5-2022

# A Mechanistic Study on the Immunological Effects of a Novel Anti-Schistosomal Compound

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## Abstract:

Schistosomiasis is a condition caused by a parasitic worm infection that is one of the primary neglected tropical diseases. It infects approximately 250 million people every year, and it causes the second largest economic impact from any parasitic disease. There exists a treatment for Schistosomiasis; however, it is ineffective against the juvenile stages of the worm. A novel drug has been developed that has been illustrated to have efficacy against all stages of worm infection. However, before this drug can be utilized, its method of action must be determined. Preliminary single cell transcriptomic data from our lab suggests that our compound is acting to enhance the immune response of granulocytes. To further characterize the response of our novel compound, we performed a series of assays to quantify how the compound affects cellular processes, such as phagocytosis, and how this compound affects the transcriptomic state of HL60, an immortalized promyeloid cell line.

# Background:

## Schistosomiasis:

Schistosomiasis, which is also known as snail fever, or Katayama fever [1], is an infectious disease caused by a parasitic flatworm from the genus *Schistosoma* [1]. The symptoms of Schistosomiasis include abdominal pain, abdominal swelling (Fig. 1), and diarrhea with long term infections leading to organ failure and cancer [2]. The World Health Organization estimates that a quarter of a billion people are infected with Schistosomiasis each year, with up to 200,000 people dying because of complications from their parasitic infections [3]. According to the Carter Center, Schistosomiasis is second only to malaria in terms of economic impact caused by a parasitic infection [4]. It is also listed as a neglected tropical disease, meaning that it is extremely common but lacks the funding that diseases such as HIV or malaria receive [5].

**Figure 1.** A picture taken in the Philippines of an 11-year-old boy with Schistosomiasis.

***Schistosoma mansoni***

While there are multiple species of *Schistosoma* worms that infect humans, our lab is primarily concerned with *Schistosoma mansoni*, which is mainly found in Africa, Brazil, Venezuela, and many other tropical countries [6]. Identified as a unique species by Patrick Manson in 1902, it has plagued the old world for 5 million years. *S. mansoni*, similar to other *Schistosoma*, is a trematode, colloquially known as a blood fluke [7]. Trematodes are flatworms that usually have a complex life cycle within two separate hosts [7]. Unlike other trematodes, *S. mansoni* are not traditional hermaphrodites—instead, the male and female worms exist in a symbiotic relationship (Fig. 2).


Figure 2. An electron microscopy image of a pairing of *S. mansoni* worms. The female worm exists within a canal inside the male worm. They are considered hermaphrodites even though they are gendered because it is impossible for the worms to mature into adulthood without "pairing up."

The life cycle of *S. mansoni* exists within two separate hosts—humans as the primary hosts (meaning that sexual reproduction takes place inside of humans) and snails as an intermediate host. Eggs are released into water from mature *S. mansoni*, which hatch in the water and release miracidia. These miracidia burrow inside of the snails and form sporocysts inside their tissue. After multiple "generations," the infected snails release cercariae, which are free-swimming and infective to humans. The cercariae pierce human skin through a hair follicle when people enter infected water. The cercariae become schistosomulae (i.e., juvenile *Schistosoma*). After this, the juvenile *Schistosoma* resides in the dermis and enters circulation. The schistosomulae circulate throughout the bloodstream and eventually migrate into the veins surrounding the left side of the heart where they pair into their monogamous relations. If the female is unable to find a suitable mate, she will stop maturing and die. After a suitable mate is found, the maturing, paired-up worms migrate into blood vessels surrounding the intestines called the mesenteric veins. Here, the female lays approximately one egg every 4.8 minutes, or 300 eggs a day. These eggs are deposited on the endothelial wall of the veins, where they will climb through and exit the host through the movement of feces (Fig. 3).

