Resveratrol-inspired Compounds: Comprehending a Novel Antiepileptic Drug Class

AP Capstone Research

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Introduction

Background

A chronic disorder of the brain, epilepsy is characterized by frequently-occuring, unprovoked convulsions (World Health Organization, 2016). The causes for epilepsy are numerous, including genetic inheritance, birth trauma, traumatic brain injury, and brain infections (i.e. tumors) (World Health Organization, 2016). The various aforementioned causes of epilepsy contribute to abnormal electrical activity in the brain, resulting in seizure-like behavior that can either elicit spontaneous, abnormal movements (i.e. shaking and stiffening of muscles), or produce changes in mental processes, contributing to symptoms such as prolonged apnea, repetitive blinking, and dementia (Nagayama et al., 2017; World Health Organization, 2016).

With over 50 million people suffering worldwide, epilepsy has become one of the most common neurological disorders on the planet (World Health Organization, 2019). Consequently, there is a pressing demand for powerful, cost-effective, and widely accessible treatments that can enhance the quality of life for those affected by it. To treat chronic neurological disorders such as epilepsy, antiepileptic drugs (AEDs) have been developed to reduce oxidative stress and hasten recovery time (Ma et al., 2013). AEDs reduce excitability in neural tissue by targeting the impulsivity of individual neurons (Meisel et al., 2015). These drug compounds alter the electrical activity in these neurons by either selectively activating/inhibiting the transport of ions via protein channels in the cell membrane, or modifying inter-neuron chemical transference by targeting specific excitatory or inhibitory neurotransmitters (Meisel et al., 2015; Paul, 2006; Sattelle et al., 1988).

Statement of the Problem

While AEDs have proven to be effective in reducing many of the seizure-like symptoms prevalent during epilepsy, the holistic efficacy and tolerability of drug treatment in regard to epilepsy have not significantly improved since the late 20th century, with AEDs demonstrating approximately 60% effectiveness in treatment (Harrington et al., 2010; Löscher & Schmidt, 2011; Mitani, 2017). Additionally, fewer AEDs have been introduced to the market primarily because of slow screening techniques: almost all AEDs have been discovered through the same conventional model organisms, specifically the maximal electroshock seizure test (MES) in rodents (Castel-Branco et al., 2009; Löscher & Schmidt, 2011). While these examinations have led to the development of useful AEDs, they have not contributed to developing those with higher efficacies, and this has been a largely unappreciated concern for various decades (Löscher & Schmidt, 2011).

There are many other challenges that arise during the drug-development phase. While rodents are a popular model organism for drug development, they can only be experimented with once before having to test a new set of subjects (Bishayee, 2009). Hence, experimenting with rodents can become very costly and time-consuming, taking months to complete an assay due to their relatively long generation cycle in comparison to other model organisms (Bishayee, 2009; Castel-Branco et al., 2009). As such, there is a pressing need to explore new avenues for drug discovery and development that can overcome these limitations and provide more effective treatments for epilepsy.

Literature Review

Introduction

When evaluating the effectiveness of AEDs, there exist two important criteria that must be considered. First and foremost, the drug should be quickly metabolized by the digestive system to promote rapid recovery times in mammalian organisms suffering from seizure-like symptoms. Additionally, the drug should be developed with minimal side effects to avoid any potential harm to patients undergoing treatment. The administration of AEDs can affect not only the targeted neurons but also adjacent neural tissue, leading to unwanted behavioral disturbances, alterations in blood sugar levels, or liver complications (Vajda, 2002). In some cases, side effects may even worsen seizure-like symptoms, rendering the medication ineffective or intolerable for the patient. Therefore, it is essential to develop AEDs that can quickly and effectively reduce seizure-like symptoms while inducing minimal side effects on the patient, ensuring their safety and tolerability. Confirming that these criteria are met is a crucial step in determining the efficacy of AEDs and more specifically, their potential for use as a treatment for epilepsy.

While rodents have been widely used in epilepsy research, their ability to accurately represent the pathophysiological mechanisms of epilepsy in humans is limited (Bishayee, 2009). Therefore, the translatability of findings to other mammals, including humans, is questionable. This highlights the need for the development of new animal models and innovative techniques for epilepsy research. As a result, further research utilizing *C. elegans* as a model organism has the potential to greatly enhance the speed of drug development and provide more representative data collection. This is because *C. elegans* have a rapid generation time, making it possible to obtain results in a more timely manner compared to using other mammal models. Therefore, conducting epilepsy research on *C. elegans* is a valuable approach that has the potential to

advance our understanding of the disease and lead to the development of more effective treatments.

Given the current lack of understanding about resveratrol's mode of action, evaluating the effectiveness of modified resveratrol structures is a crucial step in optimizing the efficacy of the drug compound. This will not only help to determine the optimal structure for resveratrol-based treatments, but it will also provide insight into the specific neuro chemotherapeutic properties of resveratrol. By conducting this research, we can gain a deeper understanding of the mechanisms behind resveratrol's neuroprotective effects, which will inform the development of more effective treatments for neurological diseases.

Caenorhabditis Elegans

The discovery of AEDs can be hastened by using *C. elegans* as a model organism. These nematode worms are a popular choice among researchers for molecular-level studies due to several attractive features.

One of the key advantages of using *C. elegans* is its non-parasitic, non-pathogenic, non-infectious, and non-hazardous nature, which makes it safe to handle and study (Alexander et al., 2014; Arya et al., 2010; Schaffner, 2000). This is particularly important for researchers who may be working with potentially hazardous materials or organisms, as it eliminates the risk of accidental exposure or infection. Additionally, because *C. elegans* is non-parasitic, it does not require specialized facilities or equipment for handling, which makes it a practical choice for a wide range of scientific applications (Alexander et al., 2014; Arya et al., 2010; Schaffner, 2000).

Secondly, *C. elegans* are hermaphroditic, meaning they possess both male and female reproductive organs, allowing for efficient reproduction (Alexander et al., 2014; Arya et al., 2010). Additionally, the hermaphroditic nature of *C. elegans* reduces the prezygotic barrier to

reproduction, as mating partners do not need to be found, leading to a more efficient use of laboratory resources (Gray & Cutter, 2014).

