Examining the Effects of Resveratrol and Other Stilbenes on Stress Granules

A Major Qualifying Project

Submitted to the Faculty of

Worcester Polytechnic Institute

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in

Biology and Biotechnology

Submitted by:

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Date: November 4th, 2021

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Abstract

When eukaryotes experience a stressor they produce stress granules due to the activation of the cellular stress response pathway via the phosphorylation of eIF2α. These stress granules are formed in response to the inhibition of mRNA translation which allows for the conservation of energy and resources that can be used in stress response pathways. Utilizing acute exposure assays and fluorescent microscopy, stress granule formation was examined in U2OS-DS and U2OS-WT cell lines that were treated with acute levels of Resveratrol and other stilbene compounds. It was found that Resveratrol, Piceatannol, Oxyresveratrol, and Pinosylvin cause stress granule formation. Piceatannol was shown to be significantly less efficient in producing stress granules, while Pinosylvin exhibited significantly more stress granule formation compared to the other stilbenes.

Acknowledgements

Professor Natalie Farny For her continuous guidance and encouragement throughout the duration of the project. Her passion and knowledge on the subject were truly inspiring and has sincerely helped me grow as a biologist. I am so thankful to have worked with such a patient and dedicated advisor, without which the research goals met in this paper could not have been fulfilled.

Dr. Nancy Kedersha and Dr. Pavel Ivanov of Brigham and Women's Hospital for donating the U2OS-DS and U2OS-WT cell lines, without which the project would not have been possible.

Worcester Polytechnic Institute Department of Biology and Biotechnology for providing the space, supplies, and equipment.

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Background

Cellular Stress Response

Eukaryotes possess an essential mechanism that allows for them to identify, adapt, and overcome an array of stressors. These stressors could include temperature shock, protein unfolding, toxic chemical exposure, or physical injury[8]. The mechanism eukaryotes utilize to combat these stressors is known as the cellular stress response (CSR), which elicits a variety of pathways depending upon the specific stressor and exposure conditions[8]. When the cellular stress response is triggered, the cell will temporarily halt cell growth and protein synthesis in order to conserve energy and redistribute resources which can then be used to address the stress condition[14]. If the cell has undergone a lethal stimulus for a prolonged period of time, and the cell growth and translational arrest was not enough to alleviate the stress, the cell will ultimately undergo apoptosis or cell death.

The CSR is an adaptable mechanism in that it can address problems depending on the period of time needed to repair the impacted cell. For situations in which a shorter response is required, the CSR will work towards repairing damaged macromolecules[14]. For longer CSR responses, the cell will spend significantly more time ensuring that cellular homeostasis has been restored[14]. When it comes to the short-term CRS response the first step is to halt mRNA translation and inhibit cell growth, which in turn stimulates the formation of cytoplasmic aggregates known as stress granules[2].

Stress Granules

Stress granules (SG) are cytoplasmic aggregates made up of non-translating messenger ribonucleoprotein complexes (mRNPs) that form as a result of the termination of translation in response to a stressor[2]. Along with mRNPs, stress granules also contain specific proteins that are involved in the cellular stress response such as the protein G3BP1[18]. Analysis has shown the stress granules are comprised of two phases: an outer less concentrated shell and internal stable core structures[3]. These two phases are formed as the mRNA from inhibited translation is released from the polysomes and packed into the SGs. Along with the mRNA, corresponding translational proteins are organized into the stress granules as well[3].

Phosphorylation of eukaryotic translation initiation factor 2 (eIF2α) is one of the most common mechanisms for activating stress granule formation[5]. eIF2α is responsible for the delivery of initiator tRNAs to pre-initiation complexes, which is required for mRNA translation

initiation. When the cell is undergoing stress, specialized kinases are activated and phosphorylate serine 51 (S51) of eIF2α, which in turn inhibits translation and cell growth[3]. eIF2α is also involved in minor initiation events including re-initiation, internal initiation, and non-AUG initiation, which are critical for translational control of specific mRNAs[13]. Through this inhibition of translation, a sudden accumulation of mRNAs occurs in the cytoplasm, thus leading to the formation of stress granules. Only four kinases have the ability to phosphorylate serine 51 of eIF2α: Heme-regulated eIF2a kinase (HRI), Double-stranded RNA-dependent protein kinase (PKR), General control non-depressible kinase 2 (GCN2), and PKR-like endoplasmic reticulum kinase (PERK)[7]. Slightly different stress granule compositions are formed depending on the stressor and which of the four pathways was activated. Distinct stressors that activate these pathways include virus infection, heat shock, iron deficiency, nutrient deprivation, changes in intracellular calcium, accumulation of denatured proteins, and induced apoptosis[5].

Stress granules are thought to be protective and conserve energy, which allows the cell to redirect that energy into stress response pathways downstream. As shown in Figure 1 below, stress granules appear as tiny fluorescent dots scattered throughout the cytoplasm.

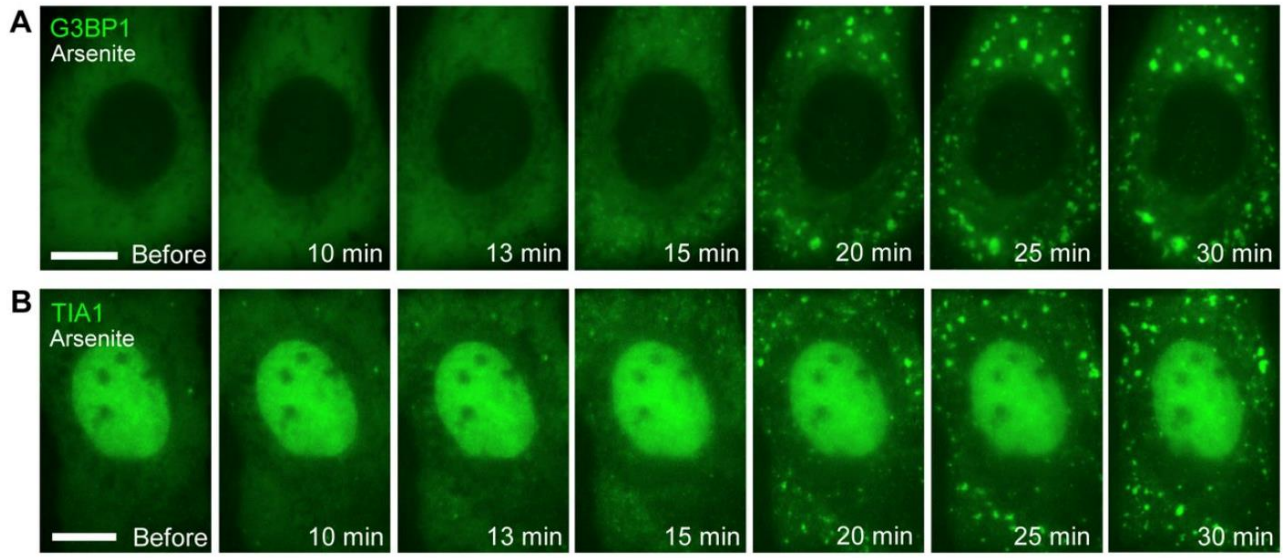

Figure 1: Example of Stress Granule Formation with Arsenite[29]

Resveratrol

The cellular stress response can be triggered by a multitude of different stressors. Resveratrol (E-5-(4-hydroxystyryl)benzene-1,3-diol) is a stilbene that has been shown at different concentrations to interact with the critical stress granule protein G3BP1 to induce stress

granules[1]. As shown in Figure 2A below, resveratrol is comprised of two phenol rings connected by an ethylene bridge with hydroxyl group substituents, and it is primarily found in a trans conformation rather than cis[20]. It is most commonly found in the skin of grapes and nuts, as well as a large variety of plant species[4]. Resveratrol is found in an abundant number of plants because it is considered to be a phytoalexin. Phytoalexins are compounds produced by plants in response to stressors such as mechanical or chemical injury[4].

Many claims have been made about the benefits of resveratrol and its ability to be a multitasking compound. Resveratrol has been shown to be anti-inflammatory, anticarcinogenic, cardioprotective, and neuroprotective[20]. It was found in one study performed on mice that resveratrol improved cardiovascular function, reduced hypertension through its vasorelaxant ability, and improve myocardial Ca^{2+} handling[24]. In terms of its neuroprotective ability, resveratrol has been shown to play several roles in neurodegenerative diseases such as Alzheimer's and Parkinson's by improving mitochondrial function and reducing damage caused by oxidative stress[21].

In addition to these findings resveratrol is highly touted for its antioxidant ability[10]. The exact mechanism for how resveratrol acts as an antioxidant is still being researched, however it was discovered that the addition of resveratrol significantly increases the activity of the protein Sirt 1[10]. Sirt 1 is part of the sirtuin family, which are NAD+ dependent enzymes that play multiple roles in the cell especially in the activation of stress resistance pathways. As seen in Figure 3, the increase in Sirt1 activity due to the addition of resveratrol has a multitude of impacts on the body and has overall been shown to increase lifespan extension in mice, fruit flies, and yeast[9]. Currently resveratrol skincare and supplements are available, however due to the low bioavailability of the compound and the need for more conclusive research, the many claims about resveratrol still need to be substantiated.