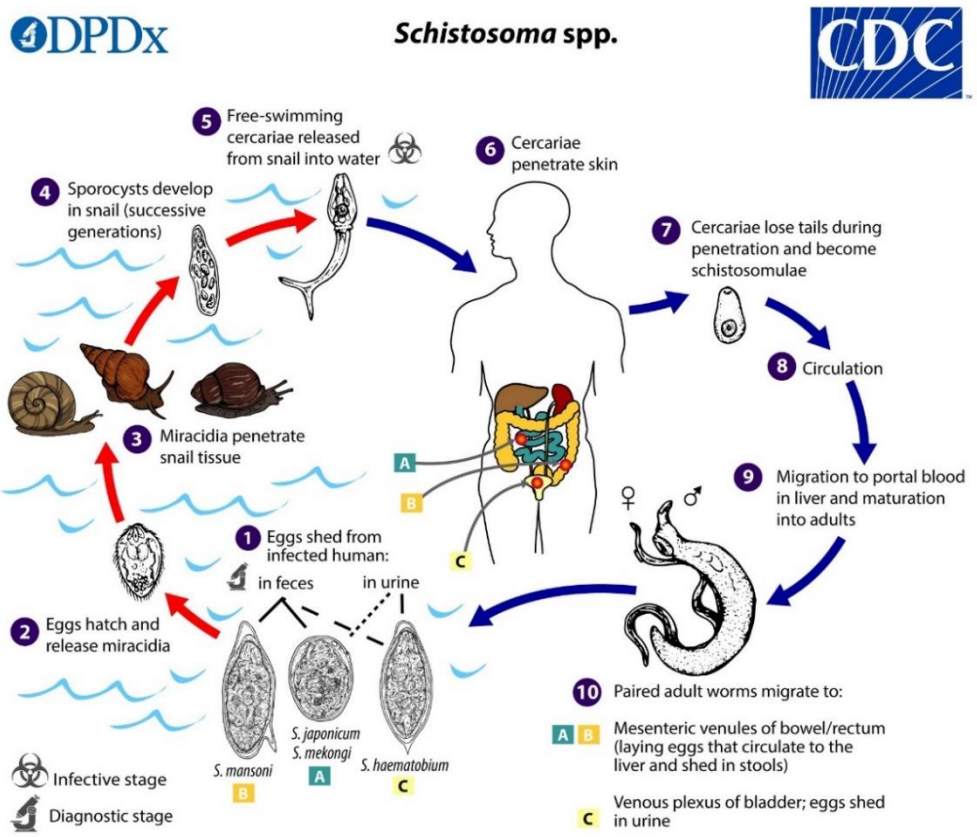



Figure 3. A representation of the *Schistosoma* lifecycle.

**Praziquantel:**

Fortunately, there exists a treatment for Schistosomiasis called praziquantel (Fig. 4). This compound is so imperative to treating Schistosomiasis that it is classified by the World Health Organization as an “Essential Medicine” [8]. The mechanism of action for praziquantel is poorly understood. The primary working theory is that praziquantel makes worms more permeable to calcium ions, which causes muscle contraction, which causes the worms to become paralyzed and the dislodged and freely flow through the host’s circulatory system. These free-floating parasites are then destroyed by phagosomes within the host organism [9].

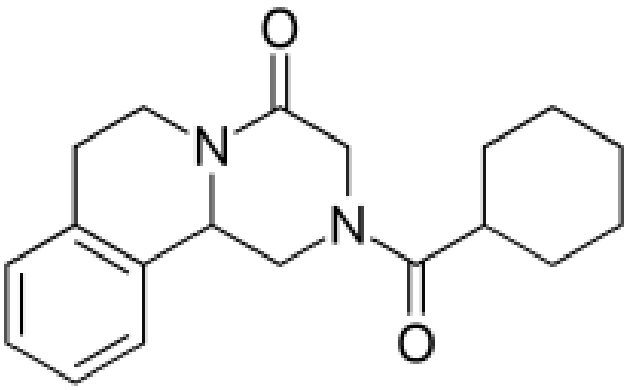



Figure 4. The molecular structure of praziquantel—an anti-Schistosomal drug

However, there are a few unfortunate downsides to praziquantel. First, praziquantel's mode of action is poorly understood. This is not a major drawback, as praziquantel has an extensive first-pass metabolism due to the fact that most is excreted from the body in the urine, and little of the drug makes its way into circulation. Second, wild-type *S. mansoni* worms have been observed to have developed resistance to the effects of the drug. This means that the drug has become less effective against parasites within the wild [10]. Third, praziquantel is ineffective against worms until they have reached maturity within the mesenteric veins [11], meaning that worms are not susceptible to the drug until six to eight weeks post-infection. Because of this, deworming campaigns almost impossible to conduct efficiently and safely, for two months after treatment has been administered to a community, Schistosomiasis can become a reoccurring issue as juvenile parasites mature into adults and lay their eggs within the water supply. Finally, perhaps the most pressing issue with praziquantel is that 80% of people treated with it would not take the drug again due to the bitter taste associated with the inactive *Sinister* enantiomer [12]. In other words, 80% of people would rather live with the possibly deadly side-effects of

Schistosomiasis than take seven to ten pills of praziquantel. To combat this issue, the World Health Organization has earmarked tens of millions of dollars to develop a chemical process that separates the racemic mixture of praziquantel enantiomers. However, this chemical process is both cost inefficient and time consuming.

**Novel Treatment:**

Therefore, there exists a clear and pressing need for a novel drug treatment that removes the unattractive qualities of praziquantel while still retaining its attractive qualities, including its oral administration, chemical stability at atmospheric temperatures, and efficacy against *S. mansoni*. The first step in finding a suitable replacement for praziquantel was looking at a molecule that has known anti-Schistosomal properties: Ro 13-3978 (Fig. 5).

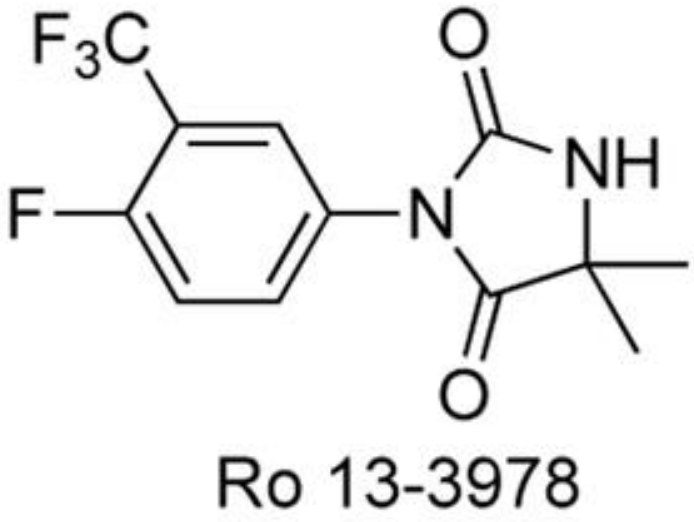



Figure 5. The chemical formula for Ro 13-3978 [13].