Thirdly, *C. elegans*' short two-week lifespan and quick three-day generation time are advantageous for researchers as it makes them a low maintenance organism (Alexander et al., 2014; Arya et al., 2010). With such a rapid life cycle, researchers can quickly observe the effects of drugs and environmental changes on multiple generations of *C. elegans*. Ultimately, these advantages make it easier for researchers to conduct longitudinal studies, while also reducing the cost of animal care (Alexander et al., 2014; Arya et al., 2010; Gray & Cutter, 2014; Harrington et al., 2010).

Furthermore, *C. elegans*' genetic similarity to humans is one of the main reasons why it has become a valuable model organism for neurological research. Research has shown that nearly 60% of the genes present in *C. elegans* have a functional human homolog, indicating a significant overlap in genetic material between the two species (Harrington et al., 2010; Löscher & Schmidt, 2011; Mitani, 2017). This similarity in genetic makeup makes it possible for researchers to use *C. elegans* to study the effects of drugs and how they may affect humans. By studying the worms, researchers can gain a more representative insight into the mechanisms of drugs and their potential side effects on the human body.

Lastly, *C. elegans* has an intricate neural network, with a large number of neurons and synapses that have been extensively mapped (Schaffner, 2000; White et al., 1986). This comprehensive mapping of the connections between neurons in *C. elegans* allows researchers to study the effects of particular drugs on the nervous system in a way that is not possible with other model organisms. This unparalleled degree of electrical circuitry and synaptic connections

in *C. elegans* makes it an ideal model organism for studying the neural mechanisms underlying various diseases, including epilepsy.

Ultimately, the safety, efficiency, low maintenance, genetic similarity, and extensive neural mapping in *C. elegans* make it a valuable model for neurological research. The aforementioned advantages of using *C. elegans* over traditional models such as rodents facilitates the discovery of new AEDs with higher efficacies and fewer side effects, ultimately making *C. elegans* a practical and more representative alternative for other model organisms in epilepsy research.

Resveratrol and Resveramorphs

Resveratrol's unique properties and potential health benefits have made it a novel compound for drug development, with researchers continually exploring its diverse range of applications and potential therapeutic uses. This section will discuss the various advantages and disadvantages of resveratrol, as well as its current state of research and development.

Firstly, resveratrol, a natural polyphenol found in various foods such as grapes, nuts, cocoa, and wine, is easily absorbed and rapidly metabolized in the digestive system, enabling the compound to quickly reach the bloodstream and exert its potential health benefits (Ates et al., 2007; Bishayee et al., 2010; Bollinger et al., 2019; Chow et al., 2005; Jang et al., 1997). To further elaborate, the natural occurrence of resveratrol in various foods means that it can be readily sourced without the need for costly extraction processes or synthetic production. This may make resveratrol a more cost-effective alternative to other drugs or supplements that require more extensive processing or manufacturing. Furthermore, the efficient absorption and metabolism of resveratrol mean that a lower dose of the compound may be required to achieve the desired health benefits compared to other drugs or supplements. This could potentially

reduce the overall cost of resveratrol supplementation, as less of the compound is needed to achieve the desired effect.

Secondly, resveratrol exhibits anti-inflammatory, anti-oxidant, and anti-carcinogenic properties and these mechanisms can vary depending on the environment (Bollinger et al., 2019; Lopez et al., 2015; Meng et al., 2021). Resveratrol's ability to adapt to different psychological contexts and target specific pathways makes it a promising candidate for developing treatments that can be tailored to an individual's needs. Additionally, the compound's diverse range of mechanisms underscores its potential as a multifaceted therapeutic agent, capable of addressing multiple health concerns simultaneously.

Unfortunately, resveratrol's aforementioned variability limits its ability to be used as a selective medicinal agent (Bollinger et al., 2019). Despite its potential health benefits, the exact mechanism of action for resveratrol remains elusive, as its function varies depending on the specific biological system or pathway being targeted (Bollinger et al., 2019). This limited selectivity of resveratrol as a medicinal agent highlights the importance of finding ways to optimize its therapeutic potential.

To address this, investigating biochemically-engineered resveratrol structures, known as resveramorphs, can help shed light on the most effective functional groups for treating specific diseases such as epilepsy (Alexander et al., 2014; Harrington et al., 2010; Ma et al., 2013). By manipulating the functional groups of resveratrol, resveramorphs can be designed with greater selectivity and efficacy. The study of these resveramorphs can not only shed light on the most effective functional groups for treating specific diseases, but also provide insights into the underlying mechanisms of resveratrol's neuro chemotherapeutic properties. This approach may ultimately lead to the development of more potent and selective resveratrol-based therapies,

offering new hope for patients suffering from a range of diseases and disorders, specifically those suffering from epilepsy.

Neuroprotection

Oxidative stress, characterized by abnormal protein accumulation and improper mitochondrial function (Bishayee et al., 2010), has been widely recognized as a significant risk factor in the development of various neurological diseases, including Alzheimer's, Parkinson's, and particularly, epilepsy (Alexander et al., 2014; Harrington et al., 2010; Ma et al., 2013). This suggests that oxidative stress plays a crucial role in the maintenance of these conditions, making it a vital area of focus for research and potential treatments (Rahman et al., 2020).

Mitochondrial dysfunction is a well-known contributor to oxidative stress, which occurs when free radicals - unpaired electrons that cause an oxidation-reduction imbalance in living organisms (Vajda, 2002) - are generated in the mitochondria during oxidative phosphorylation (Bishayee et al., 2010). This process involves the transfer of electrons down the electron transport chain (ETC) via oxygen atoms, which act as the final electron acceptors to synthesize water molecules (Bishayee et al., 2010). The energy released from electrons during these exergonic reactions is used by the cytochrome complex to create a proton gradient that drives ATP synthesis (Bishayee et al., 2010). However, as the electrons move through the ETC, some may leak out and interact with oxygen molecules, leading to the formation of free radicals. The reactivity of these newly-produced free radicals results in elevated levels of oxidative phosphorylation, leading to increased oxygen demand in the brain, ultimately leading to oxidative stress. If left unchecked, this oxidative stress can cause permanent neurological damage in living organisms (Bishayee et al., 2010; Vajda, 2002).

