous mouse study has shown that there is a good correlation between the physical size of the tumor and tumor luminescence until tumors become very large, indicating that bioluminescence is a good model to use for studying the effects of AJA on tumors, providing the evidence we needed to continue use of our intratibial model. Equally important for future studies in the lab is the creation of the 3-cell line spheroids produced in this study. These spheroids allow us to identify and study cellular pathways impacted by the drug and allow us to investigate proteins largely affected by the tumor microenvironment. There has previously been no realistic *in vitro* model in which to investigate these. The fluorescent proteins transfected into our cells used in the spheroids allow us to see the effects of our drug on the individual cell-lines; an ideal drug would

decrease viability of the cancer cells and restrict endothelial cell proliferation, leaving the fibroblasts unharmed. The data provided in this study provide rationale for the use of AJA in the treatment of Ewing's Sarcoma and enhance biological research through the addition of critical model systems in which to study disease.

Acknowledgements

I would like to thank Dr. Lori Hensley for giving me the opportunity to participate in this research and for teaching me so much throughout the course of the study. I would also like to thank Amy Eubanks for teaching me proper lab techniques and for helping keep the project organized, as well as our team at UAMS (Joseph Levy, Dr. Rob Griffin, and Nathan Koonce) for treating the mice and helping us use the IVIS imaging system to take bioluminescent photographs of the mice.

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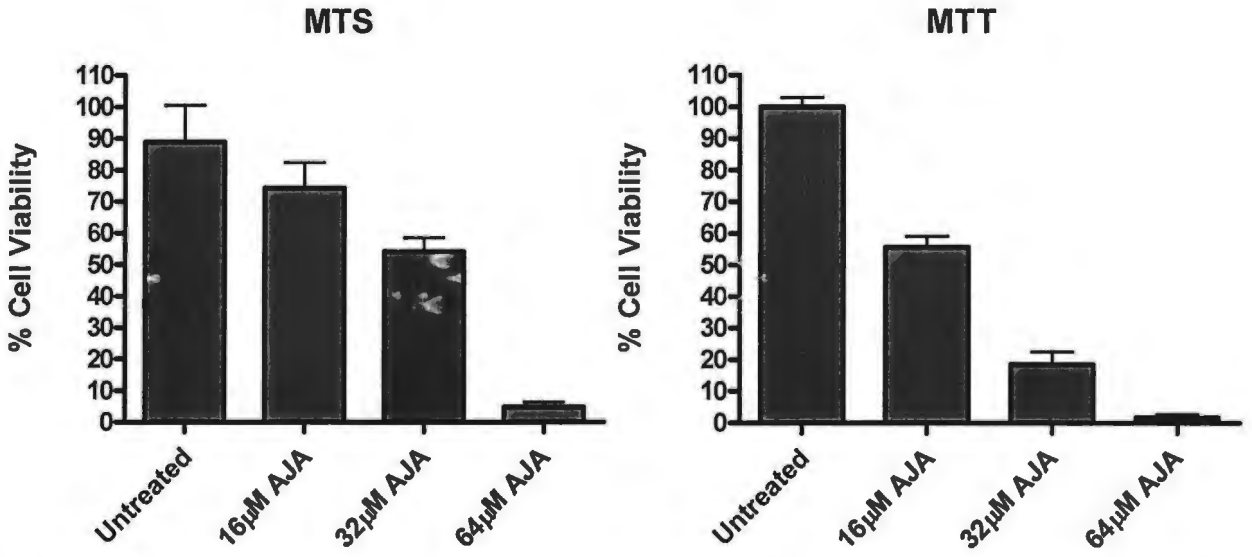

FIG 1. Cell viability is decreased in a dose-dependent manner in Ewing's sarcoma cells treated with AJA as compared to untreated controls. TC71-PM4-GR cells were plated and treated at the indicated concentrations of AJA. MTT and MTS cell viability assays were both run 72h post treatment. Standard errors are indicated.

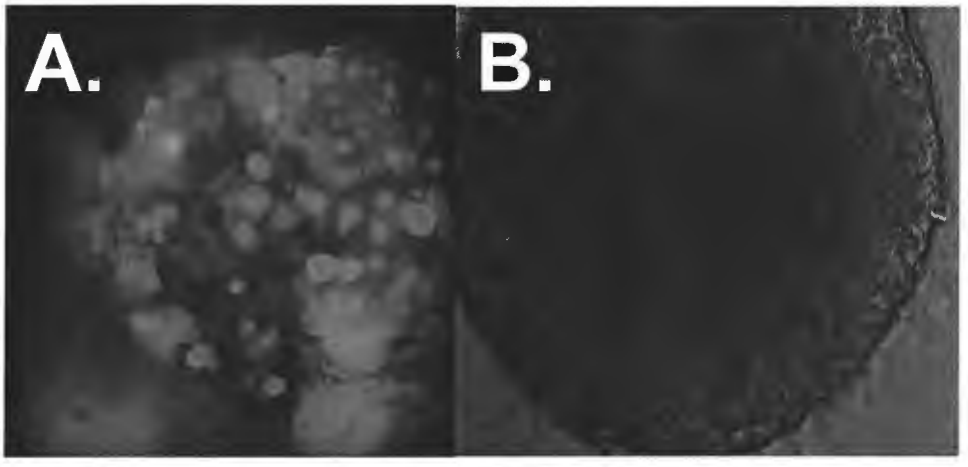

FIG 2. Spheroids were created using 2 cell-lines and 3 cell-lines. (A) EAhy-RED and TC71-PM4-GR cells were used to make fluorescent spheroids. (B) Endothelial Progenitor Cells (EPC), TC71-PM4-GR, and WI-38 cells were used to make more realistic spheroids.