Ro 13-3978 has been shown to have potent anti-Schistosomal properties and is able to clear a majority of worms in an infected mouse with a 100 mg/kg dosage (Fig. 6) [13]. However, the drug is a known androgen receptor binder, meaning that if given to males, it will induce sterility and cause chemical neutering. This is an unideal outcome; nevertheless, through a series of chemical modifications, it is possible to retain the anti-Schistosomal properties of Ro 13-3978 while removing the anti-androgenic ones. The chemical structure of this molecule is still classified, but it was created by changing a series of atoms and removing the androgenic binding properties of the new molecule. This was tested using a simple androgen receptor binding assay. Our novel compound, dubbed C-01, has been illustrated to have efficacy against all stages of infection [14]. In fact, it is more effective than Ro 13-3978 at clearing *S. mansoni* infections (Fig. 7). The observed ED50 was found to be 7.8 mg/kg.

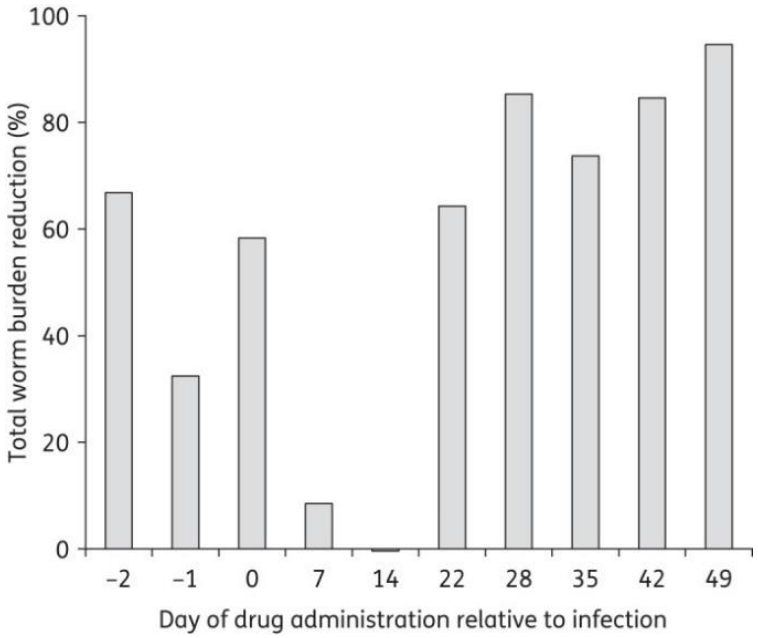



Figure 6. A worm burden reduction assay detailing the amount of *S. mansoni* worms cleared from infected mice (n=4). Courtesy of J. Keiser. [13]

