Winter 12-5-2022

Impact of ethanol on regulated cell death Crithidia Fasciculata

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2022

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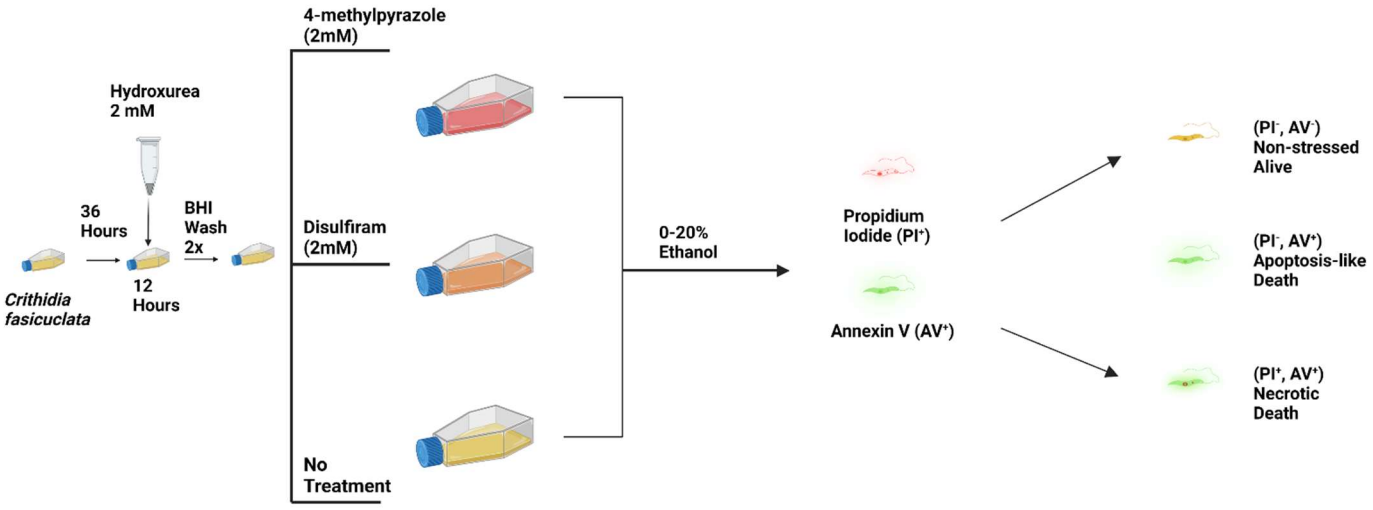
FALL 2022



Abstract

Given the early divergence of trypanosomatids in evolution and absence of genes central to "classical" cell death pathways, induction, and mechanisms of regulated cell death in these species will shed light on origin of these processes. The early divergence of eukaryotic cells can be observed with the Trypanosomatids taxa (Green, 2015)

Crithidia fasciculata is a trypanosomatid, mosquito parasite. We explored sensitivity of *Crithidia* to ethanol (≤20%) on cell death and externalization of phosphatidylserine (PS) using propidium iodide (PI) and annexin V-FITC (AV). While 20% ethanol killed cells necrotically (PI^+ /AV^+), 9% ethanol resulted elicited nonlethal, PS externalization in a subset (2%) of the population (PI^- /AV^+). To elucidate the mechanism of this cell death-like phenotype, alcohol dehydrogenases (ADH) and aldehyde dehydrogenases (ALDH) were inhibited. I hypothesized inhibition of (ALDH) with disulfiram will increase proportion of stressed, alive cells (PI^-/AV^+) because of (ALDH) is used to process acetaldehyde with the enzyme (ALDH) that produces acetate that is used in many metabolic processes. While disulfiram yielded an increase in this subset of cells, effects of inhibition were ethanol independent. In contrast, the (ADH) inhibitor 4- methyl pyrazole caused necrosis. The different patterns of cellular death can be observed by the different staining. These results suggest cell death pathways are sensitive to fermentation products produced by trypanosomatids and that early evolution of "classical," cell death pathways could have been driven by responses to sublethal stress.

Introduction

Cell death, or cessation of the metabolic processes of cell, can occur by various, regulated pathways (Shen, 2000). In this study we look at the relationship of ethanol to apoptosis-like cell in *C. fasciculata*. Ethanol-induced cell death is dose-dependent and is known to induce apoptosis in animal cells (Cardoso, 1983). Yeast experience evidence of apoptosis-like cell death from exposure to ethanol (Ishemgulova, 2017). Because ethanol causes dose dependent apoptosis cell death in both yeast and animal cells and *C. fasciculata* is from an early divergent branch in the evolutionary tree. Apoptosis is the most well studied of the regulated pathways, but there is no apoptosis causing genetic material present in trypanosomatids (Gerasimov, 2019). Trypanosomatid autophagy is well established, but alternative pathways and biological significance remain mysterious (Gerasimov, 2019).

Crithidia fasciculata is a trypanosomatid parasite non-pathogenic to humans that can be safely used as a model to understand novel cell death pathways. The parasite *C. fasciculata* is monoxenous living only a single host: mosquitoes (Ho, 2021). Mosquitos go through major life stages of eggs, larva, pupa, and adult. *Crithidia fasciculata* is acquired by the larva stage in water other infected mosquitoes live around. With each molting which occurs around three times the hindgut lining is shed as well as all the *C. fasciculata* located in the hindgut (Schneider, 2012). Once mosquitoes reach the adult stage in the life cycle *C. fasciculata* will remain in the hindgut, rectum, and papillae rectum until death occurs for the adult mosquito. Crithidia fasciculata can replicate exponentially in the host mosquito so in this study it is proposed that the ethanol that is produced as a major by-product of the metabolic pathway of the Krebs cycle is used as a regulatory molecule to halt mitosis.

