2014

Investigating the Mechanism Behind the Cytotoxic Effects of Ajulemic Acid (AJA) on Ewing's Sarcoma by Employing SMAP and Sybyl-X1.3 Surflex Dock Software Programs to Find Receptors for AJA

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SENIOR THESIS APPROVAL

This Honors thesis entitled

"Investigating the mechanism behind the cytotoxic effects of ajulemic acid (AJA) on Ewing's sarcoma by employing SMAP and Sybyl-X1.3 Surflex Dock software programs to find receptors for AJA"

written by

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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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Carl Goodson Honors Program Thesis

Investigating the mechanism behind the cytotoxic effects of ajulemic acid (AJA) on Ewing's sarcoma by employing SMAP and Sybyl-X 1.3 Surflex Dock software programs to find receptors for AJA.

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Introduction

Working on this pediatric cancer research project has been influential on me academically and personally. As a Biology and Chemistry double major, I was given the opportunity to work on this research collaboration between Dr. Lori Hensley from the Biology Department and Dr. Marty Perry from the Chemistry Department. Working with both professors allowed me to fully grasp the research, from theoretical modeling to testing out our hypothesis in lab. Based on the major principle that structure determines function, I compared protein receptors that were geometrically similar to a known receptor relevant to our study, and such protein analogues were hypothesized to function similarly by binding to the same ligand. After analyzing the interaction between the protein analogue and ligand, we tested the receptors in the lab with antibodies. From lab, I learned a great deal of key scientific principles, such as protein-ligand interaction and cell growth/proliferation. Observing, applying, and connecting key concepts learned from class and seen in the lab helped solidify my growing scientific knowledge. Many of the skills I honed in research taught me how to think critically and follow through steps accurately and precisely.

After Ouachita, I hope to pursue a medical degree and become a pediatrician. Studying Ewing's sarcoma opened my eyes to the great need for proper treatment for children in critical care. There seems to be an unfortunate difference in research funding between the adult and pediatric demographics. I hope to continue research in a clinical setting where I can also care for children in the intensive care units and hematology-oncology departments. Lastly, I have learned the importance of teamwork. The collaboration and help from Dr. Hensley, Dr. Perry, and Mrs. Eubanks made this research possible, intriguing, and exciting. Nearing the end of my undergraduate career at Ouachita, I would whole-heartedly recommend that all freshmen

seriously consider research. Through research, one will gain a more comprehensive education, a sparked interest in certain fields of study, and the value of teamwork.

Abstract

Ewing's Sarcoma (ES) is a malignant cancer characterized by the formation of tumors in bones or soft tissues of primarily children and young adults. [1] With the tendency for local recurrence and metastasis, ES is an aggressive cancer. The treatment is intensive and often yields poor long-term results. A cannabinoid derived compound, ajulemic acid (AJA), has shown strong cytotoxic effects on ES cell lines. The synthetic compound is unlike its cannabinoid counterpart tetrahydrocannabinol by lacking psychotropic effects. To investigate the possibility of utilizing AJA as a chemotherapeutic drug, the biochemical mechanism behind the cytotoxic effects of AJA needs further research. A proposed mechanism includes the binding of AJA to nuclear receptors in ES cells such as peroxisome proliferator-activated receptor (PPAR-γ). While past studies have provided great evidence for AJA binding to PPAR-γ, data from western blot analysis indicate the absence of PPAR-γ receptors in ES cells. Computational analysis was employed to find 3D structural analogs of PPAR-γ by using SMAP software program to generate a list of such analogs with PPAR-γ as the template.[2] One of the receptors most similar to PPAR-γ was vitamin D3 receptor (VD3R). Next, Surflex Dock, from Tripos Inc. Sybyl-X 1.3 program, measured how well AJA docked with VD3R by calculating total scores, crash score, C-scores, and global C-scores.[3,4] Total scores greater than 7, and both C-scores and global C-scores of 4-5 indicated a good fit. The next step in determining, if VD3R could be an important receptor through which AJA is working, is to investigate its expression in ES cells and effects on cell viability which appropriate antagonists are used.