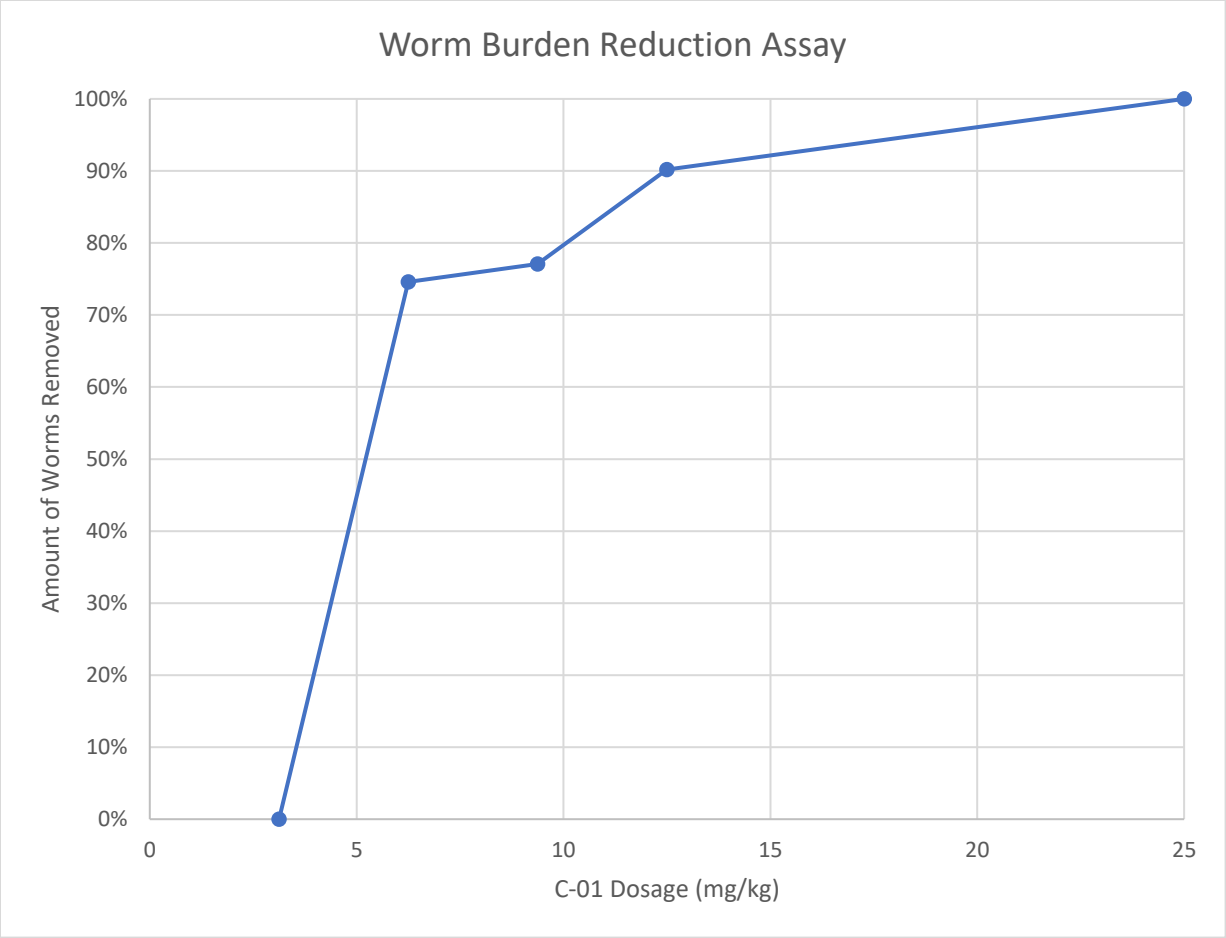



Figure 7. A worm burden reduction assay with differing doses of C-01. Full worm clearance was observed at a dosage of 25 mg/kg, which is lower than the 100 mg/kg dosage of Ro 13-3978 needed to obtain full clearance.

C-01 cytotoxicity was tested in a number of cell models through a 0.4% Trypan Blue analysis. In HFF, HEK293, and U2OS, no observable cytotoxic effects were observable until 200 uM, where 50 uM is the maximum concentration needed to achieve 100% worm clearance. There was also only a 20% decrease in viability at 200 uM, meaning that cytotoxic effects observed were found to be minimal at worst (Fig. 8).

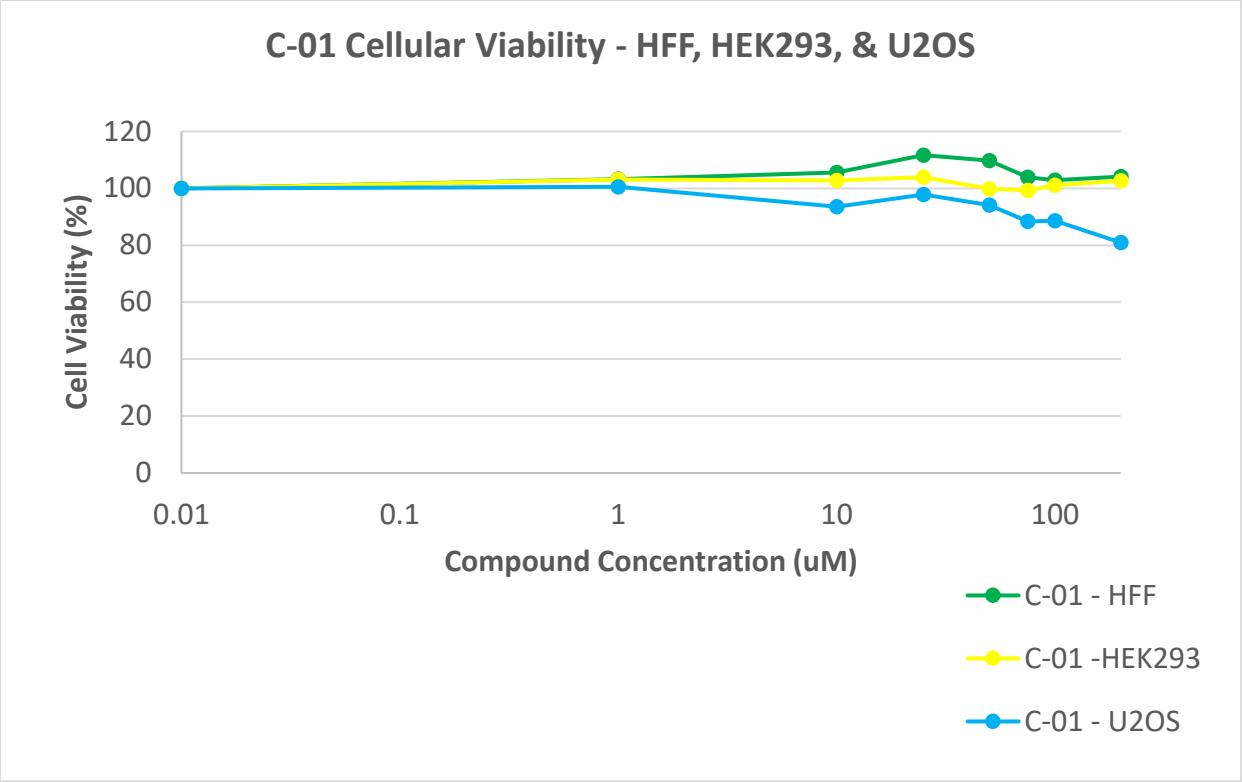



Fig 8. A viability analysis performed within 3 different cell lines using 0.4% Trypan Blue. The x-axis is logarithmically graphed to illustrate low cytotoxic effects at nominal concentration.