Interestingly, resveratrol has been found to reduce oxidative stress in living organisms like rodents and *C. elegans* by inhibiting cell proliferation through protein phosphorylation (Bastianetto et al., 2015; Khanduja & Bhardwaj, 2003; Lobo et al., 2010; Venturini et al., 2010). However, research has shown that higher doses of resveratrol (2.5 grams or more per day) may cause side-effects like liver dysfunction, nausea, vomiting, and diarrhea, although it can induce cell death in tumor tissues without affecting healthy cells at low doses (1.0 grams) (Salehi et al., 2018). Optimal intake of antioxidants like resveratrol may maximize their ability to inhibit oxidative stress while minimizing negative effects. It is, therefore, crucial to consider the balance between the effectiveness of resveratrol in reducing oxidative stress and its potential side-effects when determining its efficacy as an antioxidant.

Gap and Research Question

After this research, the major apparent gap in AED research is in the availability of AEDs that possess the ability to serve as selective treatments for neurological diseases such as epilepsy. This gap has been exacerbated by a reliance on traditional seizure models such as the maximal electroshock seizure exam (MES) which may not accurately reflect the underlying mechanisms of epilepsy and may limit the identification of more targeted AEDs (Castel-Branco et al., 2009; Löscher & Schmidt, 2011). As a result, there is an urgent need for the development of more sophisticated and disease-specific models that better reflect the underlying pathophysiology of neurological diseases, and that can help identify more effective and selective AEDs for these conditions. This raises the research inquiry: to what extent can a compound resembling resveratrol mitigate the seizure-like activity in *C. elegans*? After concluding this experiment, I anticipate that there will be a positive difference between the mean recovery time of *C. elegans* (wild type N2 strain) suffering from seizure-like behavior in a solution containing M9 saline and

in one containing Resveramorph 3 (RVM 3). In mathematical terms, I anticipate that $\mu_c - \mu_t > 0$, where μ_c is the mean recovery time of *C. elegans* in the control solution (M9 saline), and μ_t is the mean recovery time in the treatment solution (RVM 3).

Methodology

Rationale

While there is a wealth of research highlighting resveratrol's potential as an anti-inflammatory and carcinopreventive agent, limited studies have examined its feasibility as a groundbreaking AED. Therefore, an experiment is the most appropriate research design to achieve the goal of developing a highly effective and easily absorbable AED, as well as shed light on the yet-to-be-determined mechanism of action of resveratrol.

Moreover, while rodents are a frequently used model organism in drug development, they can only be tested once before requiring a new set of subjects, and the process can become both costly and time-consuming, taking months to complete an assay due to their longer generation cycle compared to other model organisms. Utilizing *C. elegans* to establish a behavioral assay for the microorganism could provide additional advancements in improving the efficacy of AEDs and potential selective medicinal agents. *C. elegans* is a small and simple organism that can be used to test drugs rapidly and at a lower cost, making it a cost-effective and more efficient alternative for supplementing research on enhancing AED efficacies and potential selective medicinal agents.

The recovery times of *C. elegans* in RVM 3 solution were compared to the recovery time of *C. elegans* in M9 saline solution to evaluate the effectiveness of the RVM 3 solution as a potential therapeutic intervention. Using a sham control (M9 saline) allowed for the control of confounding factors and the isolation of the true effect of the treatment (RVM 3) being tested. By

doing so, more accurate and reliable conclusions about the effectiveness of the treatment can be drawn, and the possibility that any observed effects are due to factors other than the treatment itself can be ruled out.

The optimal electroshock parameters (47 V and three second duration) were determined through previous studies and concentration curves, which allowed for a consistent and controlled seizure in the *C. elegans* that could be measured and analyzed (Risley et al., 2016; Risley et al., 2017). Deviations from the optimal voltage can result in overexposure, which can cause immediate death to the *C. elegans,* or underexposure, which may not sufficiently stimulate the *C. elegans.* By using these specified parameters, controlled seizures can be induced while minimizing the risk of harm to the organism, and ultimately, ensure that the experiment is conducted in a controlled and consistent manner, which can substantially improve the reliability and reproducibility of the results.

Finally, the *C. elegans* rested in the prepared drug for around 30 seconds before electroshock to allow the drug to penetrate the organism's cuticle and reach its nervous system, where it can have an effect on the susceptibility to seizures induced by electroshock. Additionally, the specified duration of 30 seconds was determined through optimization studies and prior experimentation in order to find the optimal duration for allowing the drug to penetrate the cuticle without causing significant harm or stress to the organism (Risley et al., 2016; Risley et al., 2017).

Ethical Considerations

One of the key benefits of using *C. elegans* is that it is not subject to many of the ethical concerns associated with the use of mammals (Kaletta et al., 2006; Kenyon, 1988; Zhang et al., 2020). *C. elegans* is a non-vertebrate organism and does not have a nervous system capable of

experiencing pain or suffering in the way that mammals do (Risley et al., 2016; Risley et al., 2017). As such, studies involving the manipulation of neural circuits and behaviors do not raise ethical concerns related to sentience. Furthermore, *C. elegans* is not covered by many animal welfare regulations, which means that researchers are free to conduct experiments that would be ethically prohibited in mammals, such as studies involving the use of high doses of drugs or toxins (Zhang et al., 2020).