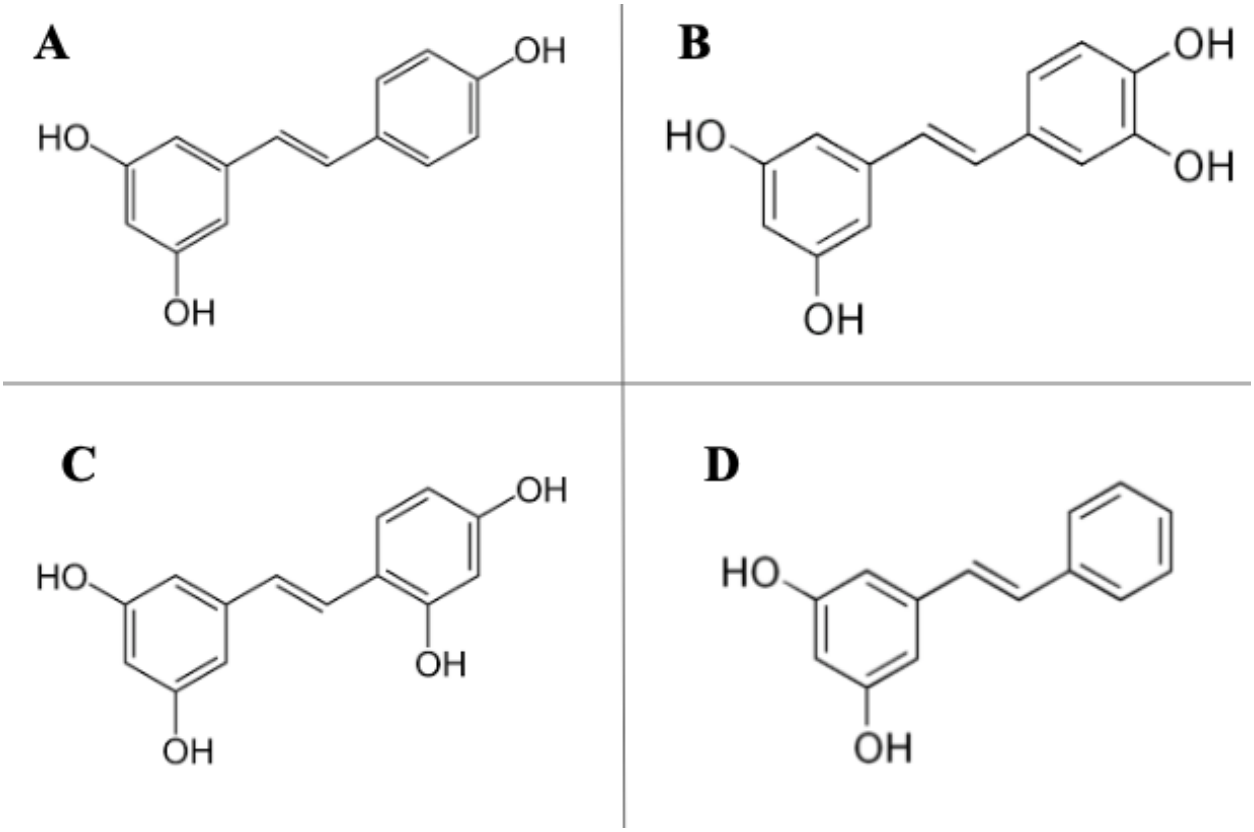

Figure 2: Chemical Structures of Stilbenes (A) Resveratrol[28], (B) Piceatannol[26], (C) Oxyresveratrol[25], (D) Pinosylvin[27]

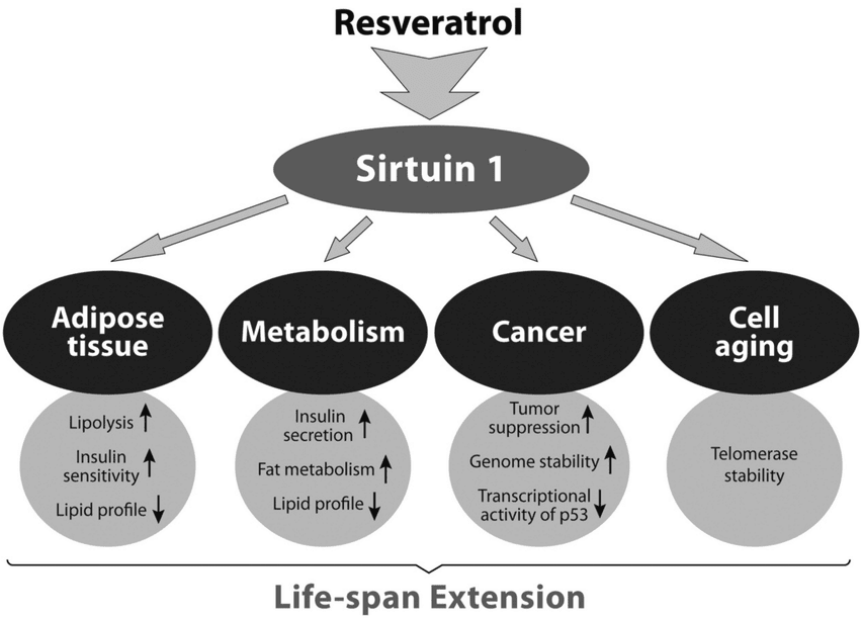

Figure 3: Resveratrol and Sirtuin 1 Pathways[19]

Resveratrol and Cancer

Looking further at resveratrol's anticarcinogenic ability, it has been shown to have strong anticancer potential because it inhibits all three carcinogenic stages of initiation, promotion, and progression[20]. Resveratrol is able to do this by indirectly activating p53, which triggers apoptosis[20]. p53 is a well-known tumor suppressor that induces apoptosis or cell death, and mutations of p53 are common among all cancers[16]. Resveratrol is able to aid in the induction of apoptosis through its interaction with the stress granule protein G3BP1[20]. G3BP1 is considered a prominent cancer protein as it has been found to be overexpressed in several human cancers including head, neck, breast, and colon cancer[30]. G3BP1 plays a role in the proliferation of cancer cells by negatively regulating p53 expression through its interaction with the protein USP10[17]. USP10 is a de-ubiquitinating protein that activates p53 in order to induce apoptosis. Essentially USP10 deubiquitinates and activates p53, which allows it to perform its function by inducing apoptosis. However when G3BP1 is introduced, it prevents USP10 from de-ubiquitinating p53, therefore keeping p53 inactive and allowing the mutated cell to spread and proliferate. One study found however that when resveratrol was introduced it would interact with a domain on the G3BP1 protein shown in Figure 4 and prevent G3BP1 from interacting and inhibiting USP10. Therefore, by preventing G3BP1 from inhibiting USP10, resveratrol helps ensure the activation of p53 and induction of apoptosis, which is incredibly significant in the field of cancer therapy[17].

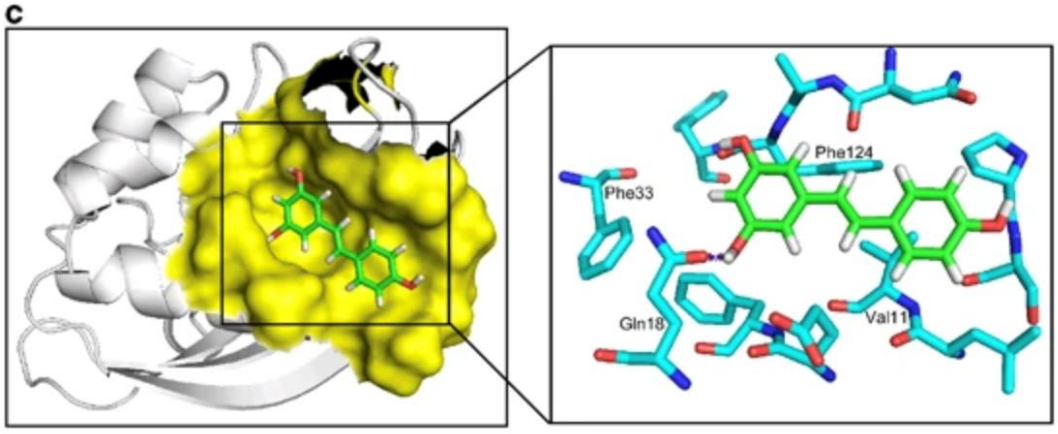

Figure 4: Image of the G3BP1 Domain that Resveratrol Interacts with to Inhibit G3BP1[17]

Other Stilbenes

While resveratrol has many impactful effects on the body, its low bioavailability makes wide distribution difficult, and therefore research on other stilbenes is essential. Therefore, analysis of the abilities of other stilbenes to bind G3BP1 or form stress granules is an important next step for this area of research. It remains unknown whether the ability of resveratrol to cause stress granules is unique to resveratrol itself or is a common feature of stilbene compounds. In this work, we examine three other stilbenes including Piceatannol, Oxyresveratrol, and Pinosylvin, for their ability to cause stress granules. All of the stilbenes have the same basic backbone of two phenol rings connected by an ethylene bridge; however the placement of the hydroxyl groups is what set them apart from one another. The structures of each stilbene can be seen in Figure 2B, 2C, and 2D.

Our collaborators at Harvard Medical School discovered that at specific concentrations (100µM, 250µM, and 500µM), resveratrol had the ability to produce stress granules in cells (S. Hofmann and P. Ivanov, Brigham and Women's Hospital, personal communication), an observation which was subsequently supported by a new publication from the Kaganovich lab at the University of Goettingen in Germany[1]. This stress granule formation was observed to be linked to the G3BP1 protein. Therefore the aim for this project was to corroborate these recent observations by measuring stress granule formation in osteosarcoma cells when exposed to different concentrations of resveratrol. A second goal of the project was to test other stilbenes Piceatannol, Oxyresveratrol, and Pinosylvin at similar concentrations (100µM, 250µM, and 500µM) to observe whether other stilbenes could cause stress granule formation, and whether the differing placements of the substituent hydroxyl groups impacted the results.