We have observed that our compound possesses efficacy against all stages of *S. mansoni* and has lost the anti-androgenic effects of its parent molecule; however, before it can be utilized in a human population, we must first understand its mode of action. To do this, our lab performed single cell transcriptomics on a murine splenosome (Fig. 9). This technique enabled us to look at the different transcriptomic states of solvent versus compound treated cells. From this plot, we observed a marked change in granulocyte populations. These cells are the primary attacking cells within the immune system. We also saw a 600% increase in reticulocytes, which are the precursors to erythrocytes. This is a key marker of inflammatory action within the

immune system called regenerative erythropoiesis, or the stress-induced regeneration of red blood cells. Together with the granulocytic activity, our current hypothesis is that C-01 is causing the immune system to become more active and aware of the foreign invaders within it. Phrased more simply, C-01 is causing the immune system to become more vigilant. In this thesis, we will further look at how C-01 is modulating the immune system.

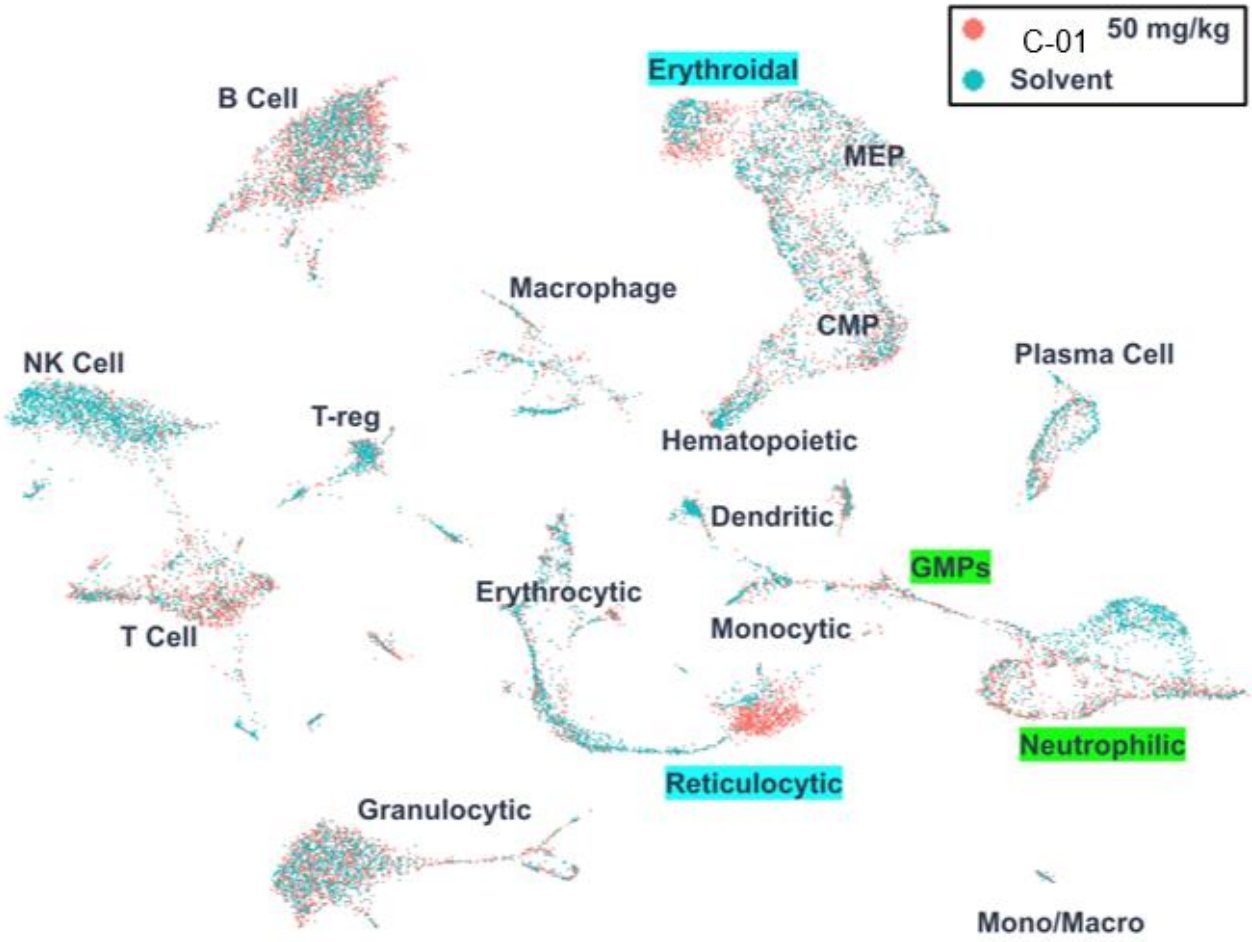



Figure 9. A single cell transcriptomic plot demonstrating the difference between the murine splenosome treated with C-01 and the control group.