Despite the ethical advantages of *C. elegans,* researchers still have a responsibility to ensure that the organisms are treated humanely. To address the ethical concerns surrounding the use of *C. elegans* in research, appropriate measures were taken to minimize harm and distress in the model organisms. To facilitate the propagation of large numbers of *C. elegans* for experiments, the worms were placed on nematode growth media (NGM) agar plates. These plates contained a nutrient-rich media, which provides essential vitamins and nutrients for the worms to grow and reproduce. The plates were then incubated at 37 ^{o}C to maintain the appropriate temperature and humidity levels to ensure healthy growth. Once the worms reached adulthood, they were carefully transferred to new NGM plates containing a fresh supply of food to ensure healthy growth. The worms were then incubated overnight at 20 ^{o}C to mature into one-day old adults. Additionally, non-invasive techniques including handwashing, sterilization of instruments and surfaces, and the use of personal protective equipment such as gloves, lab coats, and face masks were used to prevent the introduction of harmful pathogens or chemicals, which could have compromised the health of the worms. The experimental design and procedures were approved by the Institutional Review Board (IRB) to eliminate any ethical concerns.

Materials and Reagents

All materials and reagents were provided by the Dawson-Scully lab at Nova Southeastern

University (Risley et al., 2016; Risley et al., 2017). Please refer to Appendix A for the complete list of the materials and reagents used.

Chemical Recipes

All chemical recipes were provided by the Dawson-Scully lab at Nova Southeastern University (Risley et al., 2016; Risley et al., 2017). Please refer to Appendix B for the complete list of the chemical recipes.

Equipment

All equipment was provided by the Dawson-Scully lab at Nova Southeastern University (Rislely et al., 2016; Risley et al., 2017). Please refer to Appendix C for the complete list of equipment used.

Software

Open Broadcaster Software (OBS) was used for video recording of the experiments. An open source image processing program (VLC media player) was used for viewing video recordings of the experiments. Google Sheets was utilized to organize the raw data from the experiments and conduct the statistical inference procedure. Stapplet was used to extract the summary statistics for each dataset, check the assumptions for the inference test, and display the collected data. The OBS and the VLC media player were provided by Nova Southeastern University (Risley et al., 2016; Risley et al., 2017).

Data Collection Procedure

All data collection was completed at Nova Southeastern University (Risley et al., 2016; Risley et al., 2017). A detailed description of the data collection procedure can be found in Appendix D. Below is a brief description of the data collection procedure.

To prepare for data collection, the recording equipment was connected with appropriate

cables, and the timer output stimulator and additional stimulator were set up to control the duration of stimuli. Next, matured *C. elegans* were loaded into the 9 mm plastic tubing with prepared drug solutions and sham controls. The *C. elegans* were then shocked, with the electroshock parameters set to 47 V and three seconds duration. The experiment was recorded for ten minutes using the setup equipment, and this process was repeated for each trial.

Data Analysis Procedure

Before administering the shock, each worm in the test tube was labeled from left to right (e.g. Worm 1, Worm 2, Worm 3, etc.). To assess the recovery of *C. elegans* after electrical shock, a manual process was employed utilizing the VLC media player. Each worm was individually tracked and analyzed in the test tube throughout the experiment recording. The onset of the shock and the recovery time for each worm was manually recorded. To be deemed fully recovered, the *C. elegans* must exhibit a sinusoidal movement pattern similar to their pre-shock movement, specifically three continuous sinusoidal movements. If a worm did not exhibit any movement before the shock was applied or was caught in a bubble formation caused by the electrolysis, it was labeled as "NA". If a worm showed normal behavior before the shock but did not recover within 120 seconds after the shock, it was labeled as "NR". Worms labeled as either “NA” or “NR” were excluded from statistical analysis.

Each worm's recovery time is considered one data point, and at least 30 data points are collected to form a complete dataset. Dotplots and adjacent boxplots were utilized to visualize the datasets, while summary statistics were presented in tabular form. To determine the statistical significance of the mean recovery time for *C. elegans* in RVM 3, an unpaired t-test (also called an independent t-test) was conducted to compare the mean recovery times between the M9 saline and RVM 3 groups. The t-statistic was computed, and the p-value was calculated using the

t-distribution cumulative distribution function (tcdf) and appropriate degrees of freedom. After calculating the p-value, it was compared to the predetermined level of significance to determine the statistical significance of the inference procedure results.

Limitations

One of the main limitations of the study is the exclusion of worms from analysis due to bubble trapping resulting from electrolysis, which creates an uneven distribution of worms across different experimental conditions. This selective exclusion of worms can potentially result in biased samples and limit the generalizability of the findings. Additionally, the reduced sample size may affect the statistical power of the study, making it more difficult to detect true differences between groups. This can impact the accuracy of the results and should be taken into consideration when analyzing the data.

The analysis also doesn't take into account the speed or frequency of sinusoidal movement in recovered *C. elegans*. The frequency of sinusoidal movement in *C. elegans* is relevant because it can provide insights into the worm's behavior and physiological state. *C. elegans* are known for their characteristic wave-like movement, and the frequency of this movement can be used as an indicator of the worm's activity level and overall health. Changes in the frequency of sinusoidal movement can indicate a response to a stimulus or a change in the worm's environment. Additionally, changes in the frequency of sinusoidal movement can also be used to monitor the effects of drugs or other treatments on the worm.

Finally, determining when a *C. elegans* has recovered is a subjective process that can introduce significant statistical bias. Human observers may mistake muscle spasms for normal sinusoidal movement or track the wrong *C. elegans* as it collides with others in the test tube. These occurrences can result in significant misrepresentation in the data, which may affect the

accuracy and reliability of the study's conclusions. The subjective nature of the assessment based on visual observations can make it difficult to ensure consistency across observers, further exacerbating the problem of statistical bias.

Findings and Analysis

Findings

The recovery times (in seconds) for the *C. elegans* in both the M9 saline and RVM 3 solutions were visually displayed below using a combination of dot plots and boxplots, where the dotplots provide an individual observation for each worm, and the adjacent box plots illustrate the distribution of the data (Stapplet, n.d.). Additionally, the summary statistics for both the M9 saline and RVM 3 groups are summarized below. Please refer to Appendices E and F for the raw data collected from the experiment recordings.

