Loss of Akt expression protects Drosophila larva from diadzein-dependent death

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ABSTRACT

The fruit fly model, *Drosophila melanogaster*, has been utilized in different scientific fields spanning from developmental biology to cell signaling to toxicology aiding researchers in the discovery of novel mechanisms and therapeutics. Here, we demonstrated that wildtype w^{118} fruit fly larval survival is not affected by ingestion of the isoflavone genistein, in contrast to the increased lethality seen when w^{118} flies were fed the isoflavone daidzein. Decreased survival rates following daidzein consumption are attributed to activation of the Akt signaling pathway by daidzein binding to the estrogen-related receptor (ERR) within the larval digestive tract. The Akt knockout, *yw;P[GMR-Akt1],* flies were protected from daidzein consumption-induced larval lethality, supporting the hypothesis that the activation of the Akt pathway is the cause of death.

INTRODUCTION

Drosophila melanogaster, a fruit fly species, has been a viable model organism for many decades and has led to many breakthroughs such as the discovery that chromosomes carry heritable traits. Many of the genes required in the development of the fruit fly have also been found to be essential in mammalian development. Once its entire genome was sequenced, homologies with the human genome strengthened its role as a model organism for human biological and disease mechanism understanding (Pandey and Nichols, 2011). The sequenced genome also along with *Drosophila*'s characteristic malleable genetics allows for specific mutations of known genes for understanding the effect when there is a loss of function.

Isoflavones are phytoestrogens, plant-derived compounds similar in function to estrogen. They are generally found in legumes such as soybeans and green beans but can exist in other plant tissues. Isoflavones are normally water-soluble, secondary metabolites which aid in both the plant immune defense and commensal rhizobial bacteria (Van Rhijn and Vanderleyden, 1995). Two widely available isoflavones that we used in our study were genistein and daidzein. These compounds have been shown to provide relief to those suffering from various ailments such as cancer, menopausal symptoms, cardiovascular disease and osteoporosis; however, the underlying mechanisms to how isoflavones promote health are not generally well understood (Setchell and Cassidy, 1999).

When they are consumed by an organism, isoflavones can cross the plasma membranes of cells through simple diffusion due to their hydrophobicity. Once within the cell, they bind to a receptor. Current research with the isoflavone, daidzein, has been shown to bind to the estrogen-related receptor (ERR) within the digestive tract of fruit fly larvae. ERR has been shown to interact with estrogen signaling and involved with metabolism (Bardet *et al*. 2006). ERR is currently an orphan receptor meaning that it does not have a known naturally-occurring ligand. The ERRα of *Drosophila* are closely related to the ERR receptors of mammals (ERRα, ERRβ, and ERRγ). These receptors are classified as nuclear receptors and are present on the nuclear membrane within the cell. When activated, these receptors lead to transcription via estrogen response element (ERE) and ERR-response element (ERRE). Both the ERE and ERRE are the genome sequences directly affected by the ERR transcription factor complex. Of note, *Drosophila* have two isoforms of ERR, a long form and a short form. The isoforms differ by the presence or absence of an alternative exon near the ligand-binding domain. They function similarly in the activation of ERE transcription; however, the difference lies in ERRE transcription where the long isoform is a dominant negative transcription factor over the short isoform. The long ERR isoform binds to a ERRE as a monomer while it binds to a ERE as a homodimer. This dimerization is caused by protein kinase C δ-driven phosphorylation of ERRα changing from a monomeric state into a dimeric state. Dimerization appears to be necessary for ERRE and ERE transactivation. Understanding ERR function can be indispensable in the discovery of breast cancer therapies. Researchers have shown that ERRs can activate the *pS2* gene in human breast cancer cell line MCF-7. The *pS2* gene encodes a secreted protein with an unknown function; however, it is structurally similar to growth factors. The *pS2* gene also contains a functional ERRE within its promoter region. ERRs, specifically ERRα, are active within the MCF-7 cell line and directly activate the ERRE of the *pS2* gene promoter region. Inhibition of ERR transcriptional activity results in lower *pS2* gene expression (Lu *et al*. 2001). Therefore, a better understanding of ligand binding to ERRs may provide novel therapeutics for human breast cancer treatments.