Background

Ewing's sarcoma (ES), a member of the Ewing's sarcoma family of tumors, is the second most prevalent pediatric bone cancer.[1] ES is an aggressive cancer that affects primarily caucasians, and more males than females. The median age of ES patients is 15 years old.[1] In 95% of ES cases, the genetic mutation is a translocation of either chromosomes 11 and 22 or chromosomes 21 and 22.[5] There is no current research to provide evidence that ES is inheritable. Common histologic features of this soft tissue sarcoma are small, spherical, blue cells with possible origins from neural crest cells or mesenchymal.[5] Tumor masses are mostly found in the lower extremity (41%), pelvis (26%), chest wall (16%), and upper extremity (9%).[1] Prior to chemotherapy, the long-term survival rate was 10%. Modern multi-disciplinary treatments have increased the prognosis such that patients with localized tumors have a 75% survival rate. Despite all the medical advancements, almost half of the treatments given are either ineffective or unnecessary.[6] The prevailing treatment could result in short-term or long-term toxicities and greatly compromise the patient's immune system. Surgeries are also a treatment option, but may require amputation.[7] Furthermore, prognosis for metastatic and recurrent tumors remain poor. Metastases occur in 25% of the patients, with the most common sites found in the lungs (50%), bone (25%) and bone marrow (20%).[8] Once the cancer has metastasized, the overall survival is 30%.[9] Recurrent tumors typically occur within two years of diagnosis, resulting in an extremely low overall survival rate of 7%.[10]

Growing evidence supported by recent studies suggests that cannabinoids, specifically ajulemic acid (AJA), can be a viable treatment for ES.[11] There are three major types of cannabinoids- herbal, endogenous, and synthetic. Herbal cannabinoids, such as

tetrahydrocannabinol (THC), are extracted from the marijuana plant *Cannabis sativa L.* and are known for producing psychotropic effects. Endogenous cannabinoids, e.g. anandamide, are naturally produced in humans and bind to cannabinoid receptors CB1 and/or CB2. [11] Ajulemic acid is a synthetic cannabinoid that structurally similar to THC. Cannabinoids in general have displayed anticancer properties in many current studies.[12] AJA, in particular, is promising treatment because it effectively kills tumor cells and does not show any psychotrophic effects when administered.[1] In Dr. Hensley's lab, substantial data have revealed that AJA effectively induces apoptosis (i.e. programmed cell death) in ES cells. However, her results indicate that the typical cannabinoid receptors, CB1 and CB2, are not the targeted receptors for AJA. [10] The exact mechanism behind the cytotoxic effects of AJA remains a mystery.

Ambrosio et al. revealed that AJA binds exceptionally well to the ligand binding domain of the human peroxisome proliferator-activated receptor-γ (PPAR-γ). PPAR-γ is a member of the nuclear receptor (NR) superfamily of ligand-activated transcription factors that are involved in a variety of cellular processes such as adipose differentiation and inflammatory and immune response. Since AJA binds well to PPAR-γ and is associated with several important cellular processes, it is plausible that PPAR-γ is a major component to the cytotoxic mechanism. However, western-blots revealed that ES cells do not express PPAR-γ. [13]

Despite the negative results, the data was an impetus for investigating structurally similar PPAR-γ receptors using computational software. Studies have supported the idea that similar three-dimensional (3D) shapes, rather than identical amino acid sequences, mainly determine similar functions between proteins. Accordingly, a Functional Site Analysis Resources program called SMAP was utilized to cipher through the human proteome in order to find analogous 3D

structures using PPAR-γ as the template. [13] The SMAP software generates such analogs based on scores as measured by overall protein structure, binding sites, and chains comparison. According to the protocol, p-values less than $1x10^{-4}$ and a Tanimotto coefficient greater than 0.5 were the two favorable scores employed to find proteins with a similar ligand binding domain (LBD) to PPAR-γ. [3] Of the four chains, PPAR-γ C-Chain provided a list of nuclear receptors with the best scores. This NR list included vitamin D3 receptors (VD3R).

Once a promising receptor was identified, the interaction and binding of the AJA ligand to the receptor was calculated using the Surflex Dock and protein flexibility of Sybyl-X 1.3. The Surflex Dock and protein flexibility measured how well AJA docked with VD3R by generating ligand based protomols and calculating the following scores, listed in order of hierarchy- total scores, C-scores, and global C-scores. Ligand based protomols are computer generated maps of of possible interaction with ligands in the proteins LBD, i.e. the region specified as the active site of the protein. Numerous conformations of the ligand were performed in an attempt to include as many interactions between the ligand and residues in the LBD. The total score is a numerical value equal to the ligand-protein interaction expression as -log(Kd). The C-score is the comprehensive score. Total scores greater than 7 and both C-scores and global C-scores of 4-5 indicated a good fit.