Materials and Methods

Cell Line Maintenance

The cells utilized for the acute exposure assays were double-stable (U2OS-DS) cells containing stable integrations of GFP-G3BP1 and RFP-Dcp1, as well as wild type (U2OS-WT) osteosarcoma cells[11]. These cell lines were a kind gift from Drs. Nancy Kedersha and Pavel Ivanov of Brigham and Women's Hospital, Boston, MA. Cell lines were maintained in a T75 flask using DMEM media with 10% FBS, 1% Penicillin Streptomycin, and 1% Glutamine, and were incubated at 37°C with 5% CO_2. Both the U2OS-DS and U2OS-WT cells were split every

other day, the ratio in which they were split was dependent on the confluence of the cells on that given day (1:4, 1:6 or 1:8). Splitting the cells was vital to ensure that they were not subjected to any growth-related stress before the assays were run.

Acute Exposure Assay

U2OS-DS or U2OS-WT cells were plated in a 12-well plate with coverslips at $1.3x10^5$ cells per well. Each well contained 1mL of master mix, which was composed of the cells and media. To ensure the right concentration of $1.3x10^5$ cells per well, a hemocytometer was used to determine the correct number of cells and media needed. The well plates were then incubated for 24 hours to allow the cells to grow. After 24 hours the drug mixtures were created. These mixtures were made by recovering 0.5mL of media from each well (preconditioned media). This media was then mixed with the respective drug concentrations as shown on the well plates of Figure 5. The stock concentration for all the stilbenes and other drugs employed was 100mM, and the stilbenes were dissolved in ethanol. A concentration of 100µM of arsenite and ethanol were used as positive and negative controls respectively. Once all of the drug mixtures had been created, the remaining media left on the well plates was aspirated off of each well, and 0.5mL of each of the drug mixtures was applied to their respective wells as shown in Figure 5. The plates were then immediately incubated at 37° for 1 hour.

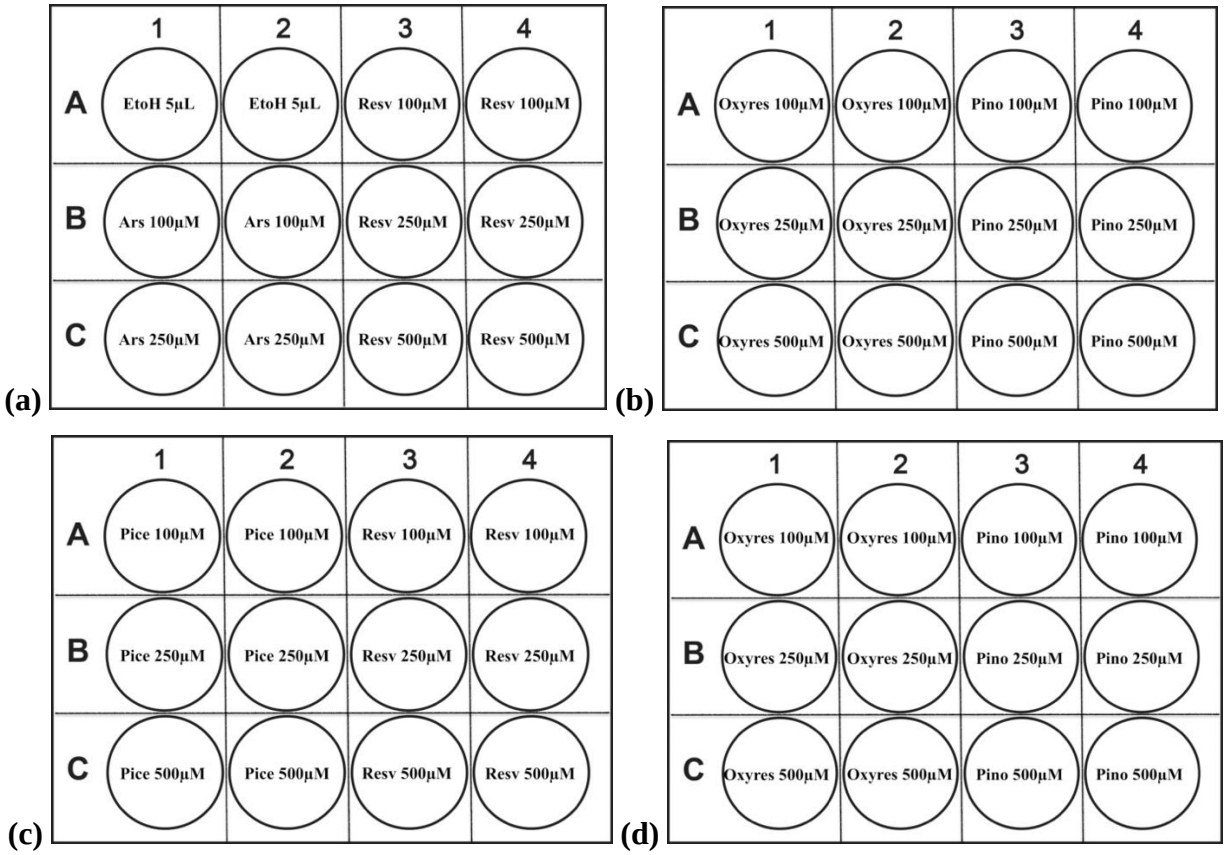

Figure 5: Acute assay exposure plate arrangements used for (a) U2OS-DS cells with resveratrol and controls, (b) U2OS-DS cells with oxyresveratrol and pinosylvin, and (c) U2OS-WT cells with piceatannol and resveratrol (d) U2OS-WT cells with oxyresveratrol and pinosylvin

Once the incubation period had run its course the fixation process began. The media was aspirated off, and all the wells were washed with 1X PBS. The 1X PBS was removed and 0.5mL of 4% paraformaldehyde was applied to each well. The plates were then placed on a shaker at room temperature for 10 minutes. After 10 minutes the 4% paraformaldehyde was removed and discarded into the proper waste container. Then 0.5mL of 100% methanol was added to the wells, and the plates sat on the shaker at room temperature for another 10 minutes. The 100% methanol was removed and placed into a designated waste container. The wells were washed one more time with 1X PBS, and then after any staining procedures that needed to be performed (see staining procedure below) the coverslips were mounted on glass slides with polyvinol mounting medium, 2 coverslips per slide.

Staining Cells for Fluorescence Microscopy

Following the completion of the acute exposure assay and fixing the U2OS-WT cells with 4% paraformaldehyde and 100% methanol, each well was treated with 0.5mL of 5% bovine serum albumin (BSA) in PBS blocking solution. The plates were then set on the shaker for 1 hour at room temperature. The blocking solution was removed from all wells, and the primary antibody solution (Table 1) was added to each well. The plates were placed on the shaker for 1 hour at room temperature. The primary antibody solution was removed, and the plates were washed three times with 1X PBS, allowing them to sit 5 minutes in between each wash. After the third wash the secondary antibody solution (Table 1) was applied to each well. The plates were then covered with foil and placed on the shaker for 1 hour at room temperature. The secondary antibody solution was removed, and the plates were wash a final three times with 1X PBS, waiting five minutes in between each wash. The coverslips were then mounted using the same procedure as described above.

Microscopy Analysis and Imaging

The percentage of cells positive for stress granules was calculated based on manual counts of fluorescence microscopy. The slides were blinded in order to reduce bias and viewed under a Nikon AxioObserverA1 inverted fluorescence microscope at 40X magnification. The criteria for counting stress granules involved counting a minimum of 250 cells, observing a minimum of 3 fields per sample, and only cells that were fully visible within the field were counted. The results were collected and analyzed using Microsoft Excel, and the statistics were calculated using VasserStats (available from http://vassarstats.net/anova1u.html). Within each stilbene concentration, a one-way ANOVA followed by Tukey posthoc testing was performed to look for differences between compounds at the same concentration. ANOVA results are available in Appendix A. A CMOS camera that was controlled by MicroManager open-source software was utilized to take representative images of the slides. These images were then processed using ImageJ software.

Table 1: Antibodies and Dilutions Used in this Study

Antibody	Application	Dilution	Manufacturer	Product Number
Rabbit -anti-G3BP	Immunofluorescence primary stain	1:2000	AbCam	Ab181150
Anti-rabbit IgG Alexa Fluor RT 488 (green) stain	Immunofluorescence secondary stain	1:1000	Cell Signaling Technology	4412S
Hoschst 3342 nuclear stain	Immunofluorescence secondary stain	1:5000	Life Technologies	1642791

Results

Acute Exposure of U2OS-DS Cells to Stilbenes Causes Stress Granules

In order to determine whether the stilbenes resveratrol, oxyresveratrol, and pinosylvin had the ability to form stress granules, an acute exposure assay was performed on U2OS-DS cells. Arsenite was used as a positive control and ethanol was used as a negative control. As seen in the positive and negative control experiment in Figure 6, when viewed under the fluorescence microscope, stress granule would fluoresce as tiny dots throughout the cytoplasm of the cells (Fig. 6B and 6C). Cells that did not contain any stress granules would appear like the cells in Figure 6A with no punctate fluorescence in the cytoplasm. This distinction was utilized when counting the number of cells that contained stress granules for each stilbene treatment condition.

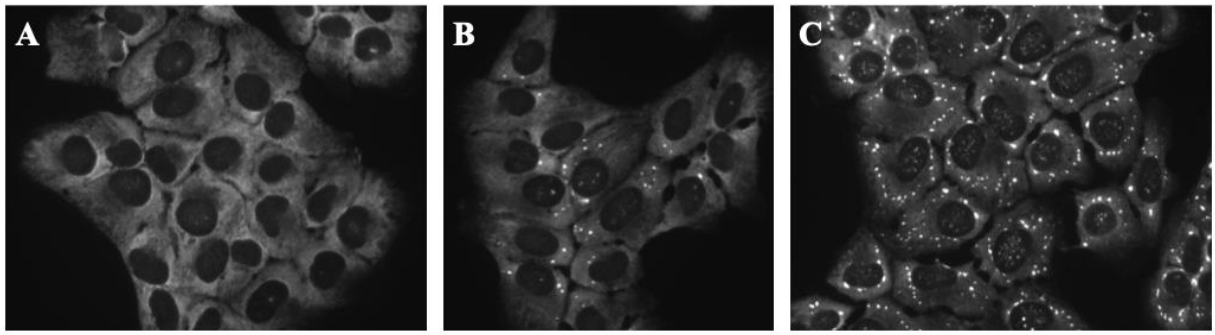

Figure 6: Composite images of U2OS-WT cells viewed at 40X magnification on the green channel of a fluorescence microscope, following a 1-hour treatment with (A) Ethanol 5µL, (B) Arsenite 100µM, and (C) Arsenite 250µM. The white dots in panels B and C represent individual stress granules.