The mechanism behind how daidzein and the ERR come into contact remains largely unknown. One current idea is that after daidzein diffuses through the plasma membrane, it is shuttled by a protein to the ERR located on the nuclear membrane of the cell. We propose that this protein is Akt. Akt, otherwise known as protein kinase B (PKB), is an important effector signal within multiple cell signaling pathways. Some of the pathways that Akt functions in are: cell

survival, cell cycle continuation, metabolism, and angiogenesis, the growth of new blood vessels (Manning and Cantley, 2007). We theorize that Akt dimerizes with daidzein and relocates to the nucleus where it binds to an ERR which translocates to the DNA causing the transcription of an ERE or ERRE which leads to cell apoptosis and organismal death. We used wildtype w^{118} fruit flies containing a functional Akt gene and *yw;P[GMR-Akt1]* fruit flies missing a functional Akt gene to determine the role of Akt in larval survival following isoflavone ingestion.

Our results show that the isoflavone, genistein, does not induce *Drosophila* larval death. Larval death through the consumption of daidzein can be rescued through the knockout of Akt preventing activation of its signaling pathway. As the ERR is still an orphan receptor, establishing a specific isoflavone ligand and characterizing its function can allow for the creation of therapeutics that could potentially aid in breast cancer disease progression.

METHODS

Drosophila Stock Maintenance

w^{118} and *yw;P[GMR-Akt1]* fruit fly stocks were kept in clear cylindrical vials containing the food media for flies and larva. The food media was a combination of agar, corn starch, corn syrup, yeast, and antimicrobial growth agents. The stocks were maintained in an incubator at 25°C. Flies would be dumped into the morgue every Thursday to prevent food media exhaustion. New vials would be created when the media becomes brown and exhausted. Flies from preexisting vials would be dumped into these fresh vials for egg laying.

Larval Survival Assay

The larval survival assay was a weeklong process with the following outline:

Monday – Egg laying chambers were set up using conical tubes with holes in the top and grape juice plates with yeast paste connected at the bottom with Parafilm. A mix of male and female fruit flies were placed within for egg laying.

Tuesday – For the genistein experiment with wildtype w^{118} flies, three survival chambers were set up with one control yeast paste group, one 500µM group, and one 1000µM group. 20 eggs from the egg laying chambers were placed in each of the survival chambers. For the daidzein with *yw;P[GMR-Akt1]* Six survival chambers were set up with two control yeast paste groups, flies, two 200µM

daidzein yeast paste groups, and two 500µM daidzein yeast paste groups. 50 eggs from the egg laying chambers were placed in each of the six survival chambers.

Wednesday-Friday – Instar larvae were removed from the seeded survival chambers from the previous day and placed into new ones with their respective experimental groups up until Friday, when third instar larvae were placed into a new tube where they would be allowed to pupate until Monday.

Protein Quantification

Ten second instar larvae were removed from the survival chambers from each group and placed into a 1.5ml microcentrifuge tube containing 1x PBS for washing. After 5 minutes, the larvae were washed with a 70% ethanol solution for 1 minute followed by a final wash within 1x PBS for 1 minute. The cell extraction buffer was created with protease inhibitor cocktails and EDTA. These inhibitors diluted at 1:100 within the cell extraction buffer. The 1x PBS solution was removed from the microcentrifuge tube and the larvae were then suspended in 100µl of the cell extraction buffer. The larvae were crushed within the cell extraction buffer then 400µl of the cell extraction buffer was added to each tube. The tubes were vortexed for 10 seconds and incubated on ice for 30 minutes with occasional vortexing every 5 minutes. After the incubation period, the tubes were placed within a centrifuge for 2 minutes at 10,000 rpm. Lysates were removed and placed within new microcentrifuge tubes. The protein working solution for the Qubit 2.0 Fluorometer was created using the following equation: (# of samples) x (200µl) = amount of protein buffer required. The dye was added to this solution at a 1:200 dilution. Standard protein concentrations were used to create a standard curve. These standards were created by using 190µl of the working buffer and 10µl of the standard and vortexed. Samples were diluted 1:100 into 1x PBS then 2µl of the dilution and 198µl of the working solution were added to a Qubit tube and vortexed for reading. These samples could incubate for 15 minutes at room temperature in the dark and then read using the fluorometer.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electorophoresis (SDS PAGE)

After the proteins were quantified, 190µg of protein from the stock solution was placed into a new microcentrifuge tube. The amount of solution required for this was matched with 2x gel loading buffer. These solutions were allowed to boil for 5 minutes in water. A gel electrophoresis rig was set up using a solution containing 90ml of 10x

Tris/Glycine/SDS buffer and 810ml of deionized water. 50µl well precast gels were used to run the gel electrophoresis. 10µl of the Precision Plus Protein Marker was added to well #1 and #7. The samples were added to wells 2-4 and 8-10 and were placed in the following order: control, 200µm, and 500µm for each section. The gel was run at 200V for approximately 45 minutes to allow for protein separation based on size.