VD3R, also known as calcitriol receptors, could be the receptor to which AJA binds in ES cells. Calcitriol is a lipid soluble steroid that regulates several major cellular functions such as "cell growth, differentiation, antiproliferation, apoptosis, and calcium/phosphate homeostasis." [14] This steroidal nuclear receptor is a ligand-activated transcriptional regulator, just like PPAR-γ.

In addition, VD3R is expressed in a variety of cancers, e.g. pancreatic, lung, breast, and even osteosarcomas. [13] Therefore, VD3R is a promising receptor for AJA in ES cells.

Purpose

(1) Dock AJA to VD3R generated from SMAP list using Sybyl-X 1.3 programs Surflex Dock and protein flexibility to measure the induced fit. (2) Western blot to determine the presence of pertinent receptors in ES cell lines. (3) Perform a MTT assay to calculate the cytotoxic effect of AJA on cells containing VD3R with good docking scores and appropriate antagonists.

Experimental Design/Procedure: Computational Analysis

A list of 3D structural analogs to PPAR-γ was generated and imported into a Microsoft Excel spreadsheet. Nuclear receptors with the best scores were identified using RCSB Protein Data Bank (PDB). VD3R that were not human proteins were ignored. Receptors with ligands already bound to the LBD were preferentially analyzed. Next, the proteins were individually prepared, Surflex Docked, and measured for protein flexibility using Tripos Inc. Sybyl-X 1.3 program. The preparation involved adding hydrogen atoms to the protein, Gasteiger-Huckel charges, and were minimized with 10,000 iterations. At the end of the minimization, the protein were named with their respective "PDB ID_MIN." After minimization, everything was deleted on the Sybyl screen since nothing needed to be opened to run a Surflex Dock. The Surflex Dock box was opened for protomol generation. The prepared protein was loaded and the protomol was created using the ligand route. The ligand naturally bound to VD3R was extracted by selecting the "Extract Ligand Substructures." After highlighting the ligand and applying it, the browser returned to the main Surflex Dock box. Under Protomol Generation, the "Ligand" option was

selected. The Mol2 File was changed to Mol Area that contained the newly extracted ligand. Then, "Generate" was selected and the protomol formed (Figure 1). Next, AJA was loaded by changing from SLN file to Mol2 file. The Surflex Dock options were modified by: imputing 3 Additional Starting Conformation per Molecule, 12 Angstroms to Expand Search Grid, 200 Max Conformations per Fragment, 200 Max Number of Roatable Bonds per molecule, 200 Maximum Number of Poses per Ligand, and 0.05 Minimum RMSD Between Final Poses. A jobname was created and the "OK" option was selected. To determine protein flexibility, the following items were selected "Hydrogen, Heavy Atoms, and Fast Protein Flex (Less Thorough Search)" and everything else was kept the same (Figure 2). Once the "Dock" was completed, the scores were analyzed in the Results Browser. The protein was loaded by selecting "View." The total score, crash score, C-score, global C-score, and polar score were all recorded. This process was repeated until sufficient numbers of VD3Rs were analyzed.

Figure 1: Protomol Generation

Uploaded 2HB7 VD3R from PDB and used Tripos Inc. Sybyl-X 1.3 software program Surflex Dock. The grey region represents the protomol, the region specified as the active site of the protein.