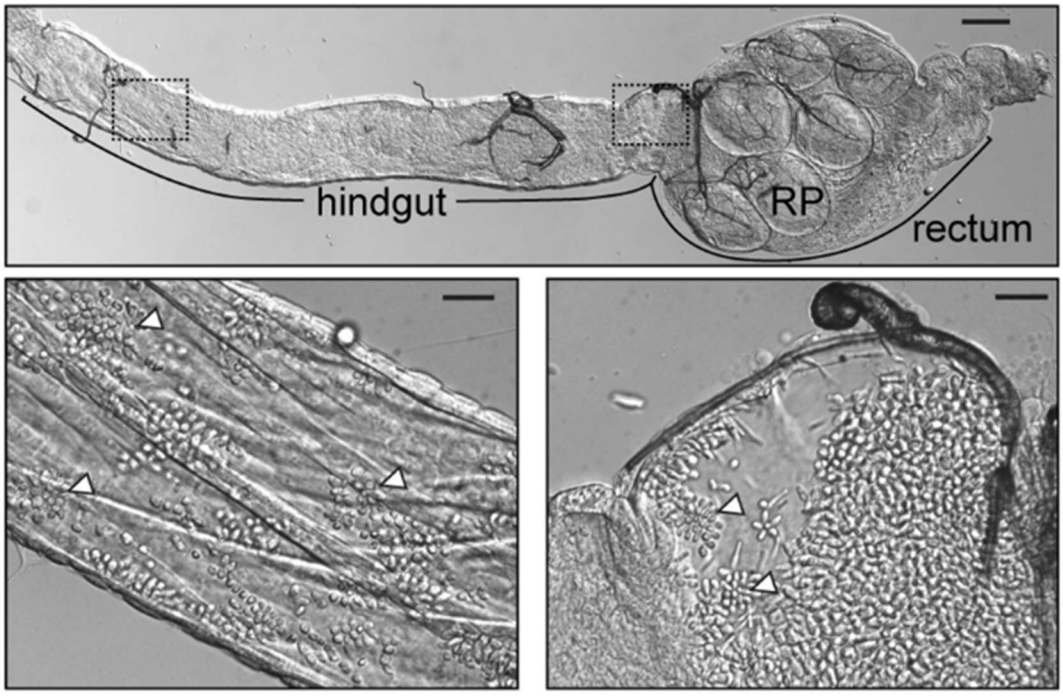


Figure 1 ***Crithidia fasciculata adheres to the mosquito hindgut, rectum, and rectal papillae (RP).*** As shown in the images above *Crithidia fasciculata* adhering inside *Aedes aegypti*. The author indicates adherence with the white arrow heads. Scale bar is 100 μm (Filosa, 2019).

Crithidia fasciculata has a dose-dependent relationship with ethanol. Carbohydrate metabolism processes observed in (figure 2) yields ethanol from pyruvate via the enzyme alcohol dehydrogenase (Castilla, 2004). In *C. fasciculata*, acetaldehyde is converted to ethanol using (ALDH). Alcohol can then be consumed using the enzyme (ALDH) to acetaldehyde. Acetaldehyde is converted to acetate via acetaldehyde dehydrogenase. Acetate is widely used in metabolism via derivatives like acetyl coenzyme or fatty acids (figure 2). One of the end pathways pyruvates take in *C. fasciculata* from (Aldehyde Dehydrogenase – Alcohol Metabolism). Crithidia fasciculata uses (ADH) to oxidize NADH via a redox reaction. Alcohol dehydrogenase in yeast have similar amino acid sequences, reactions to thiols, and reactions to gel filtrations to (ADH) in *C. fasciculata*. However, *C. fasciculata* (ADH) and (ALDH) is considered more efficient at NADH

oxidation because it has a lower K_m value then yeast so less cofactors are required for the enzymes (Castilla, 2004). Ethanol is produced and processed in *C. fasciculata.* Using inhibitors can demonstrate on how various parts of the metabolic process interact with signaling cell death pathways with the addition of ethanol as well as how does this impact mitosis and growth.

When considering an appropriate inhibitor that is used to inform about the various metabolic pathways that specifically targets the enzymes (ADH) and (ALDH). Disulfiram inhibits human and yeast acetaldehyde dehydrogenase (Schindelin, 2012) because it has a higher binding affinity then acetate. The use of disulfiram has yet to be used for *C. fasciculata* and inhibiting (ALDH). Acetaldehyde dehydrogenase is like the human enzyme from the amino acid sequences, and it is a variety of enzymes (Rieger, 2011). The inhibitor 4-methylpyrazole is also successful at inhibiting class I (ADH) oxidation of ethanol (Kitagaki, 2007).

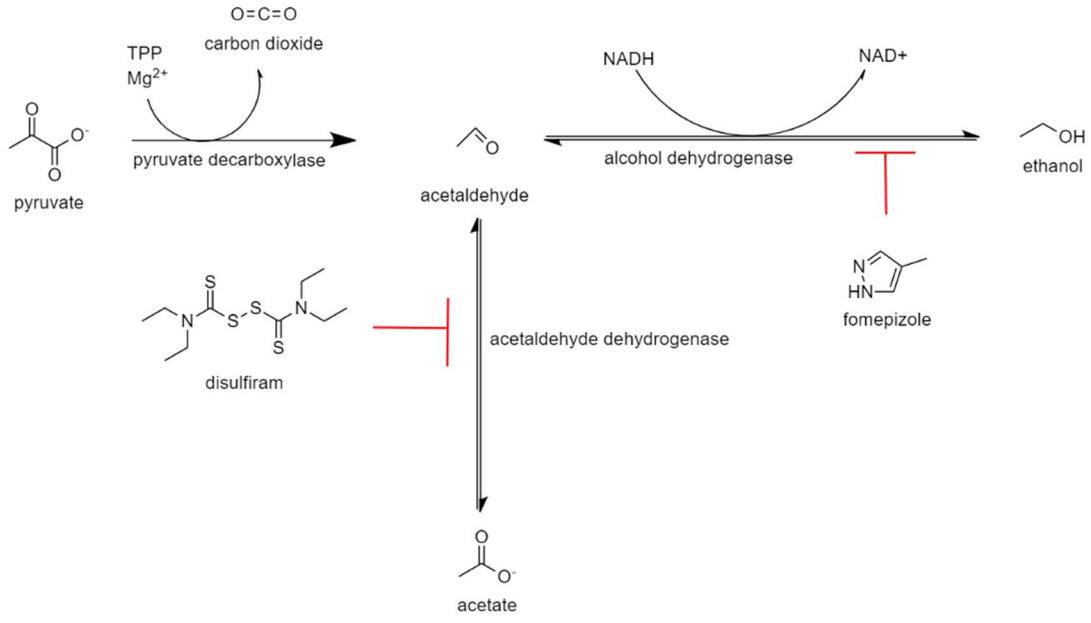


Figure 2. Metabolism of acetate and ethanol. Disulfiram and 4-methyl pyrazole inhibitors are included.