Coomassie Blue Staining for Presence of Proteins

Control lysates were run in ascending protein concentration to determine the ideal amount of protein necessary for Western blotting. A SDS PAGE was run with the following setup: Well 1 = 10µl of the Precision Plus Protein Marker, Well 3 = 42µg, Well 4 = 84µg, Well 6 = 127µg, Well 7 = 169µg, and Well 9 = 211µg. After the SDS PAGE was run, the gel was removed from its casing and washed in deionized water on a rotator for 20 minutes. GelCode Blue Stain Reagent was added after removal of the deionized water and was placed on the rotator overnight. The following day, the GelCode Blue Stain Reagent was discarded and a counter stain consisting of deionized water was added and allowed to rotate overnight.

Western Blot Visualization

After the SDS PAGE containing the experimental groups was run, the gel was placed into a plastic container with Western blot transfer buffer and put on the rotator for 20 minutes to remove the SDS buffer. Western blot transfer pads and filter paper were placed in plastic containers and soaked in Western blot transfer buffer. The Western blot transfer holder was placed into another container, black-side down with Western blot transfer buffer. An immobilon membrane was first soaked in methanol for 15 seconds and then placed into Western blot transfer buffer until needed. After 15 minutes of washing the gel, the Western blot transfer "sandwich" was created. The sandwich consisted of one transfer pad, a filter paper, the gel, the immobilon membrane, another filter paper, and another transfer pad, then the sandwich was closed. The sandwich was placed into the transfer rig. The Western blot transfer occurred in the cold room for 1 hour and 30 minutes at 85V. After the transfer, the immobilon membrane containing the proteins was removed and placed into a blocking solution overnight to prevent non-specific antibody binding. The following day, the blocked immobilon membrane was cut in half for the separation of the control (total Akt) and experiment (phosphorylated Akt) groups. The total Akt membrane was added to a

conical tube containing rabbit anti-Akt (#4691) while the phosphorylated Akt membrane was added to a conical tube containing rabbit anti-pAkt (#40545), both diluted 1:200. Both tubes were placed on a rotator in the cold room and spun overnight. The following day, the antibodies solutions were removed and stored within the freezer. The membranes were washed with tris-buffered saline solution containing Tween 20 (TBST) for 2 minutes, 3 times. The secondary antibody solution containing the alkaline phosphatase visualization enzyme was created using goat anti-rabbit diluted 1:2000 in starting block buffer. 25ml of this solution was added to each of the membranes for 45 minutes. After the addition of the secondary antibody solution, the membranes were washed with TBST for 2 minutes, 3 times. The alkaline phosphatase (AP) visualization solution was created using 48 ml deionized water, 2ml of the 25x AP Color Development Buffer, 0.5ml of AP Color Reagent A, and 0.5ml of AP Color Reagent B. 25ml of the solution was added to each membrane and observed for visualization over the next 24 hours.

RESULTS

Consumption of the isoflavone, daidzein, results in early larva developmental lethality while genistein has no effect. Experimental w^{118} fruit flies fed either 500µM or 1000µM of genistein-infused yeast paste over the course of their development. Control w^{118} fruit flies fed strictly plain yeast paste having survival rates of 98% for both transitions into 2nd instar and 3rd instar (Figure 1). The w^{118} fruit flies fed 500µM genistein-infused yeast paste had survival rates of 95% for the 2nd instar and 92% for the 3rd instar; however, the w^{118} fruit flies fed the 1000µM of genistein-infused yeast paste did not experience any death. This indicates that the deaths seen in the 500µM feeding group may not attributed to the consumption of genistein but another unknown factor. Statistical analysis of these results has p-values greater than 0.05 indicating that there is no statistical significance amongst the three groups tested.