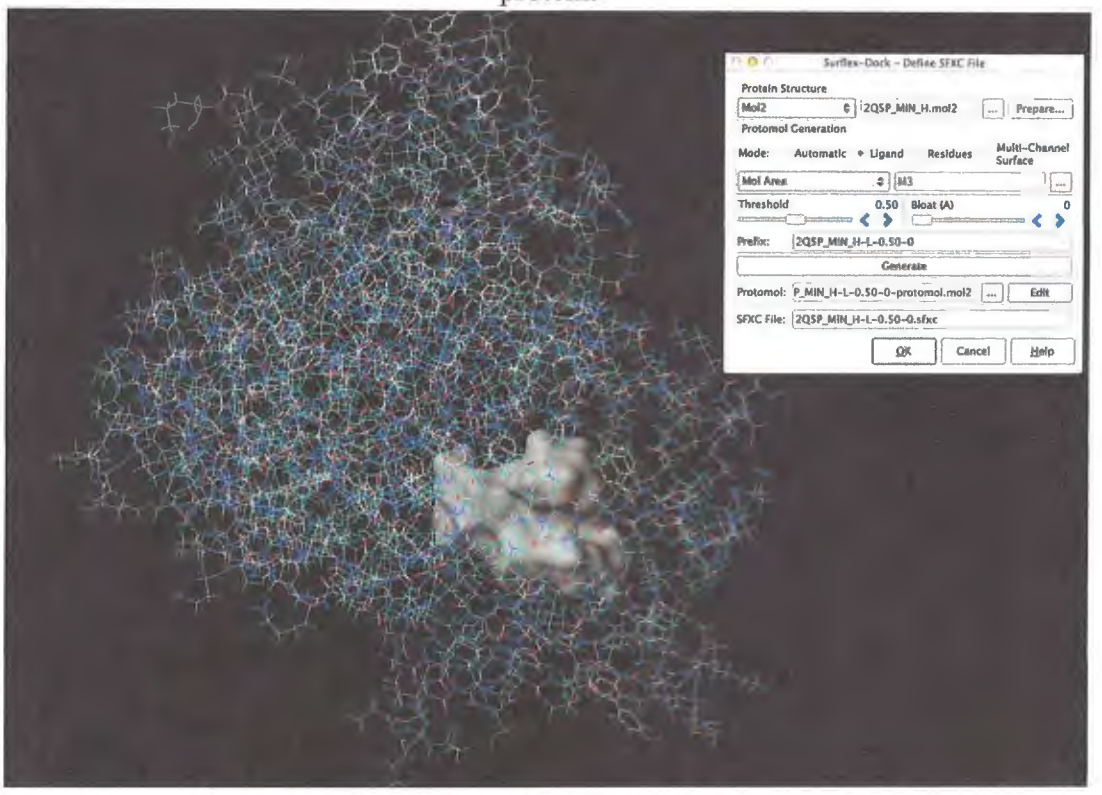
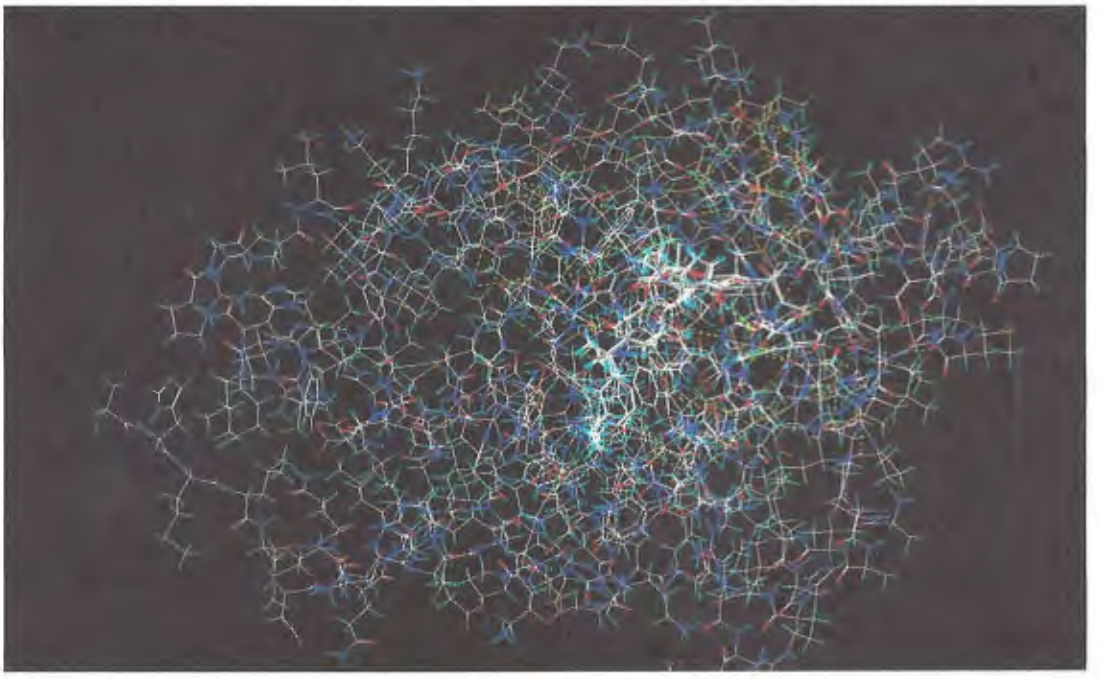


Figure 2: Protein Flexibility

Uploaded 2HAR VD3R from PDB and used the Protein Flexibility program from Surflex Dock.