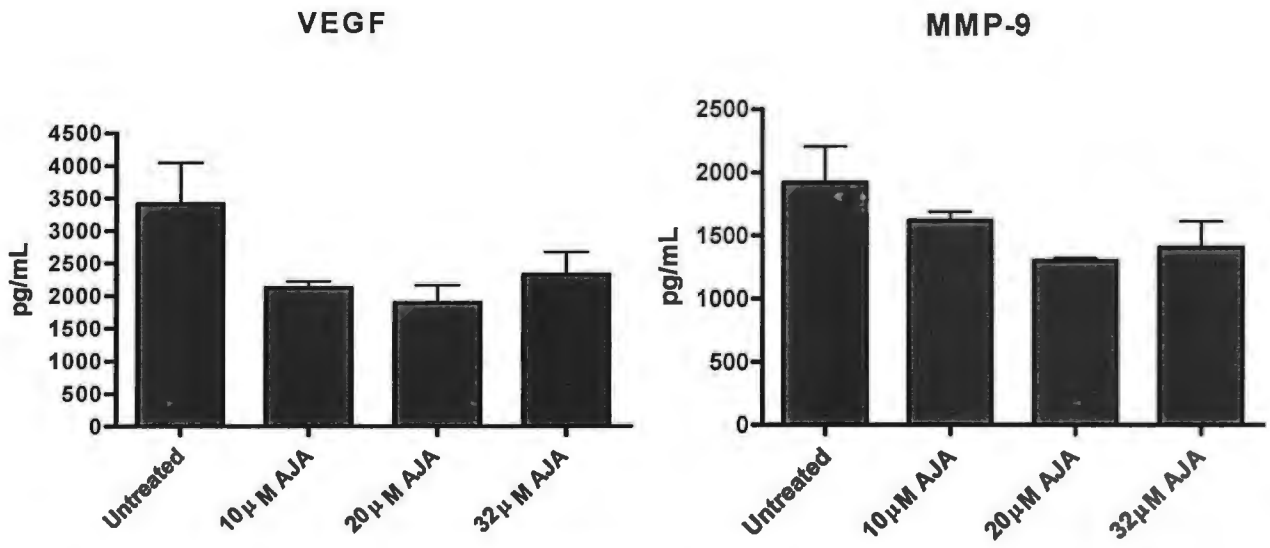

FIG 3. Proteins necessary for tumor growth are decreased in spheroids treated with varying concentrations of AJA as compared to untreated controls. Spheroids containing WI-38, EPC, and TC71-PM4-GR cells were treated with varying concentrations of AJA 6 days after being created. ELISA assays for VEGF and MMP-9 were performed 18h post treatment. Standard errors are indicated.

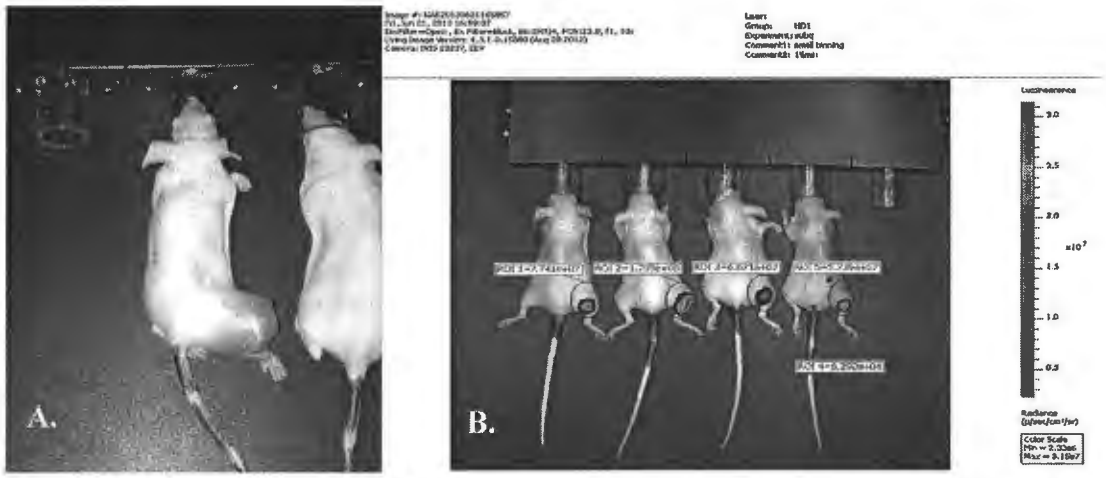

FIG 4. A novel bioluminescent model of Ewing's sarcoma was created to study the effects of AJA on tumor growth. (A) SK-ES cells stably transfected with a luciferase reporter were injected into *nu/nu* mice subcutaneously. Physical tumor growth was measured in both treated and untreated mice twice/week with a caliper. (B) Cell luminescence was also measured twice/week in both treated and untreated mice using an IVIS imaging system.