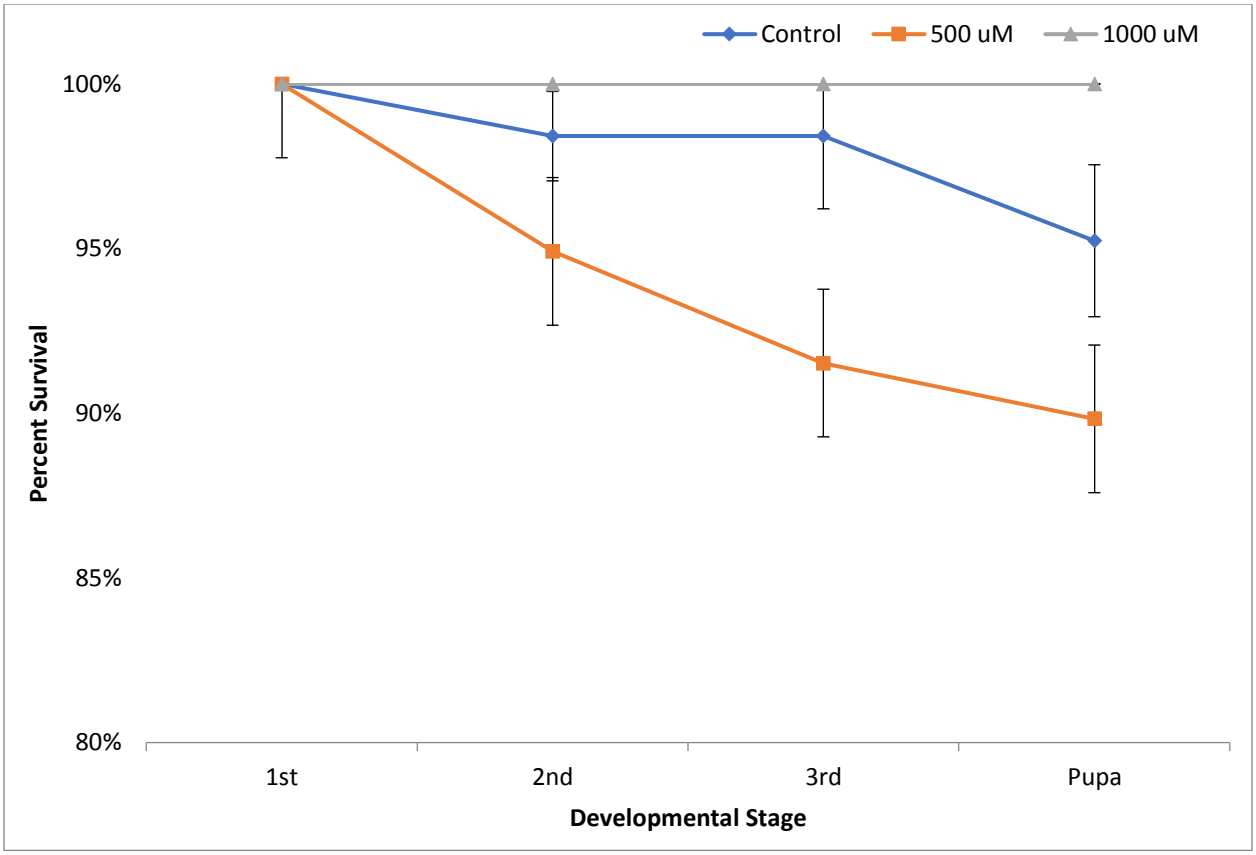

Figure 1. Genistein consumption does not significantly affect larva survival. *w*^{118} fruit fly larvae were subjected to the larval survival assay following the assay protocol described previously. *w*^{118} fruit flies laid eggs within the survival chamber from Monday to Tuesday, first instar larvae were collected on Wednesday, and they were allowed to grow until after pupation. Larvae at each interval were counted and survivability was plotted against the first instar larvae. Control *w*^{118} fruit flies experienced a 95% survival rate. *w*^{118} fruit flies fed 500µM genistein experienced a 90% survival while *w*^{118} fruit flies fed 1000µM experience an 100% survival rate. The p-value for the control and 500µM group was 0.1 while the p-value for the control and 1000µM group was 0.07. These values both indicate that there was not a significant difference between the groups.