Results: Computational Analysis

PDB Code	Total[1]	Crash[2]	C-score[3]	GC-score[4]	Polar[5]
1IE9	7.1029	-6.6530	3	3	1.0856
2HB7	8.2514	-3.0549	3	3	0.0000
2HAM	7.7245	-4.1694	3	3	0.0006
2HAR	9.3630	-3.8722	5	5	0.0082
2HAS	8.1635	-3.1904	3	3	0.0000
3AUR	6.3625	-4.6141	2	2	1.1360
3AZ1	4.3390	-8.2508	3	3	1.0294
3AZ3	7.3469	-5.4011	3	3	0.0000
2HB8	7.9446	-5.1736	3	3	1.1572
1DB1	7.5854	-5.7765	3	3	1.2919
3OGT	3.8948	-8.2414	4	4	0.0001
3TKC	5.2539	-7.6192	3	3	2.3025
2SOZ	6.5384	-5.6310	4	4	0.0001
1S19	5.6125	-5.0560	3	3	0.0006
3AZ2	8.0990	-3.7553	4	4	0.0000
[1]pKd= -log(Kd) is a measurement of ligand-protein interaction					
[2]Crash score is a measurement of the unwanted penetration the ligand has into the protein space. If the crash score is reduced from -2.00 to -1.00, the total score will increase by 1.					
[3]C-score is comprehensive score: 1 is worst, 3 is average, and 5 is the best					
[4]Global C-score is essentially the same as C-score					
[5]Polar score is a measurement of how much of the total score is due to polar interactions					

Table 1: Surflex Dock Results with Strong Scores

The relatively high total scores for different uploaded VD3Rs suggest that AJA would bind well at the LBD of the nuclear receptor, despite the unfavorable crash scores. 2HAR had the best scores overall, with the highest total score, C-score, and GC-score. 3OGT had the lowest total score, but its C-score and GC-score were 4.

Conclusion: Computation Analysis

Several VD3Rs from PDB were uploaded into Sybyl-X 1.3 to be Surflex Docked with AJA. A set of scores was compiled to measure how well AJA would bind to VD3R. In order of importance, the scores recorded were highest total score, low crash score, C-score, GC-score, and polar score. Total scores that were 7 and above indicate good ligand-protein interaction. Ten VD3RS from the PDB had total scores greater than 7. VD3R from PDB with the best overall scores were 2HB7, 2HAR, 2HAS, 3AZ3, 2HB8, 3CS6, and 3AZ3 respectively (see Table 1). Low crash scores, calculated by the absolute value of the number given from Surflex Dock, were not attained. The C-scores and GC-scores were fairly average. VD3Rs with higher total scores seemed to have smaller polar scores. Overall, the scores suggest AJA would bind well to VD3R *in vivo*. If so, VD3R could be the receptor that mediates the signal for apoptosis in ES.

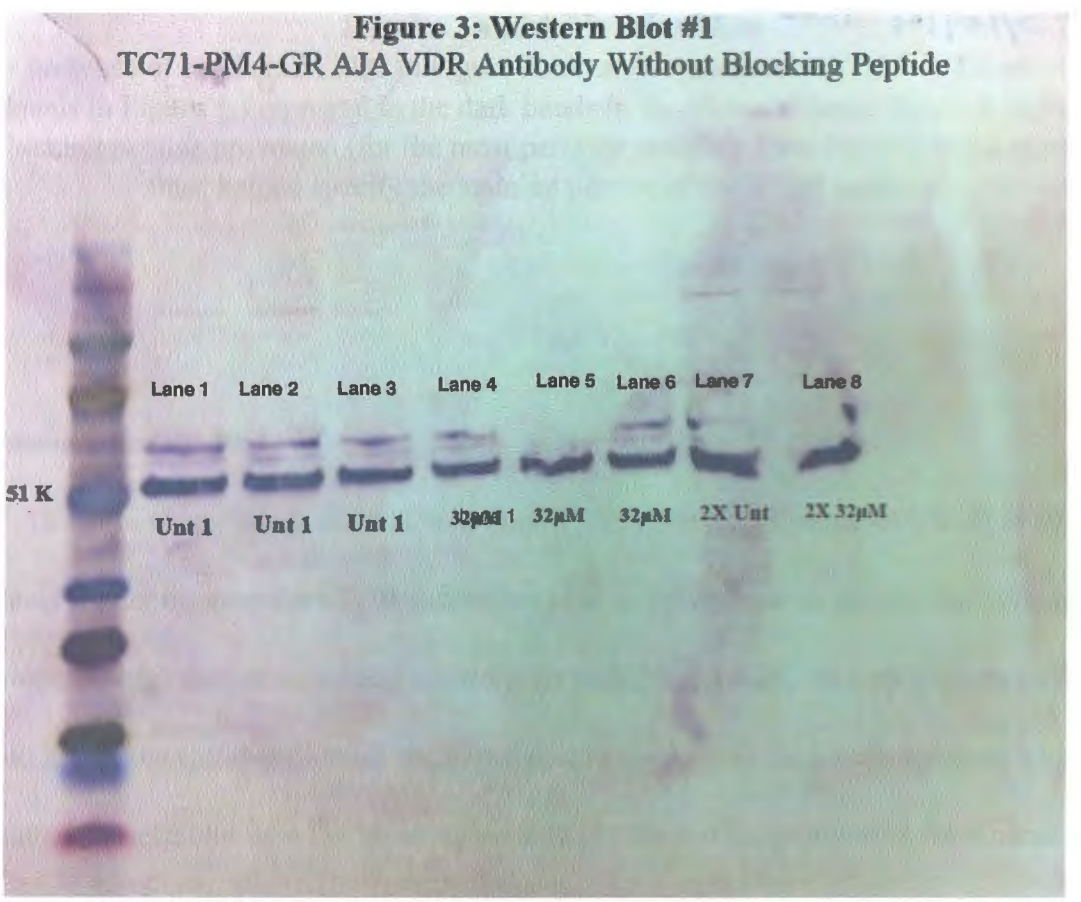
Experimental Method: Western Blot for VD3R Expression