## Materials and Methods:

### Compound Dilutions and Usage:

For *in vivo* work, our compound was diluted in a solution of 3% Tween-80, and 7% Ethanol. C-01 is only administered orally through proper oral gavage techniques. *In vitro*, C-01 is dissolved in dimethyl-sulfoxide (DMSO), which, due to its cytotoxic properties, is further diluted with the proper cell medium.

### Superoxide Dismutase Assay:

Superoxide anions are free radical reactive oxygen species (Fig. 10) of which the body has two uses: either it uses the energy stored within superoxide anions for cellular respiration, or it uses these reactive oxygen species as a method of attacking within an immune response [15]. We are most concerned with how these reactive oxygen species react within the immune system. Reactive species are a potent part of the cellular killing mechanisms, specifically against helminths like *Schistosoma* [16]. As this is the typical response observed against helminths, we believe that our compound could be affecting either the quantity of reactive oxygen species or the readiness of the immune system to create reactive oxygen species [17].

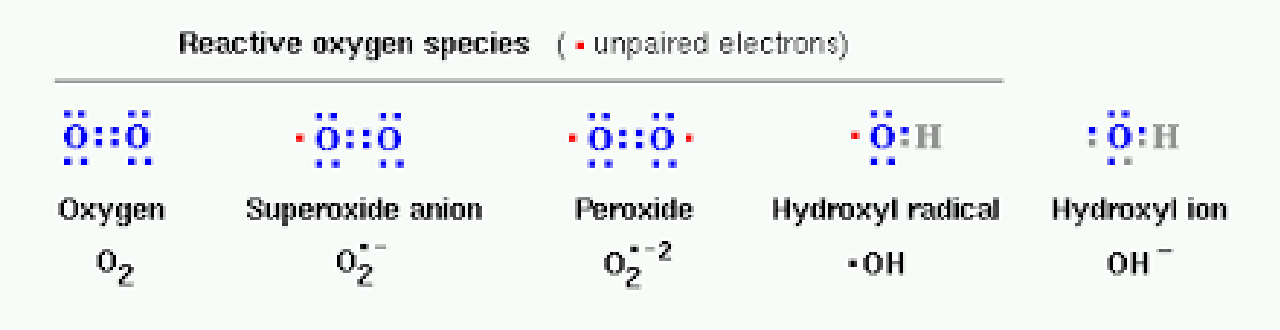



Figure 10. Chemical formulas of different reactive oxygen species. Blue dots indicate paired electrons, whereas red dots indicate unpaired (reactive) elections. Courtesy of Richard Bowen, Colorado State University.

To test this concept further, we performed a superoxide dismutase assay. Superoxide dismutase is an enzyme that reduces superoxide anions. We can color-metrically measure the amount of reduced superoxide at 550 nm [17] (Fig. 11). For this experiment, spleens were extracted from 3 BALB/C mice (n = 3), homogenized using a Tissue-Tearor, and enriched using Miltenyi Biotech magnetic separation. This was used to lessen the amount of traditional splenic immune cells and increase the number of specific cells—mainly eosinophils, which we believe to be the primary acting cell [18]. These cells were cultured throughout the assay in RPMI1640 with 20% fetal bovine serum (FBS). Cells were diluted down to 1,000,000 cells/mL with 100 uL used per group. For our superoxide dismutase assay, each group was performed in duplicate with a 100-uM cytochrome c control (to illustrate that the reduction is caused by reactive species and not another source). We took readings in real time with a three-minute gap between reads for each well for a total of ninety minutes. We used C-01 treatments of 150 mM, 30 mM, and 5 ng/mL of Phorbol 12-myristate 13-acetate (PMA) as a positive control. Cellular viability was assessed via a Trypan Blue stain at 99.3% viable after extraction from the mouse and 73.5% viable six hours after the completion of the assay.

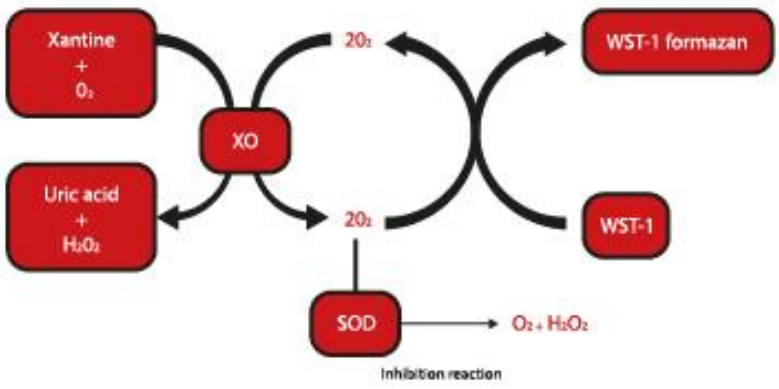



Figure 11. A graphical representation of the chemistry behind the superoxide dismutase assay. Courtesy of Themo Fischer Scientific.