To determine the role of Akt in previously documented daidzein-dependent larval death, *yw;P[GMR-Akt1]* fruit flies were fed either 200µM or 500µM daidzein-infused yeast paste over the course of their development (Figure 2). Control *yw;P[GMR-Akt1]* fruit flies were fed strictly on plain yeast paste had survival rates of 97% for the transition into the 2nd instar and 92% for the

transition into the 3rd instar. The *yw;P[GMR-Akt1]* fruit flies fed 200µM genistein-infused yeast paste had survival rates of 95% for the 2nd instar and 91% for the 3rd instar while the *yw;P[GMR-Akt1]* fruit flies fed the 500µM genistein-infused yeast paste experience survival rates of 94% for the 2nd instar and 91% for the 3rd instar. The differences in survival rates between the *yw;P[GMR-Akt1]* fruit flies fed the control yeast paste and those fed daidzein-infused yeast paste are small indicating that the consumption of daidzein without the presence of Akt prevents larva death. Statistical analysis of these results has p-values greater than 0.05 indicating that there is no statistical significance amongst the three groups tested.

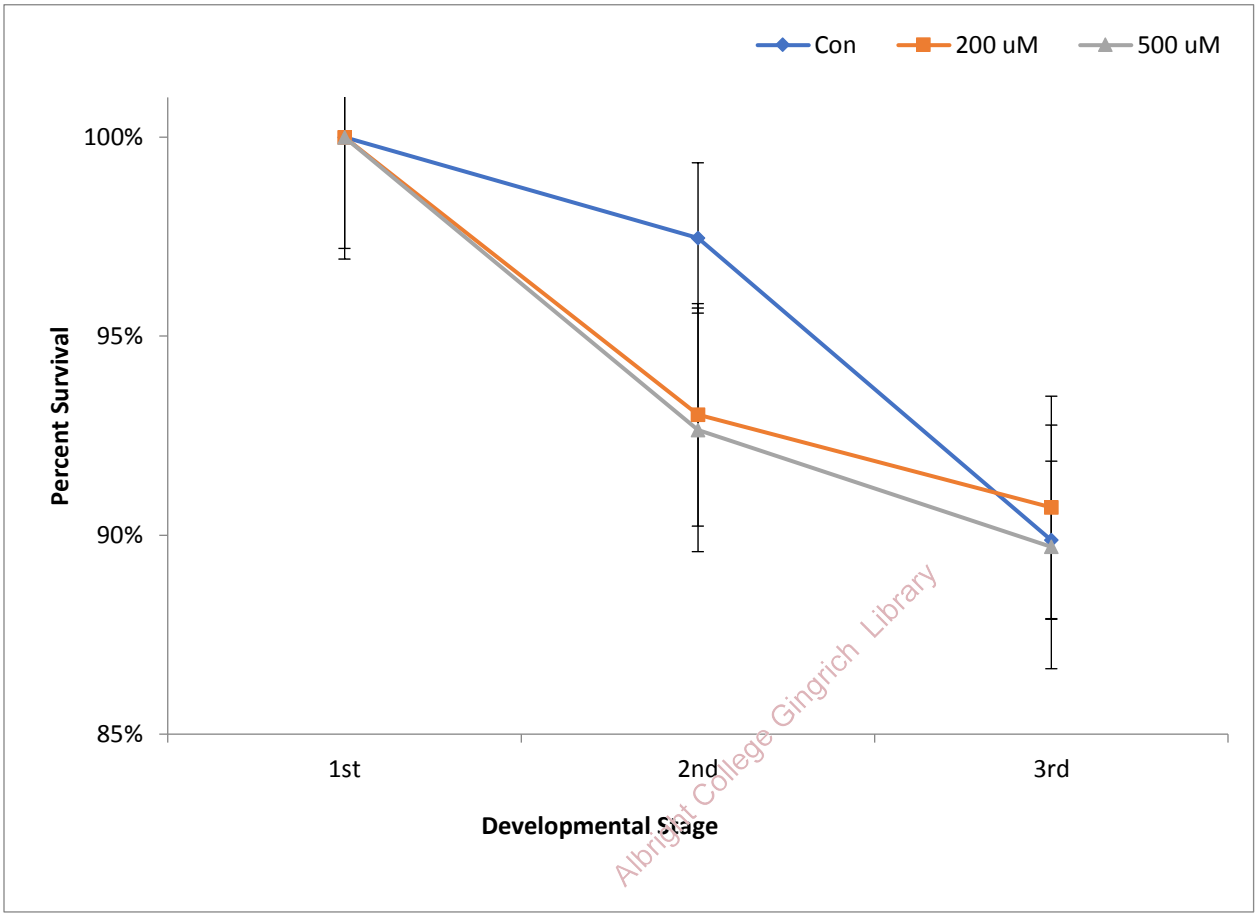

Figure 2. Akt mutant flies are protected against daidzein consumption lethality. *yw;P[GMR-Akt1]*fruit fly larvae were subjected to the larval survival assay previously described. *yw;P[GMR-Akt1]*fruit flies laid eggs within the survival chamber from Monday to Tuesday, first instar larvae were collected on Wednesday, and they were allowed to grow until after pupation. Larvae at each interval were counted and survivability was plotted against the first instar larvae. Control