To examine stress granule formation in response to stilbene compounds, three different concentrations of each stilbene were applied to the U2OS-DS cells for one hour. These concentrations specifically used were 100µM, 250µM, and 500µM. The combined results from the acute exposure comparison assays with U2OS-DS cells can be seen in Figure 7. The average stress granule formation for each stilbene concentration was plotted with error bars that represent standard error of mean (+/-S.E.M.) (n=6). For each of the concentrations shown in Figure 7 there are broad similarities in stress granule formation across all three of the stilbenes tested. The 100µM concentration never produced above 10% stress granule formation for any of the stilbenes. The 250µM and 500µM concentrations appeared to have higher variances among the stilbenes, fluctuating anywhere between a 10-20% difference in the amount of stress granules formed. Looking closer at Figure 7 the disparities in the different error bar lengths can be observed. Specifically the 250µM concentration proved to be particularly inconsistent among all three stilbenes tested, as revealed by the large error bars. This fluctuation can be further observed by examining the individual replicate data for ethanol, arsenite, resveratrol, oxyresveratrol, and pinosylvin (Figures 8 and 9). Looking closely at these two figures it is evident that towards the final replicates of the experiment the data increasingly became more variable and less reproducible, in some cases producing an abundance of stress granules and in other cases producing none at all.

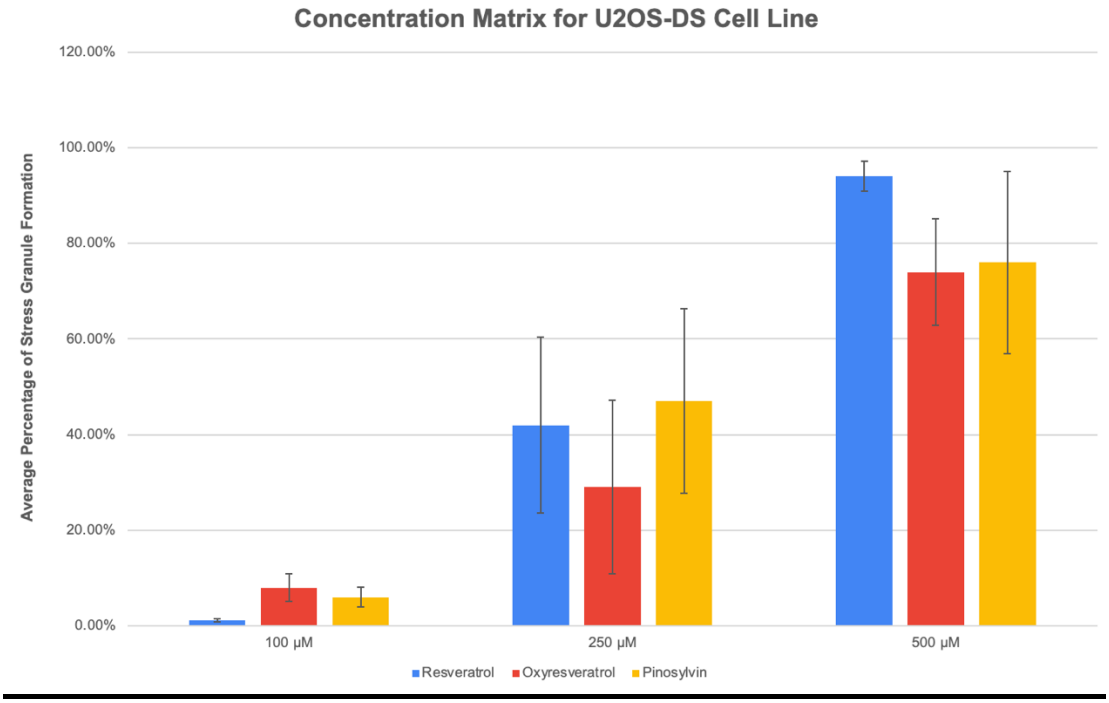

Figure 7: Stress granule formation in U2OS-DS cells when treated with resveratrol, oxyresveratrol, and pinosylvin. The three concentrations tested were 100µM, 250µM, and 500µM. Cells were treated with these compounds and incubated at 37C° for 1 hour. After 1 hour the cells were fixed and then stained with rabbit anti-G3BP1 antibody. Error bars represent standard error of mean (+/- S.E.M.) (n=6). Error bars are (+/- S.E.M.) in all of the following figures.

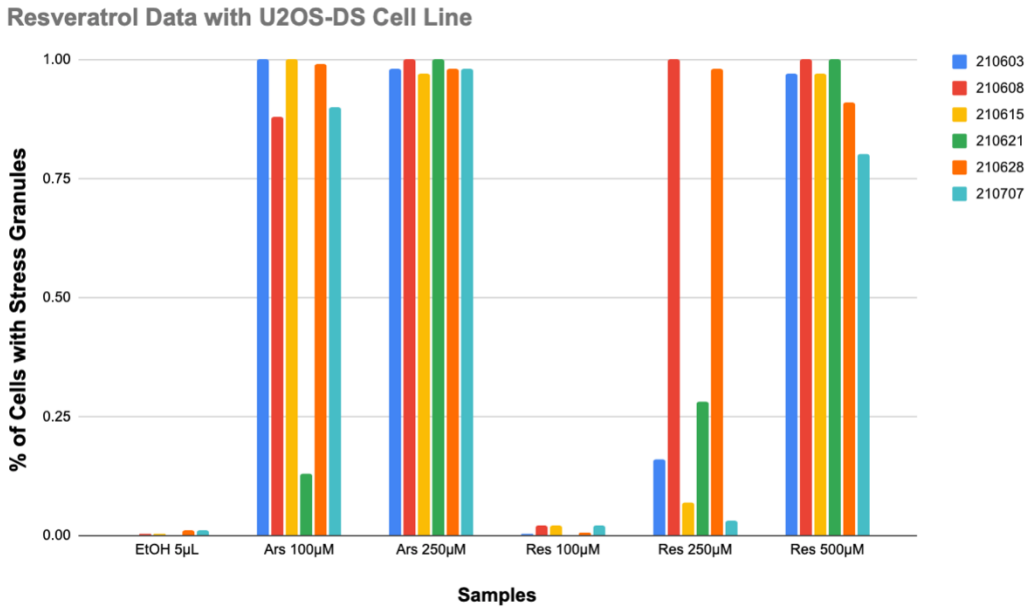

Figure 8: Stress granule formation in U2OS-DS cells when subjected to different concentrations of resveratrol, (100µM, 250µM, and 500µM). The negative control used was 5µL of ethanol, and the positive controls were

100µM and 250µM of arsenite. Cells were treated with these compounds and incubated at 37C° for 1 hour. Each of the six experimental replicates (averaged in Figure 7) are represented separately here.

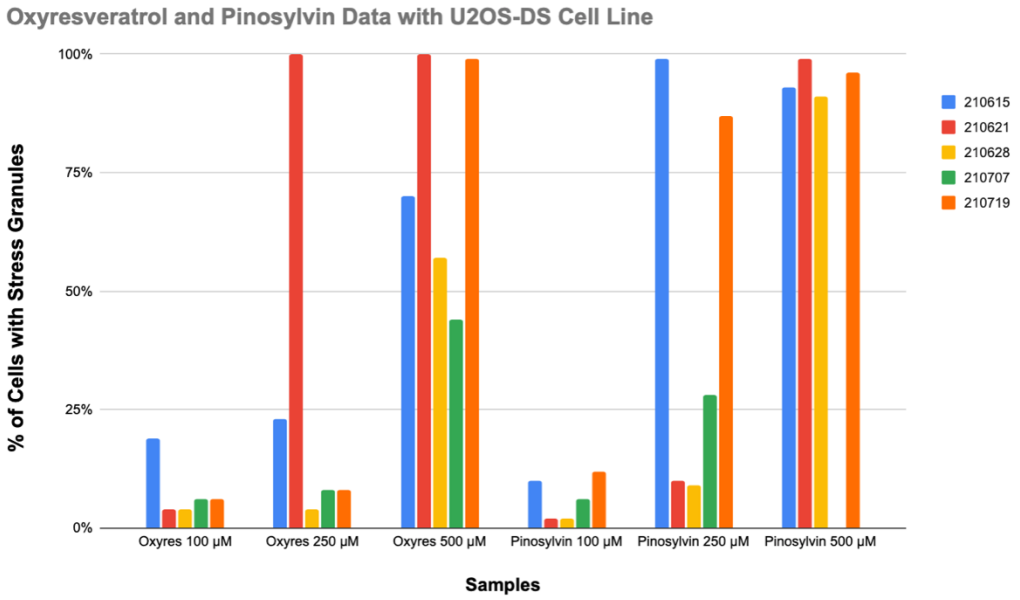

Figure 9: Stress granule formation in U2OS-DS cells when subjected to different concentrations of the homolog stilbenes oxyresveratrol and pinosylvin. The concentrations tested for both compounds were 100µM, 250µM, and 500µM. Cells were treated with these compounds and incubated at 37C° for 1 hour. Each of the six experimental replicates (averaged in Figure 7) are represented separately here.