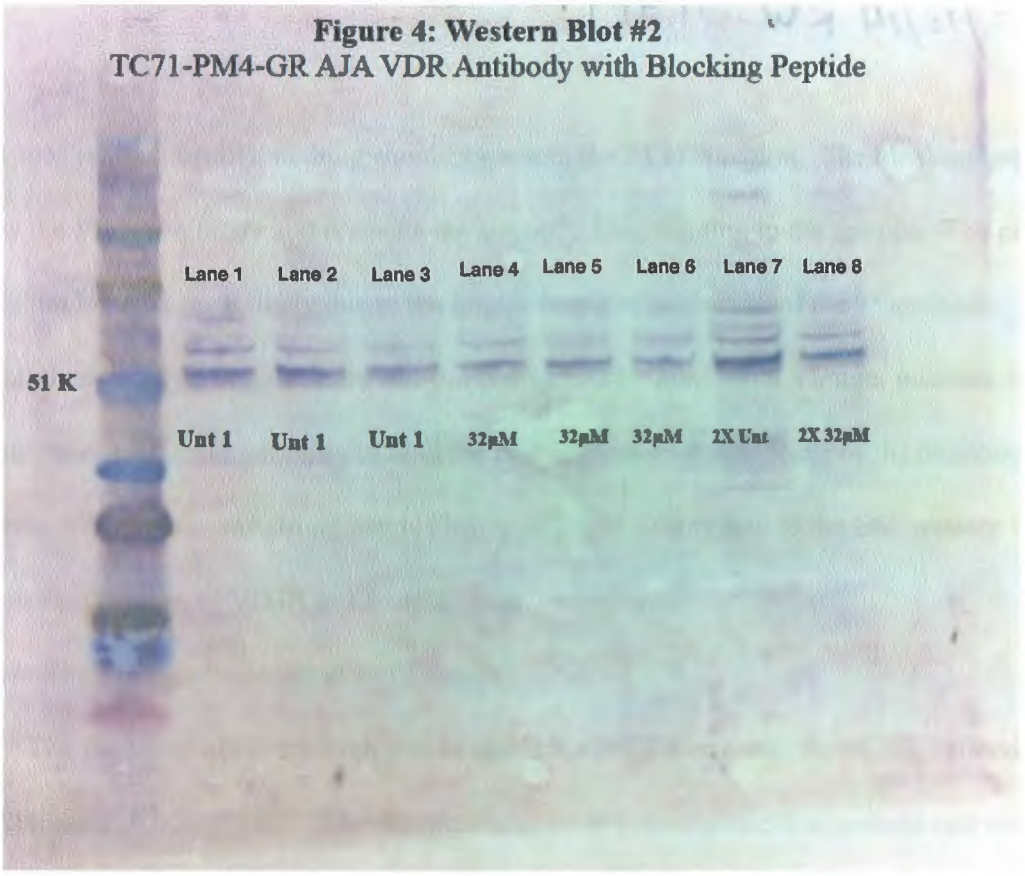
Two western blot sample sets were conducted, one with blocking peptide and one without the blocking peptide. The blocking peptide functions as a binding specificity control because they bind to the VD3R antibodies, rendering the antibodies unable to bind to the epitope. One set contained 8 total samples, half with untreated cells and the other half with cells treated for 24 hr with 32 μM AJA. Samples 1-3 of untreated cells contained 5 μL lysate, 20.2 μL phosphate buffered saline (PBS), 9 μL lithium dodecyl sulfate (LDS), and 1.8 μL dithiothreitol (DTT). Samples 4-6 of 32 μM AJA had the same volumes and concentrations as samples 1-3. Sample 7 contained untreated cells with 10 μL lysate, 15.2 μL PBS, 9 μL LDS, and 1.8 μL DTT. Sample 8 contained cells treated with 32 μM AJA mixed with 10 μL lysate, 15.2 μL PBS, 9 μL LDS, and