yw;P[GMR-Akt1] fruit flies experienced a 89.7% survival rate. *yw;P[GMR-Akt1]* fruit flies fed 200µM diadzein experienced a 89.9% survival while *yw;P[GMR-Akt1]*fruit flies fed 500µM experience an 90.7% survival rate. The pupa time interval was omitted due to lack of sufficient data points.

Larva protein was quantified using the Qubit 2.0 Fluorometer. There was some troubleshooting during this part of the procedure. Originally, the stock protein samples were diluted within cell lysis buffer; however, the concentration of the solution never went under 26µg/ml (the maximum value that the Qubit 2.0 Fluorometer can measure). The correct concentration was unable to be determined using cell lysis buffer as the diluent contained a large amount of proteins and interferes with the Qubit 2.0 Fluorometer dye absorbance readings. Moving to a 1x PBS solution allowed for the correct concentration of sample proteins as shown in Table 1.

Table 1. Protein quantification from 10 larvae per experimental condition.

Group	1:10 Dilution (ug/ml)	Stock (ug/ml)	Stock (ug/ul)	Added to gel (ul)
Control	860	8600	8.6	22.1
200uM	1020	10200	10.2	18.6
500uM	1030	10300	10.3	18.4

*Values were calculated via the Qubit 2.0 Fluorometer.

With the correct concentration known, it was possible to determine if these proteins can be separated within SDS PAGE. A Coomassie Blue stain tests for the presence of all protein within a gel. It was necessary to run this test to determine the appropriate protein concentration to run on the Western blot. The Coomassie Blue result revealed the presence of proteins within the gel (Figure 3.). The following information allowed us to determine that a higher concentration of protein is necessary for a clearer signal on the Western blot; therefore, we loaded 190µg protein per well during SDS PAGE. The protein concentration of our samples was not sufficient for Western blot visualization.