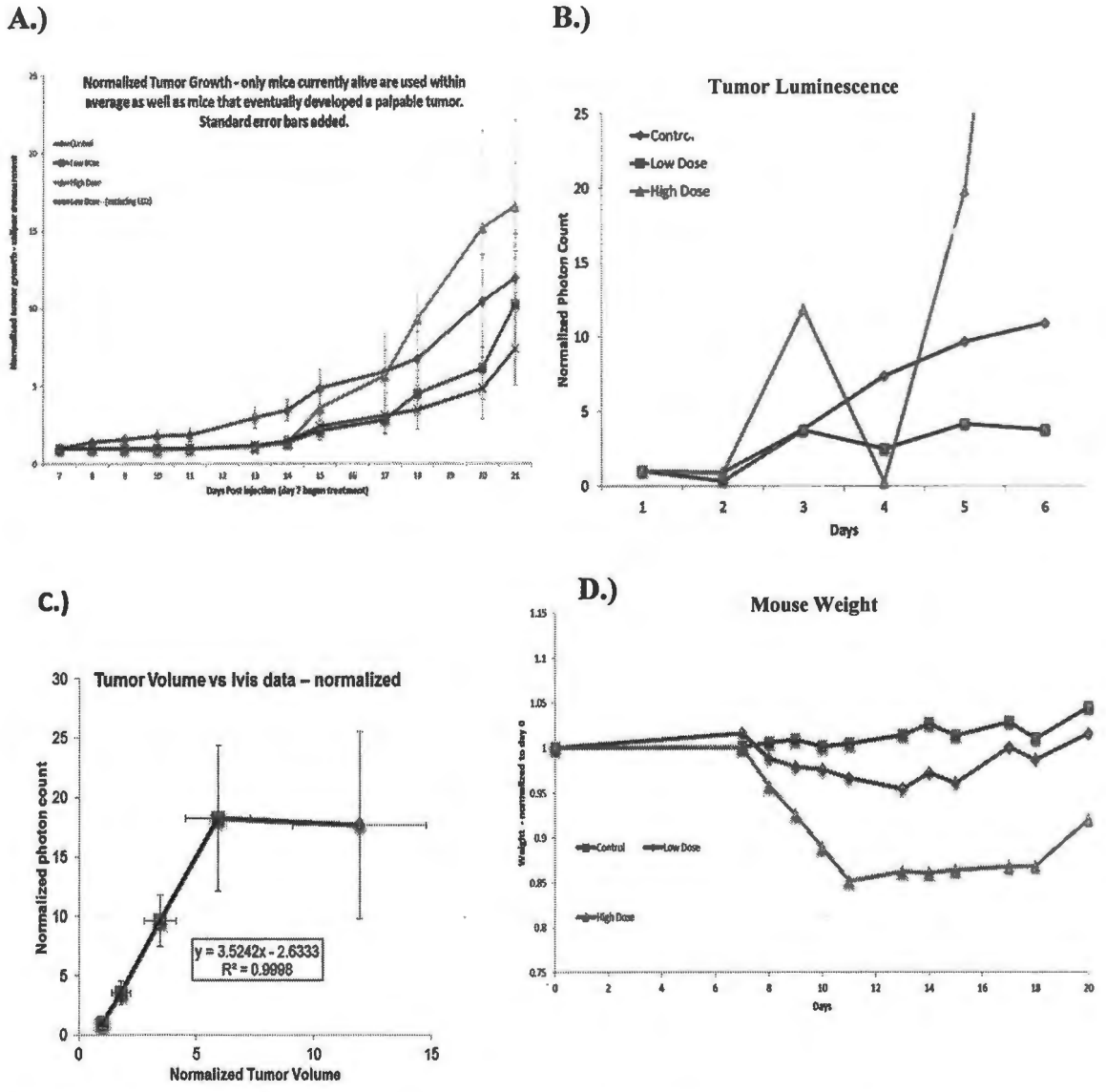

FIG 5. AJA decreases tumor growth *in vivo*. (A) Tumors were measured twice/week with a caliper to study physical growth. AJA inhibits tumor growth in a dose-dependent manner. (B) Cell luminescent data corresponds most closely with physical measurements in the control and low-dose treated mice. (C) Relationship between physical tumor growth and cell luminescence. (D) Mouse weight was measured 5 times/week. Low-dose treated mice weight was decreased within acceptable limits. Weight loss in high-dose treated mice showed evidence of drug toxicity.