Common laboratory protocols rely on necrotically killing cells with 70% ethanol, but lower concentrations may cause less traumatic, regulated cell death or differentiation processes. Ethanol slows growth of *C. fasciculata*, growth rate resumes after 9 days with a treatment of 2% ethanol (Cazzulo, 1985). A stress mechanism is the *ZC3H30* gene in *Trypanosoma brucei*, an RNA-binding protein. This RNA binding protein when knocked out of *Trypanosoma brucei* and was exposed to only 1% ethanol for an hour, a change in the replication time of the cells was observed (Cevallos, 2017).This is believed to cause a stress related response that could eventually lead to the understanding of how apoptosis-like cell death occurs. This gene is identified in *C. fasciculata* (carter).

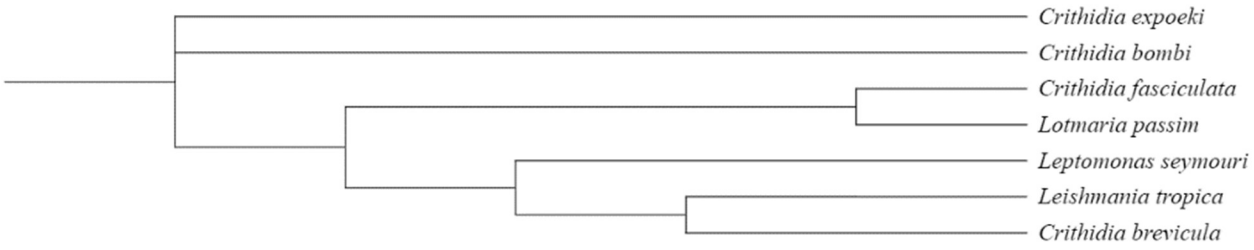


Figure 3. The phylogenetic tree shows the early splitting of the heteroxenous and the monoxenous parasites. Using 18S rRNA to compile the phylogenetic tree.

Methods and Materials

Cell Culture

Crithidia fasciculata (ATCC 11745) was incubated in (BHI, BD BBL™ Brain Heart Infusion) brain heart infusion media containing hemin (2.5 μg mL^{-1}) and penicillin-streptomycin (diluted 1:100, Corning®). Experiments were all started using 5 mL cell cultures at a density of 10^6 cells $mL^{-1}$and grown in a shaking incubator at 27°C. Maintenance cultures were passed every five days at a starting density of 10^6 cells mL^{-1}. Cell lines only existed for 1 month before proper disposal and new cells were unfrozen.

Optimizing Cell Culture Synchronization

Synchronization of cell cycle with hydroxyurea ensures that experiments are performed with a population that remains in the same replication cycle of *C. fasciculata.* Optimal conditions were determined for hydroxyurea exposure time. Cells were incubated in 2mM hydroxyurea for 12 hours before washing twice with fresh BHI. Vortexing during each wash step breaks up the pellet. Additionally, BHI should be added to resuspend the washed cells to a final volume of 5 mL. Cell density doubles every ~4 hours following removal of hydroxyurea.

Stress and death caused by ethanol

Phosphatidylserine (PS) externalization is an indicator of apoptosis in mammalian cells (Aldehyde Dehydrogenase, 2012). This was assayed in *C. fasciculata* using FITC-labeled annexin V (BD Pharmingen™). Prior to exploring the impact of ethanol on cell death, positive and negative

controls for (AV) staining were performed using a thermal stress protocol developed previously in our laboratory (unpublished results). Briefly, 100 µL of cells were warmed to 62 ℃ for 30 minutes in a water bath (positive control), followed by cooling in an ice bath for 5 minutes. Cells without heat shock were used as a negative control. Using (PI) to identify dead cells and (AV) to see if any cells experience apoptosis. Fluorescence of (AV) ($\lambda_{excitation}$ = 560nm; $\lambda_{emission}$ = 595nm) and propidium iodide ($\lambda_{excitation}$ = 535nm; $\lambda_{emission}$ = 615nm) results were measured using an epifluorescence microscope (Zeiss Axio Imager.A1). All cells in the thermal treatment were (AV^+/PI^+) necrotic. Imaging settings were carefully controlled. An exposure time of 65ms and gain of 4.1 were used for phase contrast and propidium iodide. Annexin V-FITC was imaged with exposure time of 135ms and a gain of 4.1.

Crithidia fasciculata is a parasite that is from an early branching of the eukaryotes. Ethanol causes a dose dependent cell death that in lower concentrations causes an apoptosis-like cell death and in higher concentration necrosis occurs. This is investigating how does ethanol impact apoptosis-like cell death using the inhibitors of (ADH) and (ALDH) which are a part of different parts of the metabolic pathway. For ethanol treatment, a stock solution of 90mL of BHI with a cell density of 10^6 cells mL^{-1}. The stock solution was divided equally into eighteen flasks. Cell cultures were incubated at 27 °C with shaking for 36 hours. Triplicate flasks were treated with 0% (negative control), 9%, and 20% ethanol (positive control). In concert with fluorescence imaging, density of cultures was measured. If needed to ensure sufficient number of cells in a field of view, sample density was adjusted to a density of 10^7 mL^{-1}. Cells were washed twice in 90 µL PBS. For each wash, cells were centrifuged at 2500 rpm at 20 ℃ for 5 minutes, supernatant was removed, cells resuspended in 90 µL of (AV)binding buffer, and vortexed for 5 seconds. Cells were stained at a final concentration of 1.5 µM propidium iodide and by addition of 10 µL (AV) Prior to imaging,

cells rested for 20 minutes. Image analysis was conducted using ImageJ and GraphPad version 9.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. to obtain percentage of necrotic cells (PI^+, AV^+) as a function of the total. Data were analyzed using a one-factor ANOVA test and t-tests for the impact of varying ethanol concentrations and the interaction on the measured cell death.