Acute Exposure Comparison Assay with U2OS-WT Cell Line

It was observed that the G3BP signal of the U2OS-DS cells (used in Figures 7, 8, and 9) was beginning to fade after the cells had been split many times. This fading meant that not all of the cells were fluorescing, even if they contained stress granules. This lack of fluorescence disrupted the ability to count the stress granules accurately. Therefore another approach was taken. This procedure involved performing another acute exposure comparison assay using U2OS-WT cells instead of U2OS-DS cells and staining with rabbit antibodies to G3BP1 shown in Table 1. The positive and negative controls for this experiment were arsenite and ethanol as shown in Figure 13. The stilbenes tested were piceatannol, resveratrol, oxyresveratrol, and pinosylvin. The same three concentrations of 100µM, 250µM, and 500µM were tested for each stilbene. A summary of the averages for this acute exposure assay can be seen in Figure 10 where the average stress granule formation of each stilbene concentration was plotted with error bars that represent standard error of mean (+/-S.E.M.) (n=4). When comparing the result error

bars displayed in Figure 10 to Figure 7 it is clear that the manual staining of the U2OS-WT cells provided much more accurate and reproducible results than the U2OS-DS acute exposure assay.

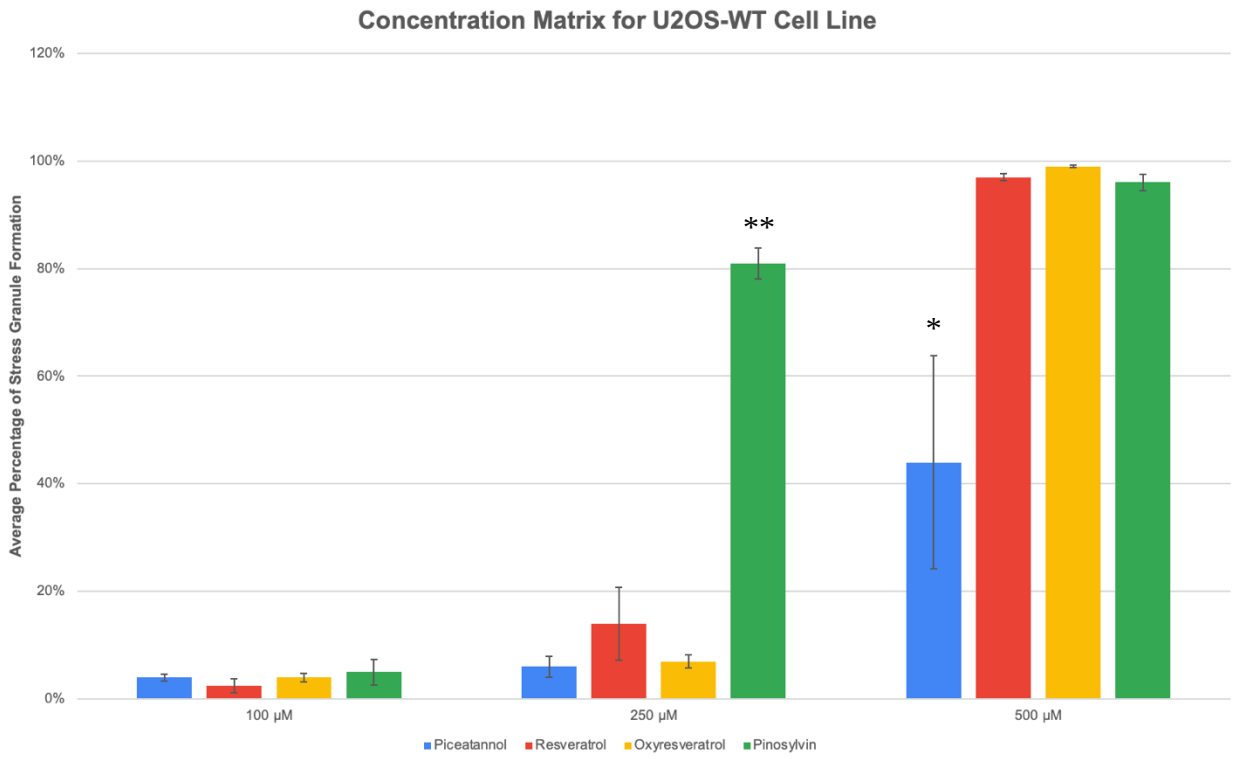

Figure 10: Concentration matrix for stress granule formation in U2OS-WT cells when treated with piceatannol, resveratrol, oxyresveratrol, and pinosylvin. The three concentrations tested were 100µM, 250µM, and 500µM. Cells were treated with these compounds and incubated at 37C° for 1 hour. After 1 hour the cells were fixed and then stained with rabbit antibody. Error bars represent standard error of mean (+/- S.E.M.) (n=4). ANOVA (one-way within concentration condition), P values for Tukey posthoc test, * $p < 0.05$; ** $p < 0.01$ compared to other stilbenes at the same concentration.

As seen in Figure 10 the 100µM concentration had consistent results, and no signficiant difference among all four stilbenes (ANOVA, P=0.689) where little to no stress granules were formed and the amount never reached above 10%. Looking further at the 250µM there were different outcomes based on the stilbene tested. The major variation from this concentration was that pinosylvin produced significantly more stress granules than the three remaining stilbenes (ANOVA, P<0.0001), as the pinosylvin produced almost as many stress granules as it did at the 500µM concentration. Piceatannol tended to have similar results at 250µM as it did at 100µM where it was less than 10%. Resveratrol had greater stress granule formation at the 250µM concentration, however the difference was not statistically significant. This result is consistent

with the data from the first acute exposure assay shown in Figure 7 where resveratrol fluctuated often between high and low stress granule formation at the 250µM concentration. Oxyresveratrol had a comparable outcome as piceatannol where the 250µM stress granule formation was similar to the 100µM concentration, hovering around 10% mark. Finally at the 500µM concentration it is apparent that resveratrol, oxyresveratrol, and pinosylvin all approached 100% stress granule formation, while piceatannol was significantly lower and averaged around 50% stress granule formation (ANOVA P=0.005). This data is further corroborated in Figures 11 and 12 which show the individual replicate data for each stilbene. As shown in Figure 12, piceatannol would vary highly, between 0-75% stress granule formation at 500µM, explaining the large error bar in Figure 10. Aside from the piceatannol 500µM results, when comparing Figure 11 and 12 to the graphs in Figures 8 and 9 the results are far more consistent and replicable for the U2OS-WT cells.

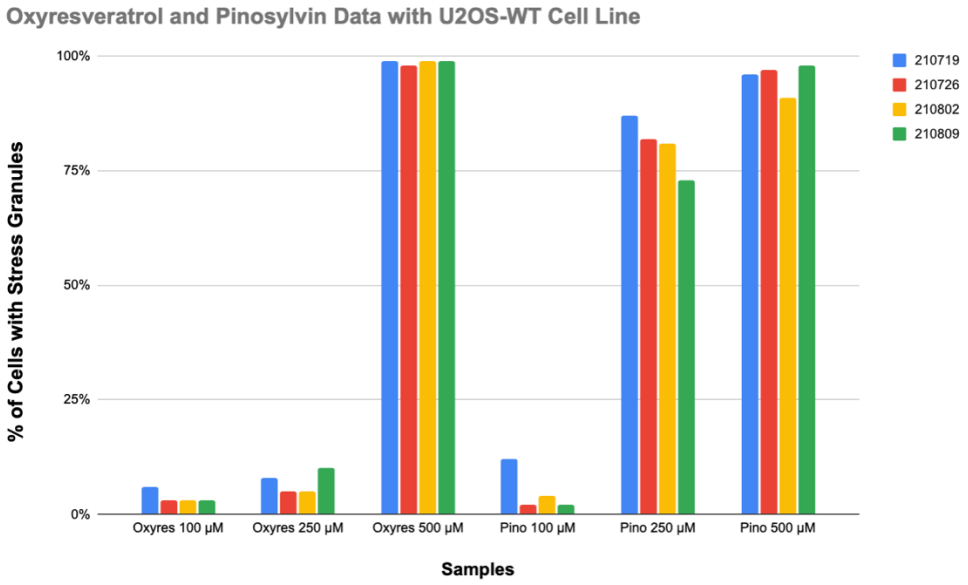

Figure 11: Stress granule formation in U2OS-WT cells when subjected to different concentrations of the homolog stilbenes oxyresveratrol and pinosylvin. The concentrations specifically tested for both compounds included 100µM, 250µM, and 500µM. Cells were treated with these compounds and incubated at 37C° for 1 hour. After 1 hour the cells were fixed and then stained with rabbit anti-G3BP1 antibody.