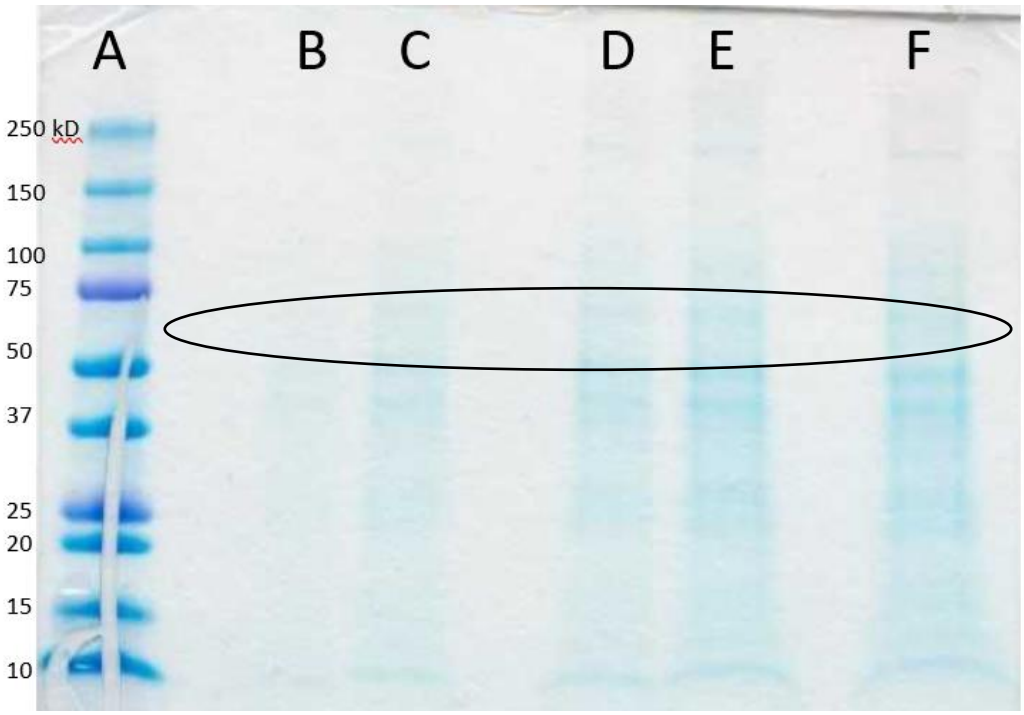

Figure 3. GelCode Blue Stain Reagent reveals presence of larval proteins within samples run through SDS PAGE. Circle indicates the expected region where the Akt and pAkt proteins are located. (A) Precision Plus Protein Marker; the numbers represent protein molecular weight in kilodaltons (kDa), (B) 42µg of protein, (C) 84µg of protein, (D) 127µg of protein, (E) 169µg of protein, and (F) 211µg of protein.

Western blot analysis did not reveal the presence of either Akt or pAkt (Figure 4). After the primary and secondary antibodies were applied to the membranes containing the proteins of interest, unbound antibodies were washed away to specify the location of the proteins in question. We expected to see a band representing Akt/pAkt at 65 kDa; however, no bands were present when the membranes were dried.

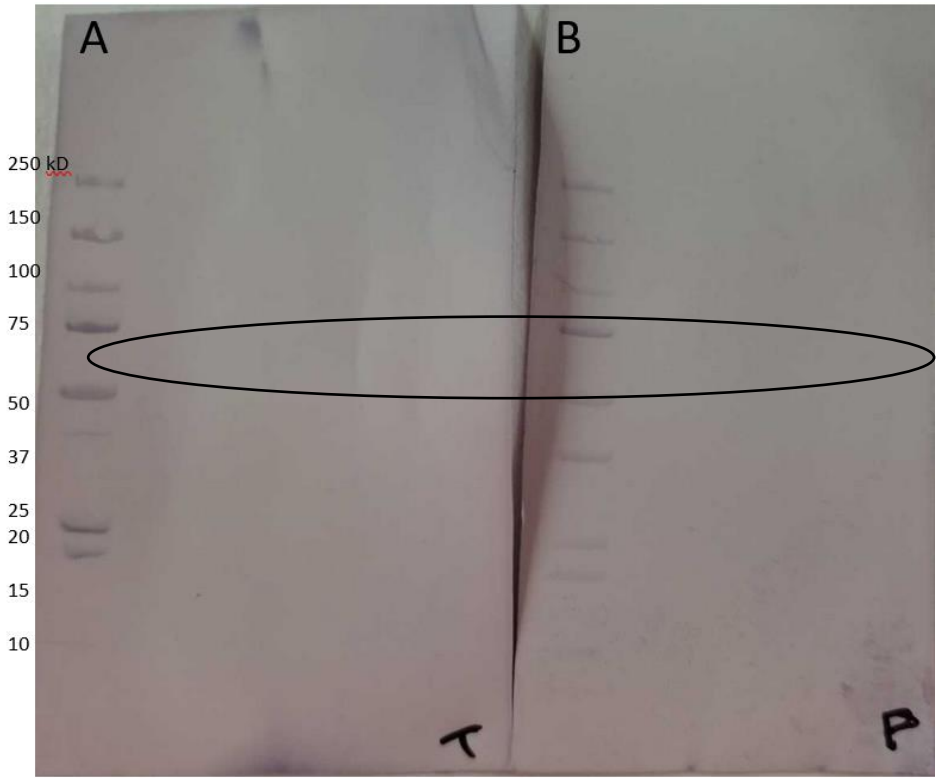

Figure 4. Western blot analysis shows no Akt/pAkt signal at expected values. Circle indicates the expected region where the Akt and pAkt proteins are located. (A) represents the half of the membrane in which total Akt was assessed while (B) represents the other half of the membrane in which phosphorylated Akt was assessed.

CONCLUSIONS AND DISCUSSION

These results show that genistein does not lead to larva death after consumption as seen with daidzein, despite both being major isoflavones. It is unknown as to why daidzein and not genistein leads to death (Figure 1). Defining orphan receptor ligands is crucial to the creation of therapeutics. Receptors have a certain specificity for their ligands which prevents molecules that do not have the same structure as the preferred ligand from ever binding. This specificity is created by the amino acids located within the active ligand-binding site of the receptor. These amino acids vary in their side chain structures. The side chain structures can exist as carbon rings, bases, acids, etc. Each of these structures play a role in the interactions with the ligand and within the protein itself. The ligand-binding site has a few important amino acids that directly correlate with ligand binding. Mutations to these amino acids can render a receptor unfunctional since the ligand is no longer able to bind. Understanding how isoflavones interact with the ERR on a molecular basis

can provide insight to which amino acids are the most important within the ligand-binding site of the receptor and how these amino acids bind to the ligand. Once this is understood, there are significant benefits in therapeutic design. Such as artificial ligands to either inhibit or activate the receptor to initiate a cell signaling cascade.