Observing stressed and dead cells from varying doses of ethanol

Synchronized cells at 36 hours of cell incubation in standard conditions were treated with 100 µM disulfiram. with resuspension during the final wash. Cells were then returned to the incubator for an hour. After pretreatment with disulfiram, ethanol was added to 9% or 20%. No ethanol was added to the negative control flasks (0% ethanol). Cells were incubated three hours after addition of ethanol.

During the incubation period, cell density was assessed so treated cells could ultimately be resuspended to a final density of 10^7 mL^{-1}. The cells are transferred to a microfuge tube. The cells were then washed with 10µL 1xPBS and centrifuged at 1,200 × g for 5 minutes at 21°C twice with the supernatant removed. Added to the solution of washed cells is 90µL of 1x (AV) binding buffer (freshly prepared) is then vortexed in the microfuge tubes to break up the pellet of cells. Then 90µL was removed and added to a new microfuge tube. Propidium Iodide with a final concentration of 1.5:100 concentration is added to the tubes. Then 1:10 concentration of (AV) is added to the tubes and set in a dark drawer for 20 minutes. The slides are prepared in a dark microscope room to avoid light bleaching, which is very common for (AV).

Images were analyzed with ImageJ (37). Pre-processing of all images was performed by adjusting the threshold using Otsu's threshold clustering algorithm or commonly referred to as OTSU, selecting a threshold, smoothing, and conversion to binary. The resulting black-and-white images are inverted to have the cells be black on a white background. Then the images are analyzed using an algorithm that recognizes a binary object that is present in contrast to the background with the pixel range set to 10-250 that can identify and measure the pixels that are different. Masking the cell images ensures all the cells are counted appropriately.

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Disulfiram, 4- methyl pyrazole, and Ethanol

The cells are started with the density of 10^6 cells mL-1 in BHI media in the shaken incubator at 27°C, which is the standard condition in triplicates. After 36 hours, the cells were grown and synchronized using 2mM hydroxyurea for 12 hours, then washed twice with fresh BHI. Cells were grown in incubation in standard conditions. Disulfiram is added to the nine designated flasks; the final concentration is 2mM in each flask, while the cells are in the falcon tubes during the rinses. Then .83 µl of the liquid 4-methyl pyrazole is added to nine designated flasks. The final

concentration of each 5mL flask with 4-methyl pyrazole added is 2 mM. The cells are added to the flasks and then returned to the incubator for an hour. After the hour, the ethanol 9% and 20% concentrations are added to the appropriate flasks. Then the three hours wait occurs.

During the three-hour wait, the cells are counted using a hemocytometer to calculate the concentrations, so the cell density used for staining is 10^7. The cells are transferred to a microtube. The cells are then washed with 10µL 1xPBS and centrifuged at 1,200 × g for 5 minutes at 21°C twice with supernatant being removed. Then 90 µL of freshly made 1x (AV) binding buffer is then vortexed in the microtubes to break up the pellet of cells. Remove 90 µL to a new microtube. Propidium iodide with a final concentration of 1.5 µM is added to the tubes. Then 10 µL of (AV) is added to the tubes, and then they are set in a dark drawer for 20 minutes. In a dark microscope room, the slides are prepared to avoid light bleaching, which is very common for (AV).

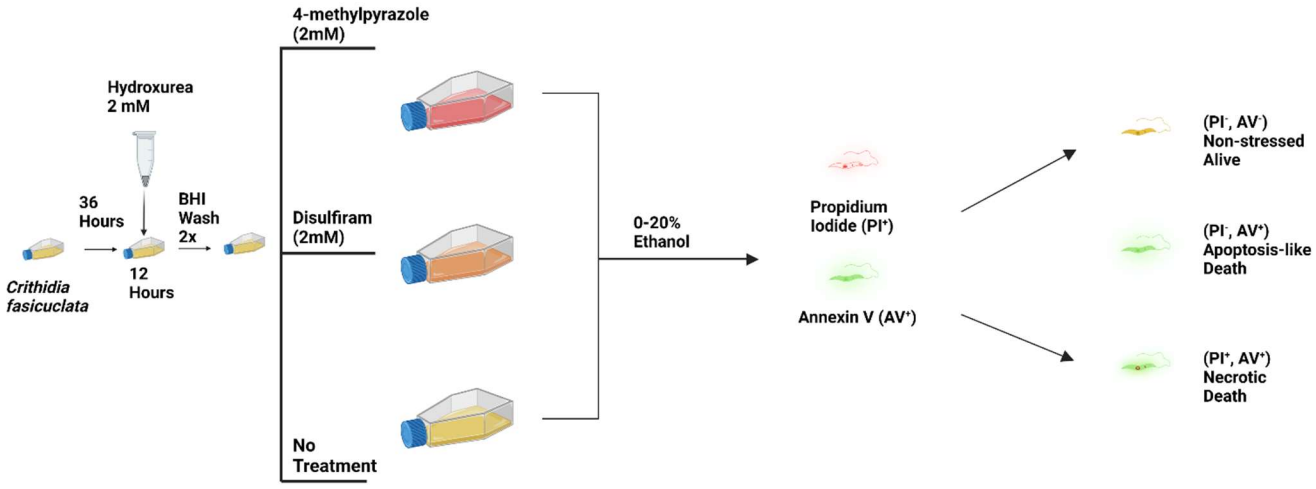

Figure 4. The role of ethanol metabolism in cell death of *Crithidia fasciculata* was assessed in a two-way design utilizing ethanol and inhibitors of pathways of ethanol metabolism.

Results

Synchronization of cell cultures was achieved using 2 mM hydroxyurea. (AV) shows the PS-binding protein flipped to the outside of the membrane when the cells are stressed, about to die, or died, which propidium iodide can indicate by penetrating the membrane (PLOS, 2014). Living cells are able to exclude PI from the cell and are (PI^-). However, the externalized phosphatidylserine indicates that the cells are in a stressed state.