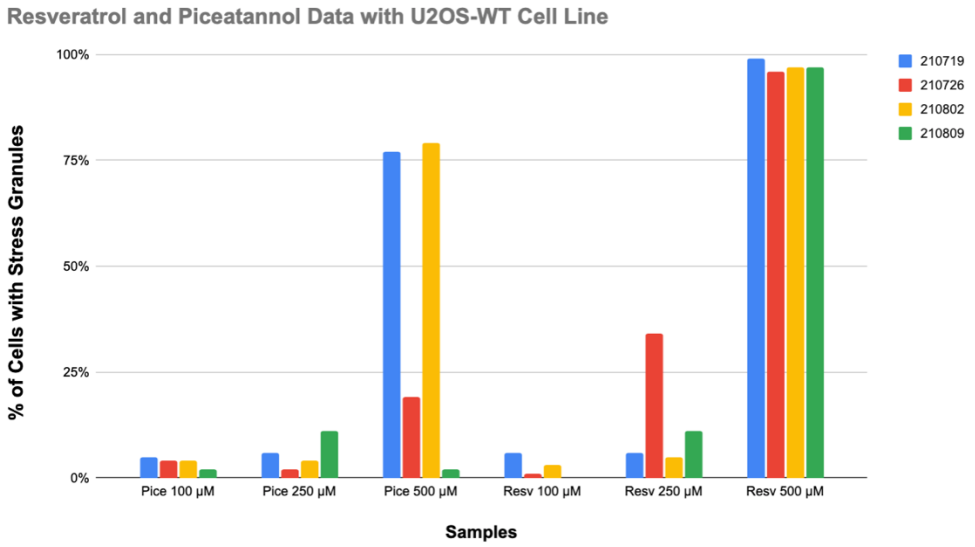

Figure 12: Stress granule formation in U2OS-WT cells when subjected to different concentrations of the resveratrol and the stilbene piceatannol. The concentrations specifically tested for both compounds included 100µM, 250µM, and 500µM. Cells were treated with these compounds and incubated at 37C° for 1 hour. After 1 hour the cells were fixed and then stained with rabbit anti-G3BP1 antibody.

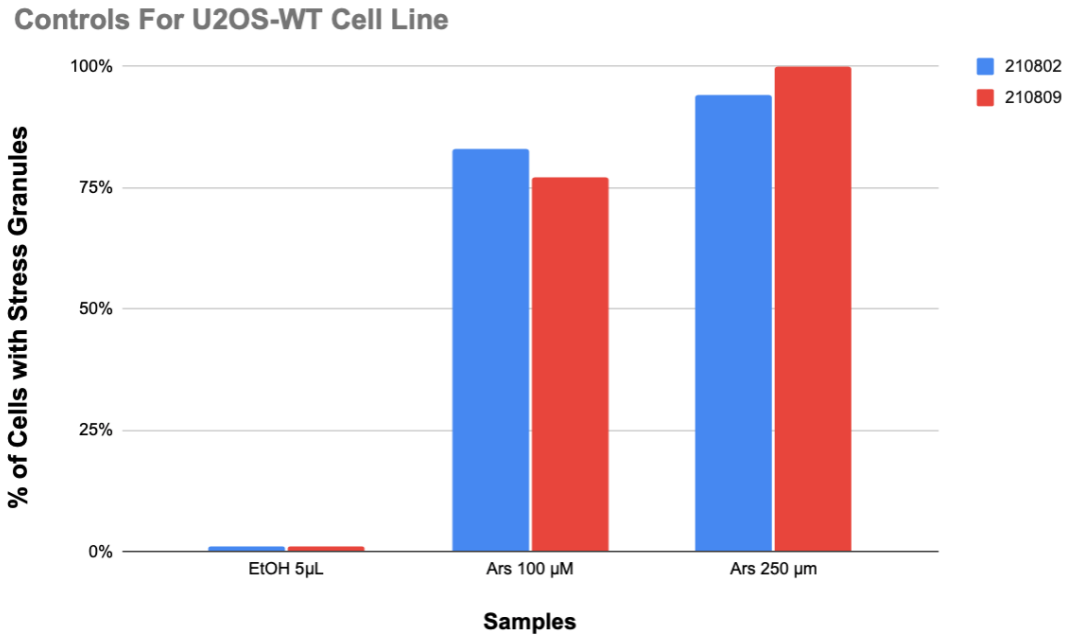

Figure 13: Stress granule formation in U2OS-WT cells when subjected to positive and negative controls. The negative control used was 5µL of ethanol, and the positive controls were 100µM and 250µM of arsenite. Cells were treated with these compounds and incubated at 37C° for 1 hour. After 1 hour the cells were fixed and then stained with rabbit anti-G3BP1 antibody.

Representative images of U2OS-WT cells treated with the four stilbene compounds are shown in Figure 14. These images represent the gradual process of stress granule formation dependent on the stilbene and the concentration applied. As shown, resveratrol and oxyresveratrol follow a similar pattern of having little to no stress granules at 100µM and 250µM, but a large number of granules at 500µM. Pinosylvin also shows no stress granule formation at 100µM but has a great deal of granules at 250µM and 500µM. Lastly piceatannol shows the least amount of stress granule formation with little to none in the 100µM and 250µM concentrations, and then only a fraction of stress granules formed at 500µM.

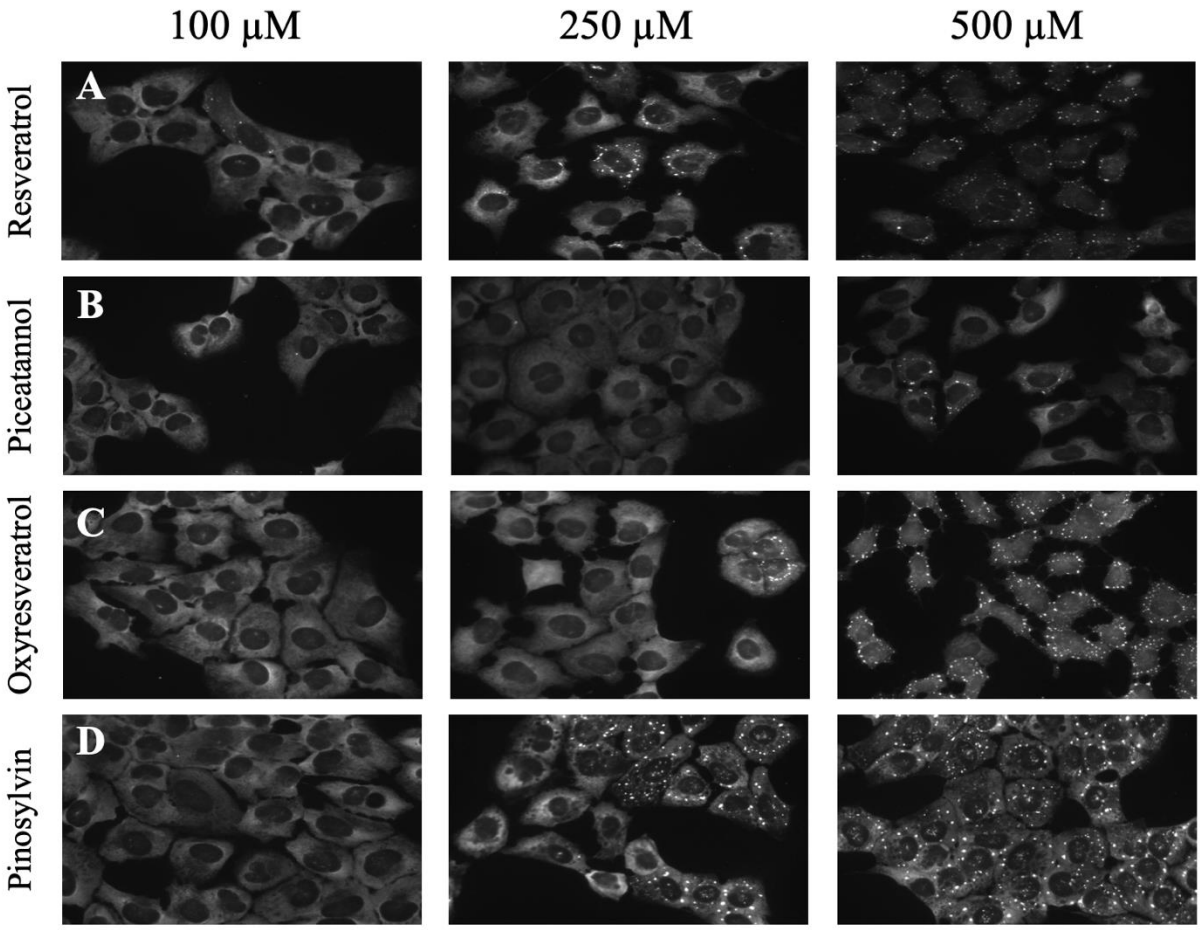

Figure 10: Composite images of U2OS-WT cells viewed at 40X magnification on the green channel of a florescence microscope, following a 1-hour treatment with (A) Resveratrol, (B) Piceatannol, (C) Oxyresveratrol, and (D) Pinosylvin. The cells were subjected to three concentrations for each compound, specifically 100µM, 250µM, and 500µM. The white dots represent individual stress granules.

Discussion

U2OS-DS Acute Exposure Assay

When observing the data collected in the U2OS-DS assays it could be seen how all three stilbenes tested possessed the ability to produce stress granules. Based upon the three concentrations tested it was concluded that experiments performed between 100µM to 500µM provided promising results to encapsulate the range in which the stilbenes would produce no stress granules to almost 100% stress granule formation. While there did appear to be trends and similarities among the three stilbenes, conclusive comparisons could not be made. These conclusions could not be drawn due to the high statistical differences among each of the stilbene trials. The large error bars for each of the stilbenes in the U2OS-DS experiments demonstrate how the data was not considered compelling. These variations were also taken seriously seeing as six replicates were performed for the experiment, yet there were still large discrepancies for all three stilbenes. It was speculated that after splitting the U2OS-DS cells repeatedly the fluorescent G3BP1 marker had begun to dissociate from the cell line and was no longer marking all the cells that had stress granules. This loss of GFP-G3BP1 may have affected the accuracy of scoring, which led to highly variable results.