The ligand-receptor interaction is only the first step of a cell signaling pathway. Cell signaling cascades can be induced from this interaction, giving rise to the activation of transcription factors and gene expression. The proteins expressed by these genes aid in other cell signaling pathways. Even with functional ligands and receptors, there could be errors within the cell signaling pathway downstream. A mutation in any part of the pathway can result in dysregulation, causing various issues within and amongst the cells of the body. Therefore, it is important to understand the full story behind cell signaling pathways. In this experiment, daidzein-induced larva death can be inhibited by the loss of Akt within the cell via gene mutation (Figure 2). This indicates that Akt plays an important role within the ERR-daidzein signaling pathway; however, the mechanism remains largely unknown. Here, a mutation within the signaling pathway rescued fly larva from daidzein consumption induced death. Further understanding of these biological pathways will help in the discovery and creation of novel therapeutics for those suffering from breast cancer. Certain breast cancer cell lines have been shown to overexpress ERR within the nuclei (Lu *et al.* 2001). By understanding how Akt can transport a ligand to the ERR will help researchers discover the natural ligand for this orphan receptor, allowing a more targeted approach in treating patients with breast cancers overexpressing ERR.

We attempted to confirm Akt's role is this process using Western blot analysis for Akt and activated Akt (pAkt), however were unable. We aid demonstrate that our methodology of protein extraction was viable through Coomassie Blue staining (Figure 3). This was an essential step in determining the necessary amount of larva required to run the Western blot analysis as well as future protein expression experiments. We loaded as much protein as possible within the 50µl well of the gel to get the strongest bands for visualization during the Western blot. Despite loading the maximum amount of protein that the well can hold; the Western blot visualization did not occur and neither Akt nor pAkt were seen (Figure 4). One explanation would be that using ten larvae during the cell lysis and protein extraction was not enough to produce a detectable signal on the Western blot. There is a possibility that *Drosophila* larva cells contain very low concentrations of both Akt and pAkt proteins. More larvae added to the cell lysis buffer would result in having a

higher protein concentration with the stock samples allowing for more protein to get loaded on the gel. One of the limiting factors to this experiment was the well size. We were restricted to using this type of well due to the supply we had on hand as well as the protein gel electrophoresis machinery we used. Another potential factor that could have led to the results seen would be antibody concentrations. In the future, the concentration of primary antibodies may have to be adjusted to detect a signal. The use of an alkaline phosphatase system also contains other inherent difficulties when being used. For example, some laboratories utilize other visualization methods that allow for the stripping of antibodies after visualization so that another type of antibody can be added to the same membrane and visualize a different protein; this is not possible with our current system. Therefore, we used multiple blots for the total Akt and pAkt which can allow error into the data due to pipetting errors.

This research expounds upon the validity of using *Drosophila* as a model organism. Their ease of use and cheap maintenance provide excellent advantages over many other organisms. Having their genome sequenced allow specific editing and manipulation of certain aspects to test various hypotheses. The protein homology between mammals and fruit fly models is astounding and sheds light on cell signaling mechanisms at the cellular level. Despite physical differences and size, fruit flies and mammals both have cells that operate in similar fashions. For example, both fruit flies and mammals have specialized and ciliated cells lining their digestive tracts to aid in food and debris movement as well as nutrient absorption.

The first initial future direction would be to run another Western blot with a higher concentration of protein. An initial test would be done to determine how many more larvae are required to increase the signal. Once various amounts of larvae are lysed, and their proteins quantified, another Coomassie Blue stain would occur after running the lysates through a SDS PAGE. We would utilize the most concentrated samples in the experimental Western blot analysis to make sure that there are enough proteins (namely Akt and pAkt) that the antibodies would be able to react and allow for visualization. After confirming what the expression levels of these proteins, another future direction would be to investigate the interactions between Akt and daidzein directly. It is unknown whether there is a direct interaction between the two or if Akt phosphorylates and activates an additional unknown protein which then directly interacts with daidzein transporting it to the nucleus.

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