Figure 1

Combined Dot Plot and Boxplot Display of M9 Saline Dataset

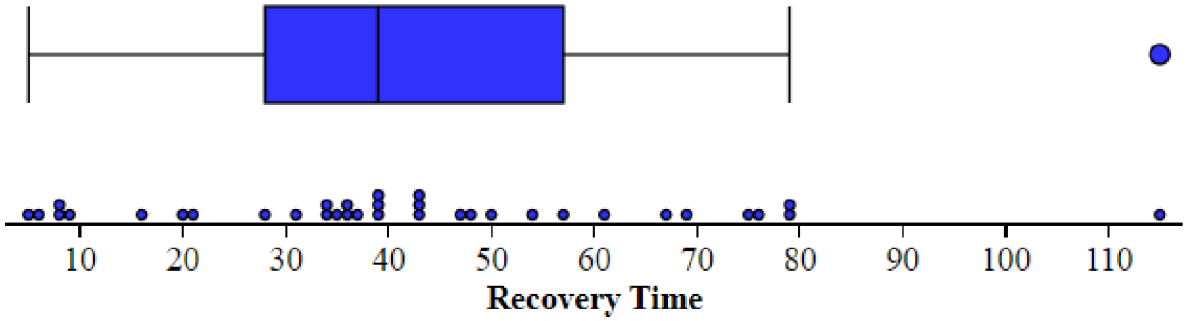


Table 1

Summary Statistics for M9 Saline Dataset

Sample Size	Mean (μ_c)	Standard Deviation	Minimum	Q_1	Median	Q_3	Maximum
35	42.486	24.734	5	28	39	57	115

Figure 2

Combined Dot Plot and Boxplot Display of RVM 3 Dataset

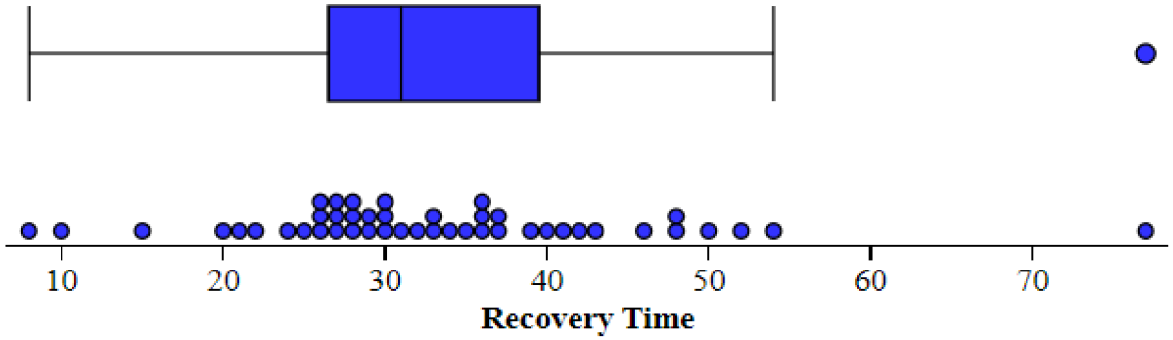


Table 2

Summary Statistics for RVM 3 Dataset

Sample Size	Mean (μ_t)	Standard Deviation	Minimum	Q_1	Median	Q_3	Maximum
45	33.244	12.143	8	26.5	31	39.5	77

Analysis

To analyze the data, the mean recovery times for the M9 saline group (control) and the RVM 3 group (treatment) were compared using an unpaired t-test with 79 degrees of freedom.

Null and Alternative Hypotheses

The null hypothesis states that there is no significant difference in the mean recovery time between the M9 saline group and the RVM 3 group ($\mu_c - \mu_t = 0$, where μ_c is the mean recovery time for the control group (M9 saline) and μ_t is the mean recovery time for the treatment group (RVM 3)), while the alternative hypothesis suggests that there is a positive difference in the mean recovery time of the two groups ($\mu_c - \mu_t > 0$).

Assumptions and Conditions

Before conducting the unpaired t-test, it is crucial to check the assumptions and conditions to ensure the validity and reliability of the results.

The recovery time of *C. elegans* in M9 saline solution has no effect on the recovery time of *C. elegans* in the RVM 3 solution; thus, the two groups are independent of one another. To assess the normality of the data, normal probability plots were employed for both the M9 saline and RVM 3 groups. The resulting plots are displayed below.