1.8 µL DTT. The electrophoresis ran at 150 V until the blue was at the bottom of the gel. The transfer ran at 200 mA for 1.5 hr. Then, 15 µL of 1° antibody (Santa Cruz Biotechnology VD3R sc-1008) was dispensed and incubated with the gels overnight in the fridge with 3 mL of blocking solution. The peptide neutralization protocol involved 75 µL blocking peptide, 15 µL VD3R antibody, and 410 µL PBS. Next in sequential order, the gel was washed, incubated in the fridge for 1 hr with the 2° VD3R antibody, washed again, and developed. For the second western blot gel, similar protocol was followed excluding the blocking peptide. The molecular weight of VD3R is 51 kDa, thus strong bands should appear in the western blot without the blocking peptide in the 51 kDa band region. However, for the western blot with the blocking peptide, the bands should be absent in that region.

Results: Western Blot

Figure 3: Western Blot #1
TC71-PM4-GR AJA VDR Antibody Without Blocking Peptide





Figures 3 and 4: Western Blots

The presence of bands at 51 kDa in Figure 3 reveal the presence of VD3R in ES cells. The faint bands in Figure 4, compared to the dark bands in the western blot in Figure 3, indicate that the blocking peptide prevented (for the most part) the antibody from binding to the epitope and thus, helped specify the staining pattern of the VD3R antibody.

Conclusion: Western Blot

The presence of bands at 51 kDa in Figure 3 confirm the presence of VD3R in ES cells. The bands appear because the VD3R antibodies bind to the epitope on the protein. The receptor was present in both the untreated and treated cells with 32 μM AJA. In both western blots, Lanes 1-4 with more concentrated proteins displayed darker bands than the less concentrated Lanes 5-8. The second western blot with the blocking peptide in Figure 4 had noticeably faint bands near

the 51 kDa region. Ideally, nothing should appear in the 51 kDa region. The blocking peptides bind to the VD3R antibody and prevents the antibody from binding to the epitope. The presence of the faint bands is most likely due to the long, overnight incubation of the 1° antibody. The incubation is usually a couple hours and can last up to 24 hours. With a longer incubation time, the antibodies had the opportunity to bind the epitope despite the presence of the blocking peptides. Nonetheless, the strong bands present in the 51 kDa region in the first western blot confirm the presence of VD3R in ES cells.

Experimental Design/Procedure: MTT assay

The final step of the research was to conduct a MTT assay with the VD3R antibody on ES cell lines TC71-PM4-GR. This colormetric assay is a measurement of percent cell viability. The mitochondrial succinate dehydrogenase reduces soluble MTT (a yellow colored tetrazolium salt) to an insoluble, blue-purple formazan. The color intensity is measured via spectrophotometer. Living cells are actively respiring, thus the color intensity is directly proportional to the number of viable cells. If VD3R is the receptor that mediates apoptosis in ES cells treated with AJA, then high percent cell viability should be expected from both VD3R antibody alone and antibody plus AJA treated cells because the antibody should block the ability of AJA to bind. A 24 well plate contained the following: untreated cells (wells 1-3); 16 μM AJA (wells 4-6); 1:50 VD3R antibody (wells 7-9); 1:500 VD3R antibody (wells 10-12); 1:50 VD3R plus 16 μM AJA (wells 13-15); and 1:500 VD3R antibody plus 16 μM AJA (wells 16-18). The wells with AJA were treated with the drug after 30 min of adding the appropriate media and VD3R antibody concentrations.