Resveratrol in Acute Exposure Assay with U2OS-WT Cells and Involvement in the eIF2α Pathway

Based upon the results from the acute exposure assay with U2OS-WT cells, the work conducted by our collaborators at Harvard Medical School (S. Hofmann and P. Ivanov, Brigham and Women's Hospital, personal communication) could be corroborated. Resveratrol was found to have a slight amount of stress granule formation at 100µM, and at 500µM had the capacity to create approximately 100% stress granule formation. However the 250µM trials produced larger inconsistencies in stress granule formation. For some of the 250µM trials stress granule formation was above 10%, while other trials remained around 5%. This result was also observed in the initial U2OS-DS assay data, suggesting that this outcome would remain persistent if more trials had been conducted. These fluctuating results for the 250µM resveratrol trials could be due to multiple factors, whether it be human error in measuring and preparing the master mix, or if the aliquot being used was slightly stronger or weaker than the previous replicate. Overall, the results support the hypothesis that resveratrol is able to produce stress granules in cells, and a concentration of 500µM is ideal for a level of 100% stress granule formation.

Taking a further look at the results from the U2OS-WT acute exposure assay, the eIF2α pathway that resveratrol participates in could also be speculated upon. The research by the Kaganovich lab showed a small amount of eIF2α phosphorylation in response to resveratrol and piceatannol treatment[1]. It remains unknown, however, whether this phosphorylation is essential for resveratrol stress granules. While resveratrol has the possibility to engage in any of the four kinase pathways, if the process is eIF2α -dependent then the PERK pathway appears to be the most likely contender. The PERK pathway is principally activated by the accumulation of misfolded proteins in the endoplasmic reticulum (ER)[6]. Being an ER transmembrane protein, PERK initiates the eIF2α pathway in order to allow the ER to repair the misfolded proteins. PERK has also recently been shown to play a role in limiting oxidative stress in cells by upregulating compounds with antioxidant and detoxifying abilities[6]. Resveratrol, being a potent antioxidant, could be one of the compounds that triggers the PERK pathway and induces stress granule formation. The PKR pathway is also another possibility as it also is triggered by ER and oxidative stress[6]. However the PKR pathway is primarily activated in response to viral infection, so its link to resveratrol may be limited. It is unlikely that resveratrol triggers the GCN2 pathway as this pathway is focused on amino acid availability and regulation[23]. Lastly, the HRI pathway helps maintain erythroid precursor cells and heme levels in the blood, which is not as likely to be activated by resveratrol compared to other pathways[23]. Further research must be conducted in order to address these speculations in order to distinctly prove which kinase pathways, if any at all, are activated by resveratrol.

Pinosylvin in Acute Exposure Assay with U2OS-WT Cells

Pinosylvin followed the same trends as the other stilbenes in which there were few stress granules formed at 100µM, and widespread stress granule formation at 500µM. However this is where the similarities end when looking at the U2OS-WT data as Pinosylvin produced significantly more stress granules, averaging around 75% formation, at 250µM compared to the other stilbenes. This increased amount suggests that there is a certain characteristic that Pinosylvin possesses such as its structure or function that induces stress granule formation at a lower dose than other stilbenes. The structure could be the chief reason for this, as the placement of the substituent hydroxyl groups differs from the other stilbenes tested. Pinosylvin consists of two hydroxyl substituent groups, both located on one of the phenol groups[27]. Comparatively

oxyresveratrol and piceatannol both consist of four hydroxyl groups, two located on each phenol group[25,26]. Resveratrol has three hydroxyl groups, one located on the right phenol group and two located on the left phenol group[28]. Pinosylvin is predominantly found in the trans conformation just as the other three stilbenes tested are, therefore it can be assumed that the confirmation of the compound is not the main reason for the increase in stress granule formation at a lower concentration[22]. Prior research from the Farny lab noted a similar pattern when examining bisphenol-induced stress granules[7]. It was observed that bisphenol-A was significantly more efficient at triggering stress granule formation than bisphenol-F, even though they are highly structurally similar. Therefore, structural similarity does not appear to be a good predictor of the strength of a compound relative to stress granule formation.

Pinosylvin is also considered a stilbenoid toxin that is created by plants in response to chemical and physical damage, specifically fungal infections[15]. Through its biosynthesis pinosylvin is able to have multiple impacts on the cell, primarily catalyzing reactions that aid in the synthesis of phenylpropanoids[15]. This could be another explanation on why pinosylvin induces stress granule formation faster, as the byproducts of the reactions it catalyzes could accelerate the inhibition of translation.

Piceatannol in Acute Exposure Assay with U2OS-WT Cells

Piceatannol had analogous results to the other stilbenes for the 100µM and 250µM concentrations with little to no stress granule formation for the U2OS-WT experiments. However piceatannol diverges from the other stilbenes at 500µM in which it only reached an average level of approximately 50% stress granule formation. There are a multitude of possible reasons why piceatannol is not as efficient as the other stilbenes, including its structure and function. As discussed earlier piceatannol contains four hydroxyl substituent groups[26]. The large number of subsistent groups could hinder piceatannol's ability to interact with certain compounds that influence stress granule formation. Yet it must be noted that oxyresveratrol is also made up of four hydroxyl substituent groups, with one group being in a different location than piceatannol[25]. Thus it could be hypothesized that the one uniquely placed hydroxyl group on the 3' carbon of piceatannol is what is causing the decreased rate of stress granule formation, or that the structure of piceatannol is not connected to the decreased rate at all.

Piceatannol has been studied as a derivative to resveratrol to combat the low bioavailability of the latter[12]. It was found through multiple studies that piceatannol possesses the same abilities as resveratrol such as its ability to activate and enhance Sirt1[12]. Piceatannol was also shown to be far more stable than resveratrol, and this stability could explain the lack of stress granules formed if it is the breakdown products of the stilbene, rather than the stilbene itself, that are inducing stress granules. However these claims must be investigated further to fully understand the mechanism and how piceatannol contributes to lesser stress granule formation at high concentrations.

Stilbene Stress Granules compared to Arsenite Stress Granules

It was discussed in a recent study how stilbene stress granules, specifically those formed with resveratrol, appeared smaller than stress granules in cells that were treated with arsenite[1]. Based upon the microscopic images taken of cells treated with arsenite in Figure 6 compared to the images of cells treated with stilbenes in Figure 10, this hypothesis can be supported as the stilbene stress granules did appear smaller and more numerous compared to the larger arsenite stress granules. This difference in size could be due to the differing structures of the compounds. Arsenite can also be considered a stronger compound in that it can produce 100% stress granule formation at a lower concentration of 250µM, while most of the stilbenes needed to be at least at 500µM to reach 100% formation. Therefore it could be speculated that if the stilbene concentration was increased it may produce larger stress granules similar to the size of the ones formed from arsenite. This leads to a broader question concerning if larger concentrations of compounds would create bigger stress granules compared to smaller concentrations. Looking at the microscopic images it is difficult to discern any distinguishable difference in the size of stress granules at 100µM and 500µM, but if more severe concentrations were employed there is the possibility it could make an impact.

Recommendations

While there has been an immense amount of progress made in the field of stress granules, there are still so many possibilities yet to be explored. Especially when concerning stilbenes and their relationship to stress granules it would be useful to test other stilbenes to see how the results compare to those results found in the U2OS-WT experiment performed. Doing further research on the stilbenes tested in these experiments could also be useful in further education on the topic,

specifically exploring piceatannol and determining at what concentration it is able to reach 100% stress granule formation. Also discovering which of the four kinase pathways for the eIF2α pathway each stilbene is involved in could aid in explaining the exact mechanisms stilbenes utilize to have their stress granule forming effect. Overall, there are endless avenues in which this research could be expanded upon, and stilbenes should remain at the forefront of the conversation.

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Appendix A: One way ANOVA and Tukey Test Results

100 µM Concentration

One-Way Analysis of Variance for Independent or Correlated Samples

[Traducción en español]

The logic and computational details of the one-way ANOVA for independent and correlated samples are described in Chapters 13, 14, and 15 of Concepts and Applications.

Procedure:

- **Initial Setup:**
  Enter the number of samples in your analysis (2, 3, 4, or 5) into the designated text field, then click the «Setup» button for either Independent Samples or Correlated Samples to indicate which version of the one-way ANOVA you wish to perform.
- **Entering Data Directly into the Text Fields:**
  After clicking the cursor into the scrollable text area for Sample 1, enter the values for that sample in sequence, pressing the carriage return key after each entry except the last. (On a Macintosh platform, the carriage return key is labeled 'Return'; on a Windows platform it is labeled 'Enter.') Perform the same procedure for the other samples in your analysis.
- **Importing Data via Copy & Paste:**
  Within the spreadsheet application or other source of your data, select and copy the column of data for sample 1. Then return to your web browser, click the cursor into the text area for sample 1 and perform the 'Paste' operation from the 'Edit' menu. Perform the same procedure for the other samples in your analysis.
- **Data Check:**
  For each sample, make sure that the final entry is **not** followed by a carriage return. (A carriage return after the final entry in a sample will be interpreted as an extra data entry whose value is zero. Importing data via the copy and paste procedure will almost always produce an extra carriage return at the end of a column.) After all values for a sample have been entered, click the cursor immediately to the right of the final entry in the list, then press the down-arrow key. If an extra line is present, the cursor will move downward. Extra lines can be removed by pressing the down arrow key until the cursor no longer moves, and then pressing the 'Backspace' key (on a Mac platform, 'delete') until the cursor stands immediately to the right of the final entry.
  If you are performing a correlated-samples analysis, also make sure that the values for each sample are entered in the appropriate sequence. Note that a correlated-samples analysis presupposes equal numbers of observations for each sample in the analysis.
- **When all** sample values have been entered, click the button labeled «Calculate.» For independent samples the default analysis is a standard weighted- means analysis. If you wish to perform an unweighted- means analysis, click the «Unweighted» button before calculating.

Note that when the number of samples is **k**=2, the analysis of variance (standard weighted-means analysis) is equivalent to a non-directional **t**-test with $\mathbf{F}=\mathbf{t}^2$.