Figure 5. These cells above are synchronized with 2mM hydroxyurea. Cytokinesis can be observed in cells by the presence of separatory anchor ends. By observing morphological changes that mitosis is occurring in synchronous with the surrounding cells with the final step of cytokinesis can be observed.

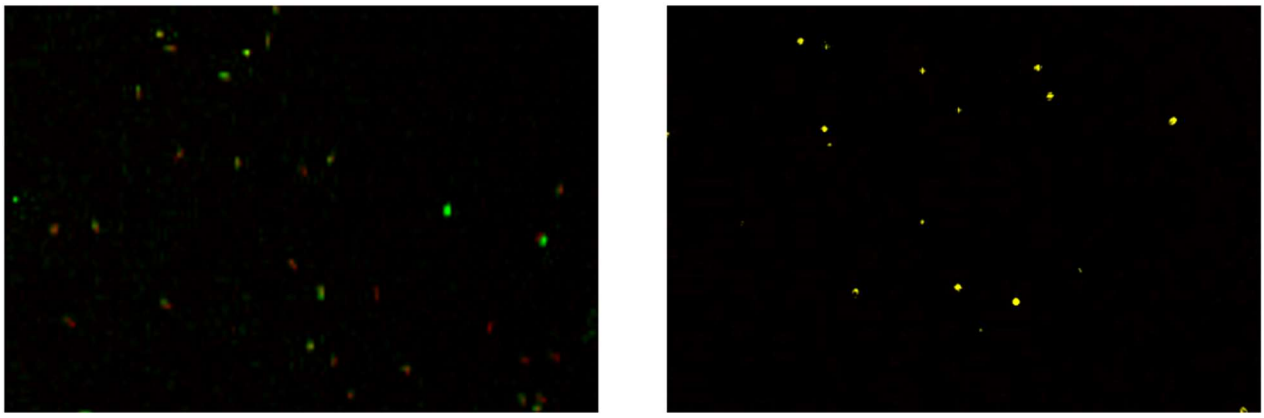

Figure 6. Cells that die necrotically stain for both PI and AV. (A) Cells heated to 62 °C for 30 minutes. (B) Control cells not exposed to high temperatures. *Red,* propidium iodide. *Green,* annexin V-FITC.

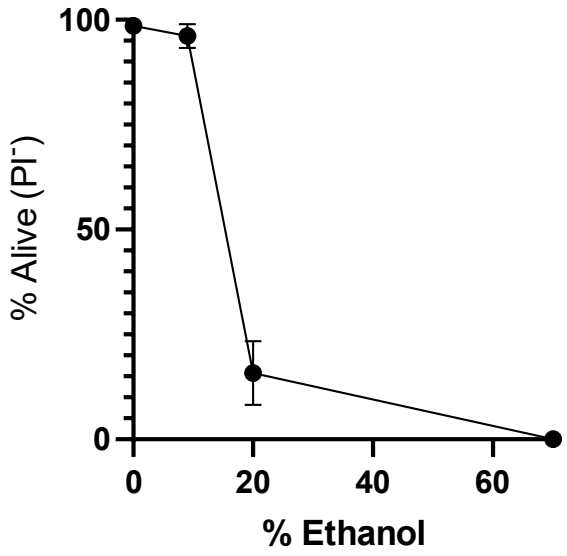

Figure 7. LC_{50} graph of varying concentrations of ethanol with C. fasciculata. After 3 hours of exposure to varying concentrations of ethanol the calculated LC_{50} is 15.17%. The formula below is how the curve is calculated for the LC_{50} graph.

Ln(Odds) = 0 = $\beta 0 + \beta 1 * X$, we can solve for X:

$\beta 0 + \beta 1 * X = 0$

$\beta 1 * X = -\beta 0$

$X = -\beta 0 / \beta 1$

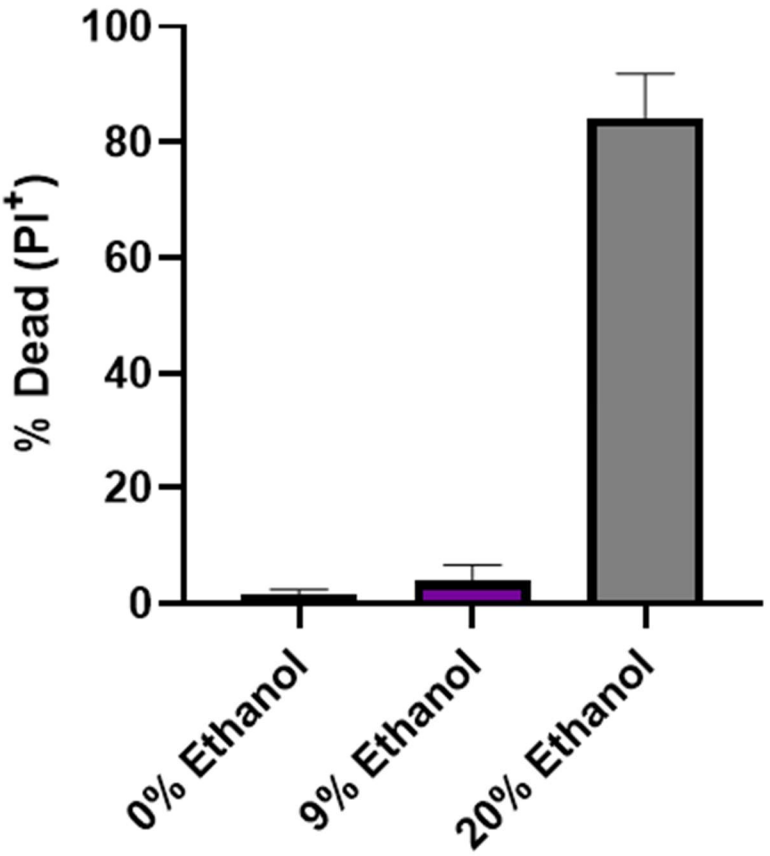

Figure 8. Cell death significantly increases at 20% ethanol. *Crithidia fasciculata* when exposed to varying concentrations of ethanol when a one-way anova is preformed it shows significance of $P < 0.0001$.