Figure 3

Normal Probability Plot of M9 Saline Dataset

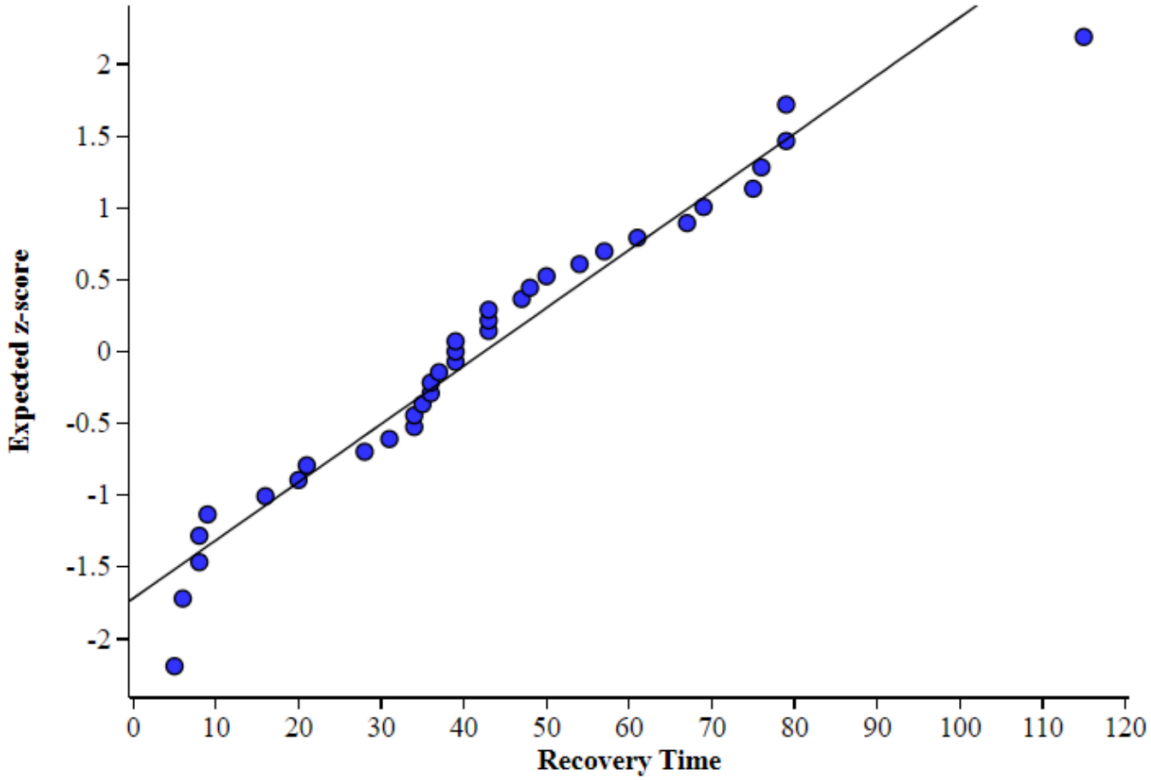
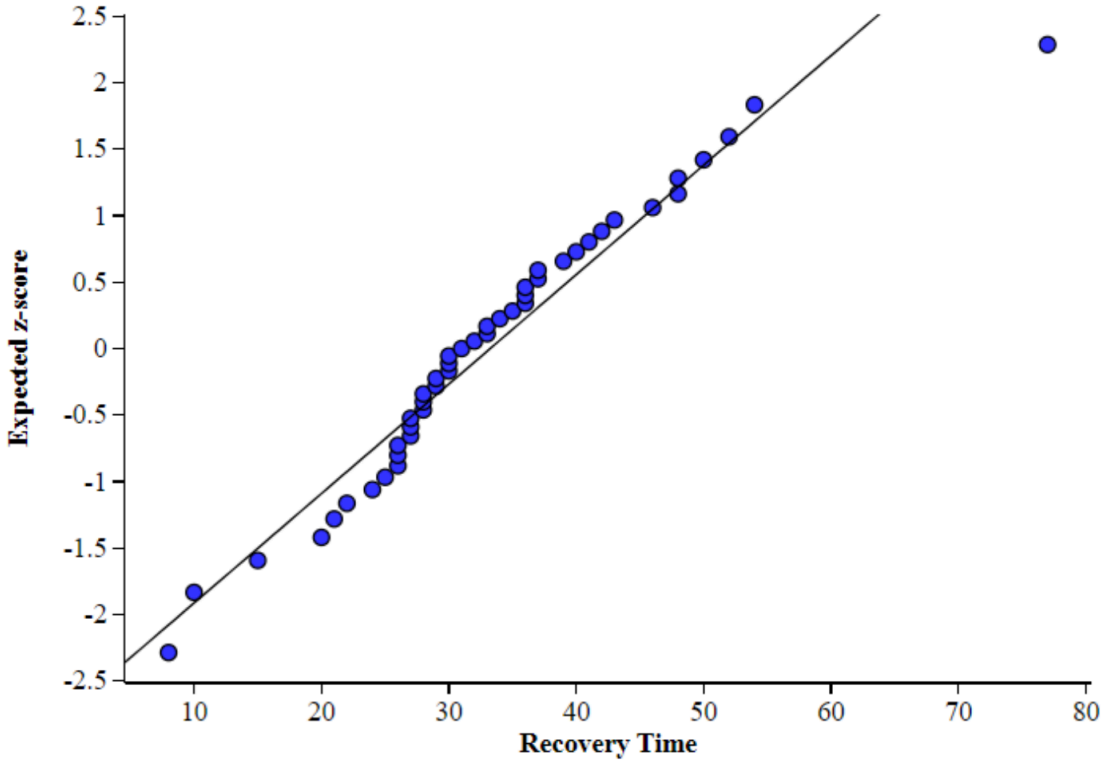


Figure 4

Normal Probability Plot of RVM 3 Dataset



Both normal probability plots show data points closely following the diagonal line with rare substantial deviations. The data points are symmetric with respect to the diagonal line, and the plots contain few extreme outliers that deviate significantly from the expected values. As a result, the data appears to be approximately normally distributed. Consequently, both conditions for statistical inference are met, and the unpaired t-test can be conducted appropriately.

Significance Level

For the sake of this experiment, a level of significance of 5%, or 0.05, will be used as reference. This level of significance is a commonly used threshold in statistical analysis, and it is widely accepted as a standard level of significance in many scientific disciplines.

Means and Sample Standard Deviations

The mean recovery time and sample standard deviation for the M9 saline group are approximately 42.486 seconds and 24.734, respectively, while those for the RVM 3 group are approximately 33.244 seconds and 12.143, respectively.

Calculating the t-Value

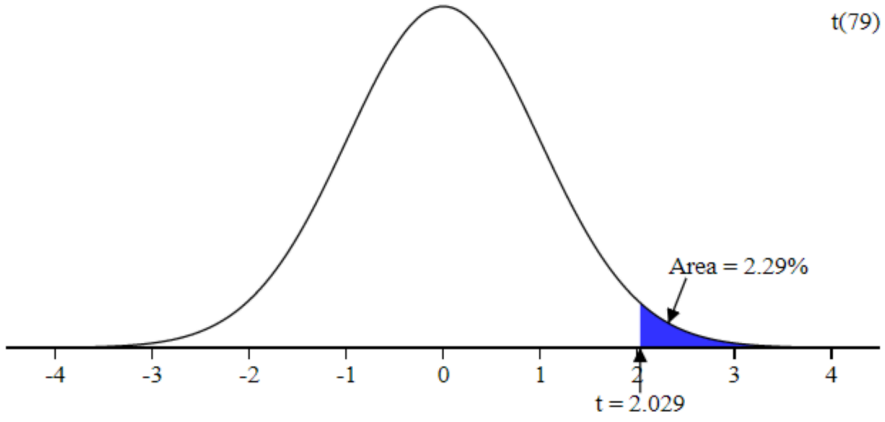
After conducting an unpaired two-sample t-test with 79 degrees of freedom using the collected sample means, sample standard deviations, and sample sizes of the two data sets, the t-value was calculated to be approximately 2.029.