Setup

Number of samples in analysis = 4

vassarstats.net/index.html 1/3

Independent Samples Correlated Samples	4 standard weighted-means analysis
Unweighted	Click this button only if you wish to perform an unweighted-means analysis. Advice: do not perform an unweighted-means analysis unless you have a clear reason for doing so.
Weighted	Click this button to return to a standard weighted-means analysis

Data Entry

Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
5 4 4 2	6 1 3 0	6 3 3 3	12 2 4 2	

Reset Calculate

Data Summary

	Samples					
	1	2	3	4	5	Total
N	4	4	4	4		16
ΣX	15	10	15	20		60
Mean	3.75	2.5	3.75	5		3.75
ΣX^2	61	46	63	168		338
Variance	1.5833	7	2.25	22.6667		7.5333
Std.Dev.	1.2583	2.6458	1.5	4.761		2.7447
Std.Err.	0.6292	1.3229	0.75	2.3805		0.6862

standard weighted-means analysis

ANOVA Summary 4

Source	SS	df	MS	F	P
Treatment [between groups]	12.5	3	4.1667	0.5	0.689269

Error	100.5	12	8.375
Ss/Bl			
Total	113	15	

Graph Maker

Ss/Bl = Subjects or Blocks depending on the design.
Applicable only to correlated-samples ANOVA.

Tukey HSD Test

This test will be performed only if K>2
and the analysis of variance yields a
significant F-ratio.

M1 = mean of Sample 1
M2 = mean of Sample 2
and so forth.

HSD = the absolute [unsigned] difference between any two sample means required for significance at the designated level. HSD[.05] for the .05 level; HSD[.01] for the .01 level.

250 µM Concentration

One-Way Analysis of Variance for Independent or Correlated Samples

[Traducción en español]

The logic and computational details of the one-way ANOVA for independent and correlated samples are described in Chapters 13, 14, and 15 of Concepts and Applications.

Procedure:

- **Initial Setup:**
  Enter the number of samples in your analysis (2, 3, 4, or 5) into the designated text field, then click the «Setup» button for either Independent Samples or Correlated Samples to indicate which version of the one-way ANOVA you wish to perform.
- **Entering Data Directly into the Text Fields:**
  After clicking the cursor into the scrollable text area for Sample 1, enter the values for that sample in sequence, pressing the carriage return key after each entry except the last. (On a Macintosh platform, the carriage return key is labeled 'Return'; on a Windows platform it is labeled 'Enter.') Perform the same procedure for the other samples in your analysis.
- **Importing Data via Copy & Paste:**
  Within the spreadsheet application or other source of your data, select and copy the column of data for sample 1. Then return to your web browser, click the cursor into the text area for sample 1 and perform the 'Paste' operation from the 'Edit' menu. Perform the same procedure for the other samples in your analysis.
- **Data Check:**
  For each sample, make sure that the final entry is **not** followed by a carriage return. (A carriage return after the final entry in a sample will be interpreted as an extra data entry whose value is zero. Importing data via the copy and paste procedure will almost always produce an extra carriage return at the end of a column.) After all values for a sample have been entered, click the cursor immediately to the right of the final entry in the list, then press the down-arrow key. If an extra line is present, the cursor will move downward. Extra lines can be removed by pressing the down arrow key until the cursor no longer moves, and then pressing the 'Backspace' key (on a Mac platform, 'delete') until the cursor stands immediately to the right of the final entry.
  If you are performing a correlated-samples analysis, also make sure that the values for each sample are entered in the appropriate sequence. Note that a correlated-samples analysis presupposes equal numbers of observations for each sample in the analysis.
- **When all** sample values have been entered, click the button labeled «Calculate.» For independent samples the default analysis is a standard weighted- means analysis. If you wish to perform an unweighted- means analysis, click the «Unweighted» button before calculating.

Note that when the number of samples is **k**=2, the analysis of variance (standard weighted-means analysis) is equivalent to a non-directional **t**-test with $\mathbf{F}=\mathbf{t}^2$.

Setup

Number of samples in analysis = 4

Independent Samples Correlated Samples	4 standard weighted-means analysis
Unweighted	Click this button only if you wish to perform an unweighted-means analysis. Advice: do not perform an unweighted-means analysis unless you have a clear reason for doing so.
Weighted	Click this button to return to a standard weighted-means analysis

Data Entry

Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
6 2 4 11	6 34 5 11	8 5 5 10	87 82 81 73	

Reset Calculate

Data Summary

	Samples					
	1	2	3	4	5	Total
N	4	4	4	4		16
ΣX	23	56	28	323		430
Mean	5.75	14	7	80.75		26.875
ΣX^2	177	1338	214	26183		27912
Variance	14.9167	184.6667	6	33.5833		1090.3833
Std.Dev.	3.8622	13.5892	2.4495	5.7951		33.021
Std.Err.	1.9311	6.7946	1.2247	2.8976		8.2552

standard weighted-means analysis

ANOVA Summary 4

Source	SS	df	MS	F	P
Treatment [between groups]	15638.25	3	5212.75	87.18	<.0001

Error	717.5	12	59.7917
Ss/Bl			
Total	16355.75	15	

Graph Maker

Ss/Bl = Subjects or Blocks depending on the design.
Applicable only to correlated-samples ANOVA.

Tukey HSD Test

HSD[.05]=16.25; HSD[.01]=21.27
M1 vs M2 nonsignificant
M1 vs M3 nonsignificant
M1 vs M4 P<.01
M2 vs M3 nonsignificant
M2 vs M4 P<.01
M3 vs M4 P<.01

M1 = mean of Sample 1
M2 = mean of Sample 2
and so forth.

HSD = the absolute [unsigned] difference between any two sample means required for significance at the designated level. HSD[.05] for the .05 level; HSD[.01] for the .01 level.

500 µM Concentration

One-Way Analysis of Variance for Independent or Correlated Samples

[Traducción en español]

The logic and computational details of the one-way ANOVA for independent and correlated samples are described in Chapters 13, 14, and 15 of Concepts and Applications.

Procedure:

- **Initial Setup:**
  Enter the number of samples in your analysis (2, 3, 4, or 5) into the designated text field, then click the «Setup» button for either Independent Samples or Correlated Samples to indicate which version of the one-way ANOVA you wish to perform.
- **Entering Data Directly into the Text Fields:**
  After clicking the cursor into the scrollable text area for Sample 1, enter the values for that sample in sequence, pressing the carriage return key after each entry except the last. (On a Macintosh platform, the carriage return key is labeled 'Return'; on a Windows platform it is labeled 'Enter.') Perform the same procedure for the other samples in your analysis.
- **Importing Data via Copy & Paste:**
  Within the spreadsheet application or other source of your data, select and copy the column of data for sample 1. Then return to your web browser, click the cursor into the text area for sample 1 and perform the 'Paste' operation from the 'Edit' menu. Perform the same procedure for the other samples in your analysis.
- **Data Check:**
  For each sample, make sure that the final entry is **not** followed by a carriage return. (A carriage return after the final entry in a sample will be interpreted as an extra data entry whose value is zero. Importing data via the copy and paste procedure will almost always produce an extra carriage return at the end of a column.) After all values for a sample have been entered, click the cursor immediately to the right of the final entry in the list, then press the down-arrow key. If an extra line is present, the cursor will move downward. Extra lines can be removed by pressing the down arrow key until the cursor no longer moves, and then pressing the 'Backspace' key (on a Mac platform, 'delete') until the cursor stands immediately to the right of the final entry.

  If you are performing a correlated-samples analysis, also make sure that the values for each sample are entered in the appropriate sequence. Note that a correlated-samples analysis presupposes equal numbers of observations for each sample in the analysis.
- **When all** sample values have been entered, click the button labeled «Calculate.» For independent samples the default analysis is a standard weighted- means analysis. If you wish to perform an unweighted- means analysis, click the «Unweighted» button before calculating.

Note that when the number of samples is **k**=2, the analysis of variance (standard weighted-means analysis) is equivalent to a non-directional **t**-test with $\mathbf{F}=\mathbf{t}^2$.

Setup

Number of samples in analysis = 4

Independent Samples Correlated Samples	4 standard weighted-means analysis
Unweighted	Click this button only if you wish to perform an unweighted-means analysis. Advice: do not perform an unweighted-means analysis unless you have a clear reason for doing so.
Weighted	Click this button to return to a standard weighted-means analysis

Data Entry

Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
77 19 79 2	99 96 97 97	99 98 99 99	96 97 91 98	

Reset Calculate

Data Summary

	Samples					
	1	2	3	4	5	Total
N	4	4	4	4		16
ΣX	177	389	395	382		1343
Mean	44.25	97.25	98.75	95.5		83.9375
ΣX^2	12535	37835	39007	36510		125887
Variance	1567.5833	1.5833	0.25	9.6667		877.2625
Std.Dev.	39.5927	1.2583	0.5	3.1091		29.6186
Std.Err.	19.7964	0.6292	0.25	1.5546		7.4047

standard weighted-means analysis

ANOVA Summary 4

Source	SS	df	MS	F	P
Treatment [between groups]	8421.6875	3	2807.2292	7.11	0.005309

Error	4737.25	12	394.7708
Ss/Bl			
Total	13158.9375	15	

Graph Maker

Ss/Bl = Subjects or Blocks depending on the design.
Applicable only to correlated-samples ANOVA.

Tukey HSD Test

HSD[.05]=41.75; HSD[.01]=54.67
M1 vs M2 P<.05
M1 vs M3 P<.05
M1 vs M4 P<.05
M2 vs M3 nonsignificant
M2 vs M4 nonsignificant
M3 vs M4 nonsignificant

M1 = mean of Sample 1
M2 = mean of Sample 2
and so forth.

HSD = the absolute [unsigned] difference between any two sample means required for significance at the designated level. HSD[.05] for the .05 level; HSD[.01] for the .01 level.