Results: MTT Assay

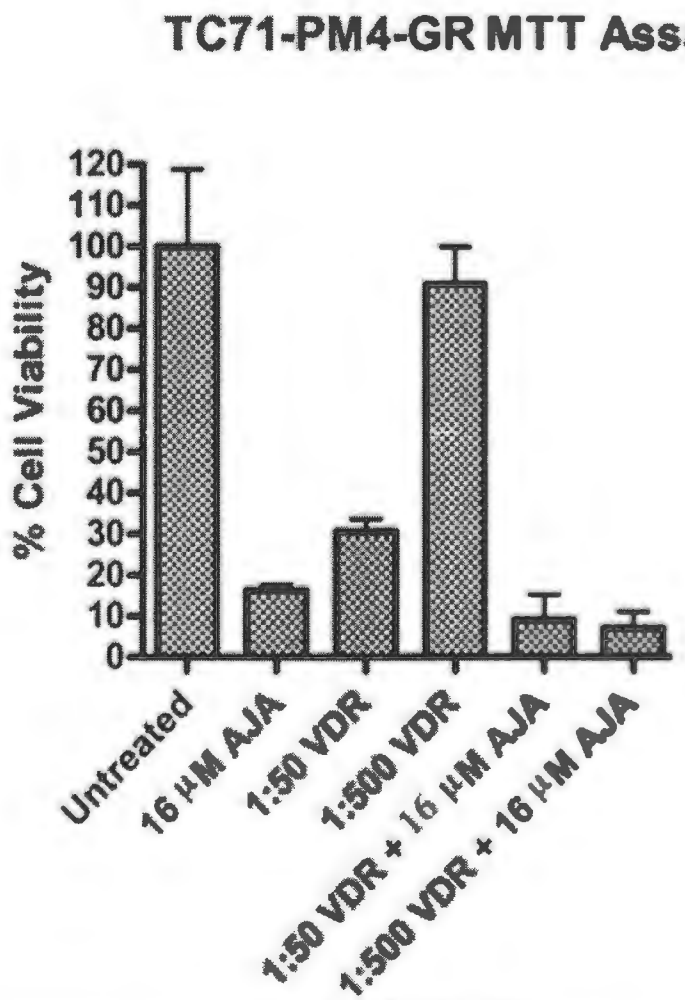


Figure 5: Cell Viability

The wells containing the antibody and AJA had unexpectedly low percent cell viability. Since 1:50 VD3R, compared to 1:500 VD3R, greatly reduces percent cell viability, there may be a correlation between dosage of antibody and magnitude of cell death.

Conclusion: MTT Assay

The pilot assay seems to suggest that the binding of the VD3R antibody to the nuclear receptor acts as a partial agonist and is further exacerbated when AJA is present. The wells with the untreated cells had the expected high percent cell viability. Similarly, the wells treated with AJA alone had low percent cell viability. However, the VD3R antibody was hoped to act as an antagonist and block the AJA from binding to the VD3R. Accordingly, a high percent cell viability was expected. Yet the percent cell viability was low for the VD3R antibody treatment and antibody plus AJA. One interesting observation was that since 1:50 VD3R, compared to the 1:500 VD3R, greatly reduced percent cell viability, there may be a correlation between dosage of antibody and magnitude of cell death (Figure 5). While this pilot assay does not definitively affirm VD3R as the receptor to which AJA binds and signals apoptosis in ES it does suggest the VD3R can mediate cell death in ES cells, but that the antibody may activate, not block, the receptor. More controlled assays are necessary, and a true VD3R antagonist is needed.

Further Directions

As mentioned previously, another controlled MTT assay is necessary. The second assay should include a positive control with vitamin D3 and a negative control with a known antagonist. With a limited amount of time and resources, the pilot assay had to be accomplished with an antibody instead of a known antagonist. Interestingly, all nuclear receptors [PPAR-γ, retinoic acid receptor alpha (RAR-α), and VD3R] tested thus far as possible receptors for AJA are heterodimers of another receptor, retinoic X receptor (RXR). In addition to testing VD3R in future assays, RXR should be tested with VD3R. If VD3R is not the main mediator in the

cytotoxic pathway, AJA-RXR interaction should be analyzed similarly using SMAP, Surflex Dock, and Protein Flexibility. Another set of western blots would be appropriate to confirm the presence of the receptor. Along with the computational analysis and western blots, a more controlled MTT assay can be conducted for future experiments.

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