P-Value and the t-Distribution

Regarding this experiment, the p-value was calculated using the t-distribution cumulative distribution function (tcdf), a mathematical function used in statistics to calculate the probability that a t-value falls within a certain range (Harper, 1985). Below is a graphical representation of the t-distribution, where the shaded blue area represents the probability of observing a t-value as extreme or more extreme than the calculated t-value of 2.029, assuming that the null hypothesis ($\mu_c - \mu_t = 0$) is true (Nahm, 2017).

Figure 5

t-Distribution with 79 Degrees of Freedom



Thus, with 79 degrees of freedom, the probability that a random variable from the t-distribution is greater than 2.029 is approximately 2.29%, or 0.0229. With a right-tailed p-value of approximately 0.0229, I can reject the null hypothesis that there is no difference between the mean time of recovery of the M9 saline solution and the RVM 3 solution ($\mu_c - \mu_t = 0$). Because my p-value of 0.0229 is less than 0.05, there is statistically significant evidence to support the alternative hypothesis that there is a positive difference between the mean recovery time of the M9 saline solution and the RVM 3 solution ($\mu_c - \mu_t > 0$).

Conclusion and Recommendations

Conclusion

My hypothesis that there will be a positive difference between the mean recovery time of the M9 saline group and the RVM 3 group was supported by the quantified data. The right-tailed p-value of 0.0229 is statistically significant evidence that *C. elegans* in the RVM 3 solution will recover quicker than *C. elegans* in the M9 saline solution.

Recommendations

The process of determining the efficacy of AEDs in *C. elegans* involves analyzing the recovery of the organisms after being subjected to a shock. This recovery is typically identified by the presence of three sinusoidal movements, but external factors such as muscle spasms can lead to significant misidentification. To eliminate observer bias and improve the accuracy of results, it is recommended to use machine-learning algorithms to minimize observer bias and objectively quantify the efficacy of AEDs (Agin, 2017). The algorithm would track individual worms and develop a mathematical model to determine when the organisms have recovered from the shock, which would result in more reliable and accurate data (Belkin et al., 2019; Bollinger et al., 2019). However, the effectiveness of machine-learning algorithms in minimizing observer

bias depends on the quality of the images captured during the experiment. To address this, computer-aided design (CAD) can be used to enhance the visual quality of the recordings by designing microscope attachments that allow for proper placement of position trackers (Memon et al., 2020). The machine-learning algorithm will then use the position trackers and previous data sets to analyze the movement of *C. elegans* and make predictions about their undulatory locomotion. By implementing these techniques, the validity of future research findings can be significantly improved.

The experiment described investigated the neurochemotherapeutic properties of Resveramorph 3, and as the name implies, there are several other resveramorphs that have not been fully tested. Based on this information, it is recommended that further research be conducted to explore the unique characteristics of these additional resveramorphs. The goal of this additional research is to discover one or more resveramorphs that have the potential to be as effective, if not more effective than Resveramorph 3, and ultimately, have the potential to be used as selective medicinal agents. This will help to expand our understanding of the neuro chemotherapeutic properties of these substances and provide new avenues for treatment in the field of medicine (Risley et al., 2016; Risley et al., 2017).

The ultimate goal of this research project is not only to develop a statistically significant AED in comparison to a control solution but also to achieve statistical significance when compared to already approved FDA AEDs. To make a comprehensive comparison between Resveramorph 3 and FDA-approved AEDs, it is recommended to establish a third comparison group. Conducting an ANOVA one-way comparison test on the treatment group, control group (M9 saline solution), and the FDA-approved drug will provide a more comprehensive view of

the research project. This approach will broaden the implications of the study and ensure greater validity and wider applications for future research projects (Shimada & Yamagata, 2019).

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Appendix A

Materials and Reagents

- Plastic tubing, cut into 9 mm segments
- 2, 20 gauge insulated copper wire, cut to 8 cm segments
- 60 x 15 mm Petri dishes
- Pipette tips
- Vacuum filter
- Two Alligator clip wires
- *C. elegans* wild type strain N2
- M9 solution (see Appendix B)
- *E. coli* strain 0P50 (see Appendix B)
- Nematode growth medium (NGM) agar plates (see Appendix B)
- LB broth powder
- LB broth (see Appendix B)
- Agar
- KP buffer (see Appendix B)
- Ethanol
- Cholesterol
- Peptone
- Sodium chloride (NaCl)
- Sodium phosphate dibasic (Na_2HPO_4)
- Potassium phosphate monobasic (KH_2PO_4)
- Potassium phosphate dibasic (K_2HPO_4)

- Magnesium sulfate solution ($MgSO_4$)
- Calcium chloride solution ($CaCl_2$)

Appendix B

Chemical Recipes

LB broth

- Add 25 g LB broth powder and 1 L distilled water (dH_2O) to a 2 L flask
- Autoclave for 30 min

Nematode growth medium (NGM) agar plates

- Add 3 g NaCl, 17 g agar, 2.5 g peptone, and 975 ml dH_2O to a 2 L flask
- Autoclave for 30 min
- Let cool to 50 °C in the water bath
- Add 1 ml 1 M $CaCl_2$, 1 ml 1 M $MgSO_4$, 5 mg cholesterol dissolved in 1 ml 95% ethanol, and 25 ml KP buffer to the agar solution, mixing well after each addition
- Pipette 10 ml of the well-mixed solution into each 60 x 15 mm Petri dish
- Cover and store at 4 °C
- When needed, seed plates with 50 µl of OP50 *E. coli* and incubate overnight at 37 °C