**Luciferase ATP Assay:**

The next assay performed was one designed to measure adenine triphosphate content (ATP). ATP is a chemical compound that is known as the primary source of cellular energy [20]. It is a well-documented phenomenon that the body uses ATP to produce reactive oxygen species [21]. From this assay, we attempted to see the immune system prime itself for the creation of reactive oxygen species. We can measure the amount of ATP within a cell using an enzyme known as luciferase (Fig. 12). Luciferase oxidizes ATP into adenine monophosphate (AMP) while also producing light. This is the enzyme that allows fireflies to light up their thorax [20]. We used three BALB/C mice (n = 3) treated with 50 mg/kg C-01 24 hours before spleen extraction; another three mice were used as a solvent control. The spleen was homogenized until a single cell suspension was created; after this, the cells were resuspended in RPMI1640 with 20% FBS. A serial dilution of cells was created on a 96 well plate. The Luciferase enzyme was added, and the plate sat at room temperature for two hours to allow the reaction to occur. Following this, the plate was read on a plate reader measuring luminosity.

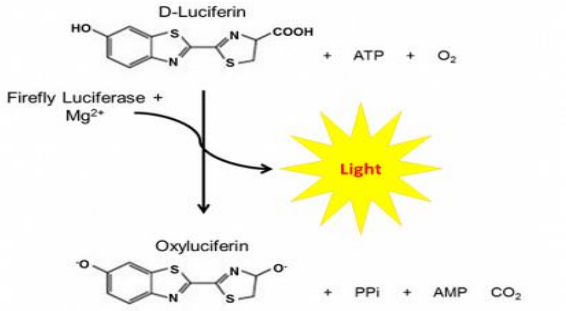



Figure 12. A representation of the luciferase enzyme reaction that utilizes ATP to produce light. Courtesy of Berthold Technologies.

**Resazurin Reduction Assay:**

Resazurin—or, as it is more commonly known, Alamar Blue—is a compound that can be reduced to form resorufin, a strongly fluorescent compound [22]. Reduction is the antithetical process to oxidation and occurs when compounds accept electrons. Electrons are the primary carrier of potential energy within a cell and are vital to the creation of ATP through cellular respiration. Resazurin is a strong electron acceptor; therefore, we can measure the number of free electrons within a cellular system (Fig. 13).

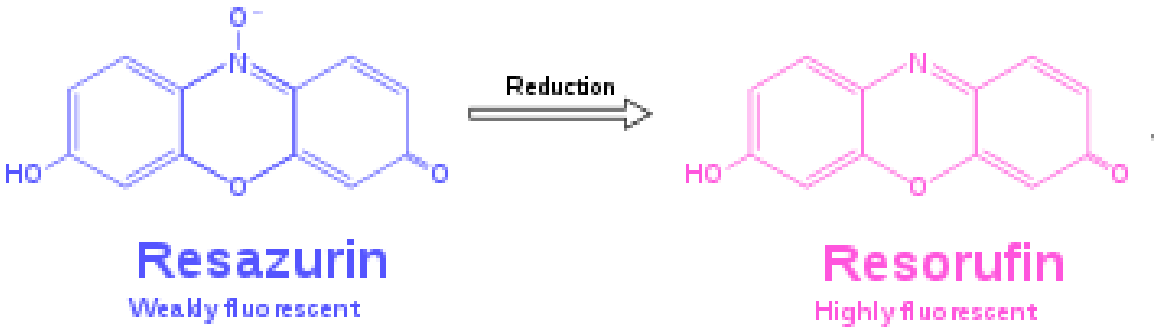



Figure 13. The chemical reaction showing resazurin being reduced to form resorufin.

For our experiment, three mice were orally administered 50 mg/kg C-01, while three mice were given the drug solvent. After 24 hours had passed, their spleens were extracted, homogenized, and filtered until a single cell suspension had been created. The cells were diluted down to a final concentration of 1,000,000 cells/mL, and 100 uL were put into each well. 100 uL of a 4% resazurin solution were added and allowed to incubate for four hours after which a fluorescent reading was taken at 540 nm.

**Endothelial Transcriptional Analysis:**

From our previous single cell transcriptomics analysis on the murine splenosome, we have begun to believe that C-01 has an immune enhancement effect. One of the ways that the body naturally does this is through the migration of specific cell types from the blood stream into the tissue (Fig. 14).