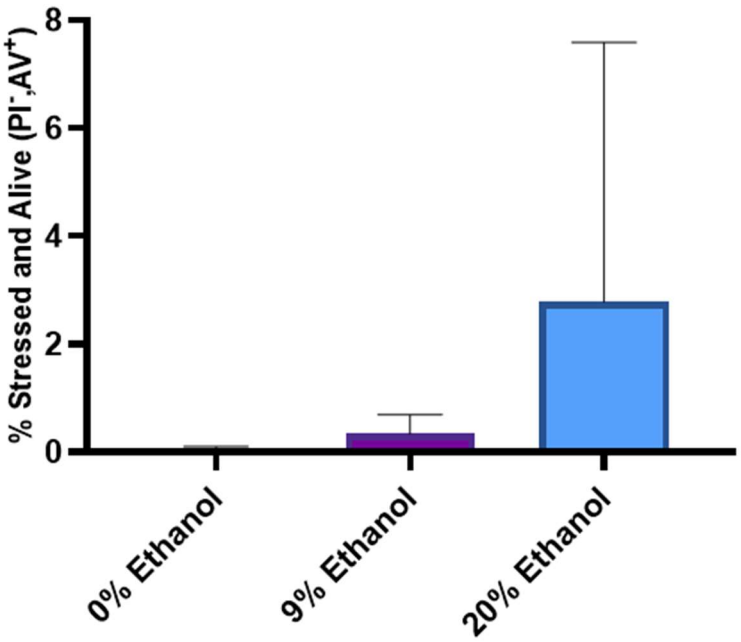

Figure 9. Using varying concentrations of ethanol with synchronized cells after 3 hours of exposure. One-way anova showed differences were marginally significant (p=.0547, ANOVA, n=3).

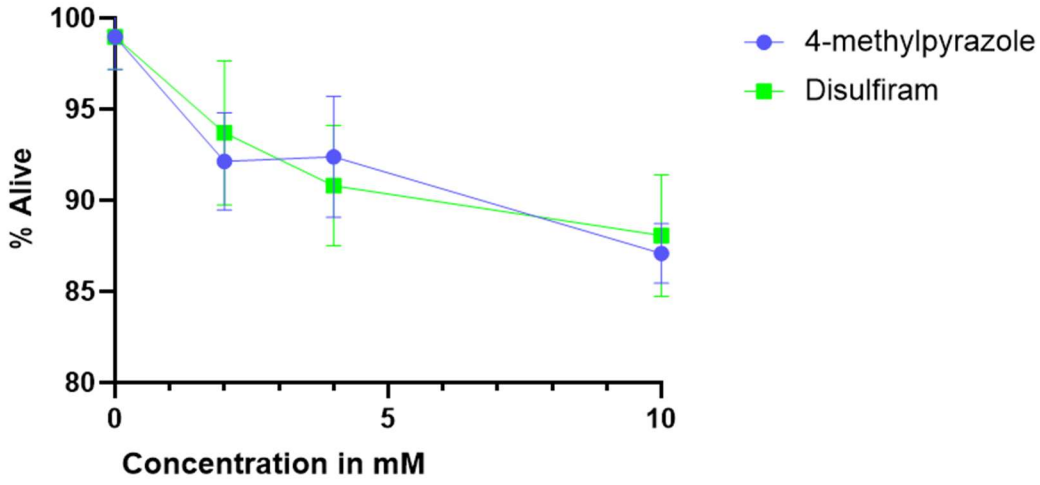

Figure 10. The lethal concentrations of Disulfiram at 2mM 4mM, and 10mM. Disulfiram and 4-methyl pyrazole (2-10 mM) only kills around 10% of *C. fasciculata* with 4 hours of treatment.

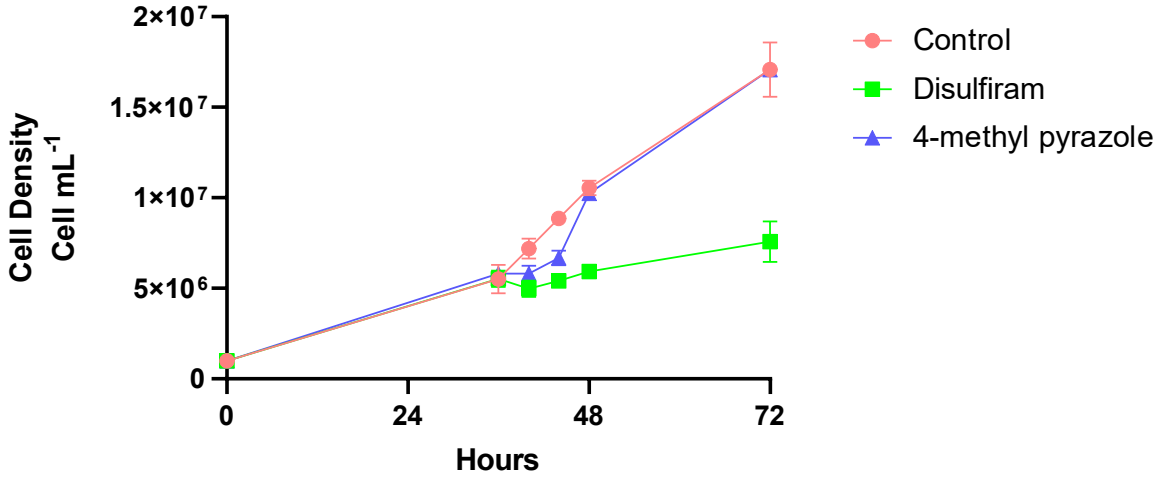

Figure 11. The growth curves of the inhibitor's disulfiram and 4- methyl pyrazole compared to a negative control. There is a 12 hour delay in the growth curve after the introduction of 4-methyl pyrazole.