KP buffer

- Add 2.46 g KH_2PO_4 and 1.205 g K_2HPO_4 to 25 ml deionized water (diH_2O) with a stir bar
- Adjust pH to 6.0 and vacuum filter

OP50 *E. coli*

- Suspend 1 colony of OP50 *E. coli* from a stock plate in LB broth
- Incubate overnight at 37 °C with the lid loosely on
- Store at 4 °C

M9 solution

- Add 3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, and 1 ml 1 M $MgSO_4$ to a 2 L flask and fill

with diH_2O to 1 L

- Autoclave for 15 min and store at room temperature

Appendix C

Equipment

- 2 L flask
- Pipettes
- Stir bar
- Dissection stereo microscope
- Dissecting stereoscope
- Timer output stimulator
- Stimulator
- CCD color microscope camera
- Television monitor
- HDD and DVD recorder
- DVD-recordable discs
- Digital oscilloscope
- Ethanol lamp
- Water bath (50 ºC)
- Incubator (37 ºC)
- Incubator (20 ºC)
- Timer
- Metric ruler
- Infrared temperature gun

Appendix D

Data Collection Procedure

Connecting the Microscope Camera, Television Monitor, and Digital Video Recorder

- To set up the microscope camera to the television monitor and digital video recorder, first connect the microscope camera to the television monitor and digital video recorder using the appropriate cables.

Setting up Experimental Equipment:

- The stereoscope (for viewing stereoscopic pairs of images), oscilloscope (for displaying and analyzing waveforms of electronic signals), timer output stimulator, recording equipment, and an additional stimulator (for controlling time duration) will be set up in preparation for the experiment.
    - Connect the output on the timer output stimulator to a cable with a banana plug splitter, connecting the stimulator to the ground.
    - Modulate the banana plug inputs on the additional stimulator to connect the timer output stimulator to the additional stimulator for proper duration modulation.
    - Set the timer output stimulator to at least 5 V and a stimulus duration of three seconds.
    - Attach one alligator clip to the positive (+) output on the additional stimulator and the other alligator clip to the negative (-) output. The alligator clip wires will connect the additional stimulator to the experimental tube.
    - Clip each copper wire to each open end of the alligator clips.

Preparing the Worms:

- 24 hours before experimentation, transfer 30 L4 stage *C. elegans* to fresh nematode growth media (NGM) plates seeded with OP50 *E. coli*.
- Place the worms in a 20 ºC incubator overnight to facilitate the maturation of L4 *C. elegans* into 1-day old adults (approximately an 18 hr process).
- Leave the NGM plates on the lab bench at room temperature (23 ºC) during experimentation the following day.

Preparing Drug Solutions and Sham Controls:

- Prepare the drug solutions on the morning of experimentation and dissolve them directly into a vial of M9 solution with a total volume between three and five milliliters.
- Prepare sham controls during this time as well.

Initiating the Experiment

- Insert a blank DVD+R into the HDD/DVD recorder. Verify that the settings on the timer output stimulator and the additional stimulator are correctly modified using the oscilloscope to measure the voltage output.
- Set the electroshock parameters to 47 V and three second duration.
- Attach a pipette tip to the pipette and fill it with approximately 20 microliters of either control solution or drug solution, depending on the trial.
- Fill the 9 mm plastic tubing with the solution, and immediately load 6-8 *C. elegans* into the tube using a platinum pick and set a timer for approximately 30 minutes.
- Gently place the tube under the camera setup 30 seconds before the timer expires; turn the light base on and plug each copper wire into the ends of the plastic tubing, making sure that the copper wires are approximately 10 mm apart.

- Verify the distance between the copper wires using the metric ruler and initiate the electroshock.
- Allow the experiment to record for ten minutes; once the duration has been completed, stop the recording and discard the tube and its contents.

Appendix E

Raw Data Table of Recovery Times in M9 Saline Solution

	Time of Shock	Worm 1	Worm 2	Worm 3	Worm 4	Worm 5	Worm 6
Video 1	31	NA	36	39	NR	NR	-
Video 2	31	NR	NR	79	68	51	110
Video 3	32	NR	89	68	NR	NR	48
Video 4	34	95	109	NR	NR	103	65
Video 5	34	NR	NR	55	NA	-	-
Video 6	30	NA	39	38	73	-	-
Video 7	33	NR	80	-	-	NA	-
Video 8	44	123	NR	159	-	-	-
Video 9	33	NR	NR	100	NR	69	-
Video 10	39	NR	78	73	67	NR	-
Video 11	38	NR	NR	NR	72	81	NA
Video 12	36	NR	86	75	79	-	-
Video 13	37	43	76	72	113	-	-
Video 14	42	NR	96	NR	NR	-	-

The values in the rows beneath each worm represent the time (in seconds) from the start of the video at which point the *C. elegans* successfully recovered. To find the post-shock recovery time for each *C. elegans*, the time associated with each worm and the time of shock associated with that experiment are subtracted.

Appendix F

Raw Data Table of Recovery Times in RVM 3 Solution

	Time of Shock	Worm 1	Worm 2	Worm 3	Worm 4	Worm 5	Worm 6
Video 1	31	66	46	NR	60	71	NR
Video 2	31	NA	62	67	85	61	-
Video 3	31	74	81	NA	70	68	-
Video 4	33	70	81	NR	60	-	-
Video 5	35	45	56	71	62	65	-
Video 6	35	55	77	NA	68	NA	NR
Video 7	35	61	NR	67	NR	-	-
Video 8	37	NR	NA	59	65	-	-
Video 9	33	NA	81	NA	74	NA	62
Video 10	34	111	62	NA	-	-	-
Video 11	38	64	72	65	-	-	-
Video 12	37	67	NR	73	83	NA	70
Video 13	37	61	63	NR	-	-	-
Video 14	36	44	NR	64	61	NR	88

The values in the rows beneath each worm represent the time (in seconds) from the start of the video at which point the *C. elegans* successfully recovered. To find the post-shock recovery time for each *C. elegans*, the time associated with each worm and the time of shock associated with that experiment are subtracted.