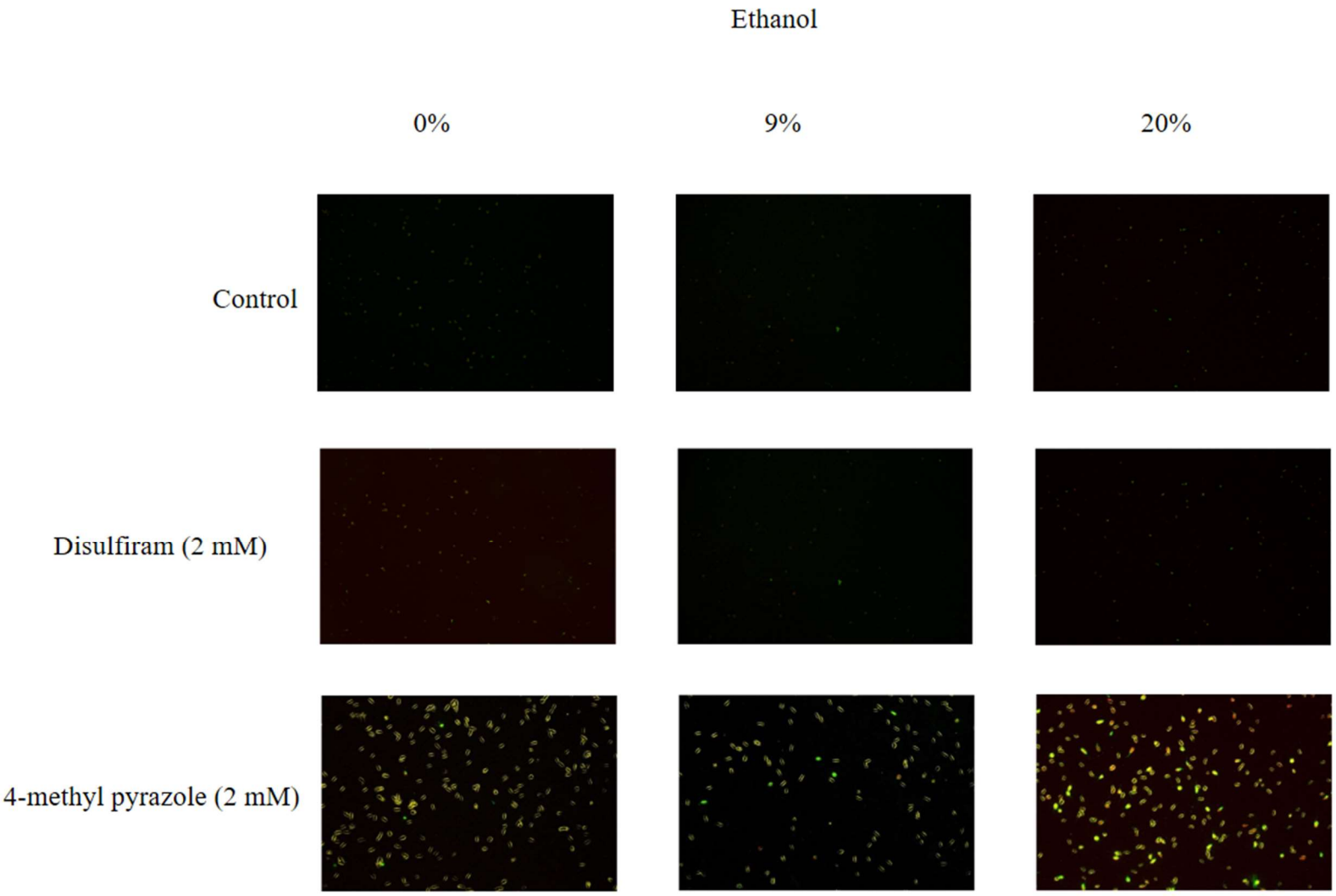

Figure 12 The (AV^+) cells are located on the left and the propidium positive cells are on the left of each image. The treatments are listed above the corresponding images. Samples viewed

under (AV) had an exposure time of 350 and gain of 4.9. Propidium iodide was viewed at an exposure time of 65 and gain of 4.9.

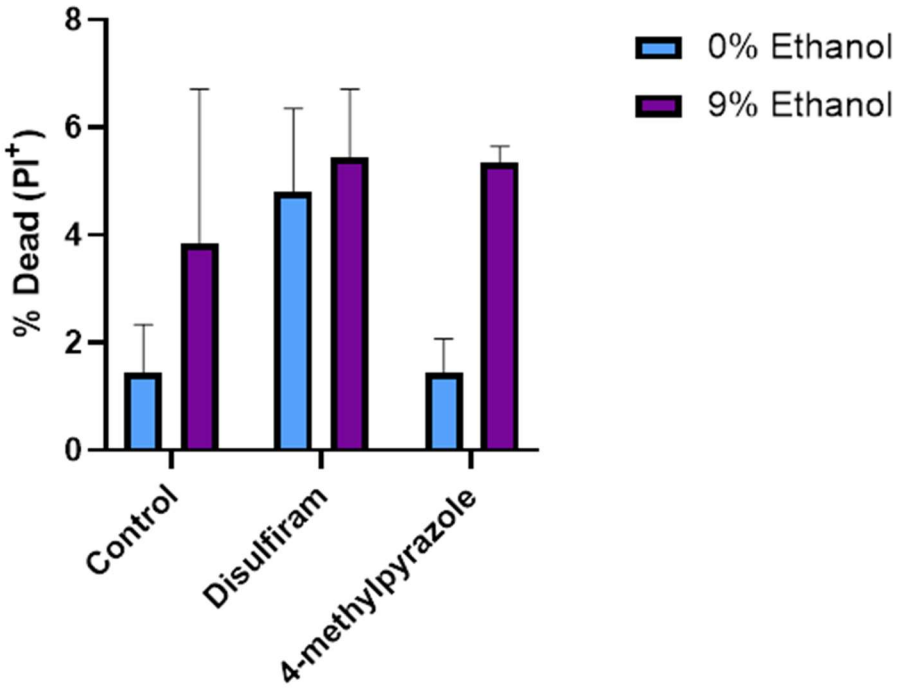

Figure 13. With a lower amount of cell death. Disulfiram 2 mM with no ethanol had more cell death then the control and 4-methyl pyrazole 2 mM. A t-test shows no significant difference caused from 9% ethanol (p= 0.1324).

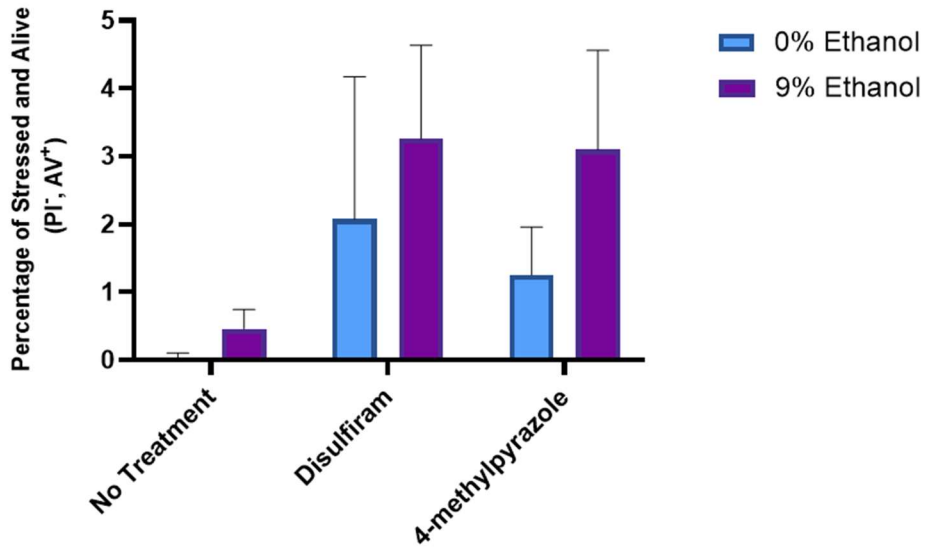

Figure 14. There is a greater amount of stressed and alive cells with inhibitors 4-methyl pyrazole 2 mM and disulfiram 2 mM than the negative control cells. A t-test showed ethanol had no significance (p=0.1923). Disulfiram and 4-methyl pyrazole had similar percentage of stressed and alive cultures (PI^-, AV^+) and both was a greater amount then control with less than 1% of stressed and alive cultures.

Discussion

Ethanol does cause apoptosis-like cell death in *C. fasciculata* also when treated with disulfiram that inhibits the enzyme (ADLH) also cells that are treated with 4-methyl pyrazole. The purpose of these experiments is to help identify reliable ways of inducing a unique cell death pathway called apoptosis-like cell death in *C. fasciculata*. This is important because this a single celled organism that can undergo an apoptosis-like cell death process. The apoptosis-like cell death is observed at exposure to low concentrations of ethanol (Figure 9). *Crithidia fasciculata* produces ethanol as an offshoot of the metabolic pathway (Castilla, 2004). The slight increase in cell death compared to the negative control cells can also be observed in the LC_{50} graph (figures 13 and 10). There is a low amounts of cell death around 10% in the graphs with the addition of ethanol. It is an interesting finding that is likely related to disulfiram impacting more than just the acetate not being broken down.

A mere 1% of ethanol causes a delay in the growth curve and yet one of the byproducts of its metabolic pathway is producing ethanol (Cevallos, 2017). There is a regulatory death pathway that has a dose dependent relationship with ethanol for 9% ethanol causing apoptosis-like cell death in 4-methyl pyrazole and disulfiram but when 20% ethanol is added necrosis is observed. Keep in perspective that the host is a mosquito, and *C. fasciculata* only get to occupy the hindgut, rectum, and rectal papillae (Filosa, 2019). Given it is such a small volume that is allowed and a limited supply of glucose, having a mechanism that signals to delay the rapid growth of *C. fasciculata*. Ethanol in small concentrations may temporarily slow the cell cycle, until ethanol has been processed, for the very possible greater survival of all *C. fasciculata* in the mosquito is just an evolutionary advantage.

Future projects that the findings of this paper can lead to is a way of reliably causing apoptosis-like cell death to be observed using 4-methylpyrazole or disulfiram and 9% ethanol to capture in detail the morphological changes of the apoptosis like cell death pathway, as well as leading to the discovery of the genes that activate the apoptosis-like cell death pathway. Thinking about the host of *C. fasciculata* there could be experiments that measure the amount of ethanol present and the amount of ethanol present and if there were behavioral changes because of the ethanol produced. This can all lead to a greater understanding of how the relationship of not just *C. fasciculata* but other trypanosomatids to the host insect.

Conclusion

Apoptosis-like cell death may have appeared early in evolution, and investigation of early diverging lineages may inform our understanding of development of these pathways. Trypanosomatids belong to an ancient lineage that lacks classical apoptosis genes yet present apoptosis-like phenotypes in response to low ethanol concentrations (Gerasimov, 2019). Additionally, inhibition of (ADH) induces a 12 hour delay in growth rate and drastically increased back to the growth rate of the cultures exposed to 4-methyl pyrazole. Yet inhibition of (ALDH) showed a greater amount of cell death, stressed and alive cells, and a delay in the growth curve that after 36 hours of exposure to disulfiram is still delayed (figure 11). Inhibition of (ADH) and (ALDH) appear to drive cell death through a path independent of ethanol, suggesting that cell death observed in the presence of ethanol may represent an alternative mechanism at work (Castilla, 2004). Varying concentrations of ethanol causes a variety of regulated cell death pathways. Because lower concentrations of 9% ethanol are shown to cause apoptosis-like cell death and 20% ethanol causes necrosis in *C. fasciculata*.

Ethanol is a byproduct of the major metabolic pathway (Cevallos, 2017). Mosquitos are the host of C. *fasciculata* and the limited space of the hindgut, rectum, and rectum papillae. In order to not overwhelm the host and killing the mosquito *C. fasciculata* possibly uses the byproduct of ethanol to slow the growth rate (Cevallos, 2017). These experiments help peel back the curtain just a little bit about the possible development of apoptosis that our human cells do every day.

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