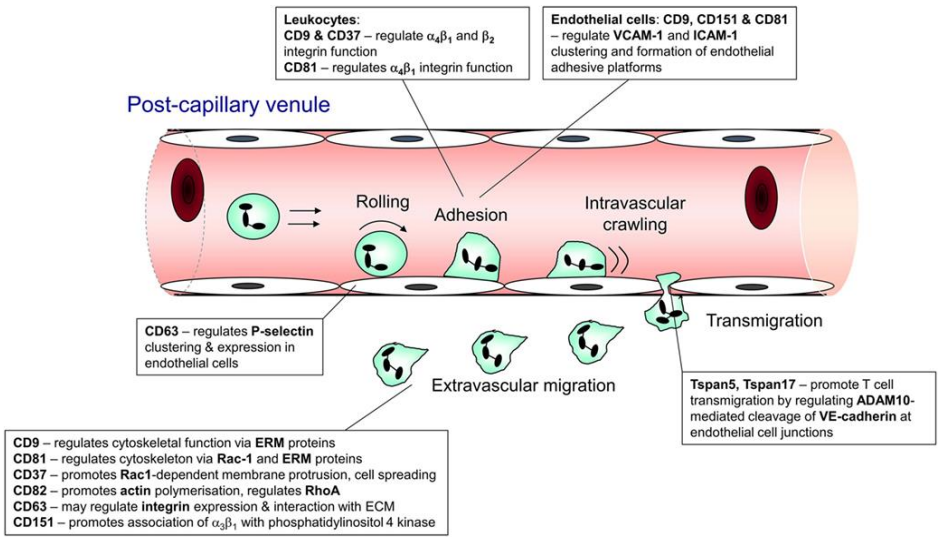



Figure 14. A representation of how cells migrate from blood vessels to tissue.

This series of rolling, adhesion, and migration is integral to attacking infections that are localized to a specific point. From our initial hypothesis that our compound is enhancing the body's ability to respond to a specific infection, we should be able to see a specific increase in migratory cytokines. We tested this by measuring the transcriptomics of C166 murine endothelial cells. This immortalized cell line was isolated from a mouse vein. Its usage allows us to measure if our drug is interacting with endothelial cells and causing the release of migratory cytokines. The cells were cultured to 70% confluence in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS at 37 ℃ in a 5% $CO_2$ atmosphere. After they had reached 70% confluence, adherence was removed using a Hank's Balanced Salt Solution with no calcium or magnesium; the cells were plated into a six-well plate and allowed to obtain adherence. 50 uM C-01 was placed onto the cells alongside 50 ng/mL TNF alpha as a positive control. RNA was extracted from these cells at 0 hours, 2 hours, 4 hours, 8 hours, and 24 hours using the New England Biotech RNA extraction kit. RNA was stored at -20 degrees Celsius for a week after which qPCR (quantitative polymerase chain reaction) was performed on the sample by a collaborator and normalized to the cells-only control.

**Phagocytosis Assay with RAW264.7 Murine Macrophages:**

Typically, from an immunomodulatory response, we also observe an increase or decrease in phagocytosis, which is the process by which cells engulf and remove foreign microbes. Inflammation is normally something that doctors look to suppress; however, we believe that our compound is acting as an immune enhancement drug. For this assay, we utilized an immortalized

murine macrophage cell line called RAW264.7. These cells were cultured in DMEM with 10% FBS at 37 ℃ in a 5% $CO_2$ atmosphere. The cells were cultured to 40% confluence after which they were removed via a 2% Trypsin wash and plated in a six-well plate and allowed to regain adherence. 50 uM of C-01 and 100 ng/mL lipopolysaccharide (LPS) were added as both drug treatment and positive control, respectively. The plates were incubated at 37 ℃ with a 5% $CO_2$ atmosphere for 4 hours. Fluorescently tagged *E. coli* bioparticles were added and allowed to incubate for two hours. Readings were taking on a plate reader at 480/520 nm, and pictures were taken on a fluorescent microscope.

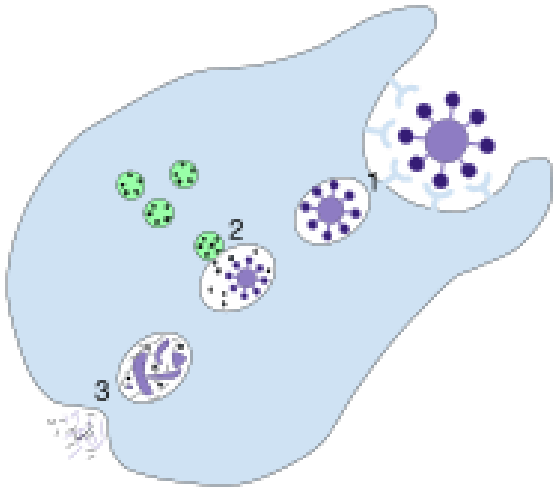



Figure 15. A picture representing phagocytosis of a microbe. The cell adheres to the microbe, engulfs it, and then digests it using the phagosome.

**Culture and Differentiation of HL-60 Human Promyeloid Cells:**

From previous single cell transcriptomic data, we know that neutrophils are the one of the most reactive cells with C-01, as we see an 80% decrease in inactivated neutrophils and a 500%

increase in activated neutrophils [19]. Because neutrophils are notorious for being unable to survive outside of the host for long, our solution was to utilize an immortalized promyeloid cell line (HL-60) that can be differentiated into a neutrophil morphology using different chemicals (Fig. 7). In essence, this means that we have the precursor cells that we can make into a neutrophil-like cell using a compound. These HL-60 were received from the ATCC and thawed according to manufacturer directions. Since these cells are frozen with DMSO as the primary cytopreservative, special measures must be taken as DMSO is a compound that will push HL-60 into a neutrophil-like morphology, after which cellular division no longer occurs and cell death becomes immanent. The cell medium was created according to the following formula: 79% IMDM (Iscove's Modified Dulbecco's Medium), 20% FBS, and 1% L-Glutamine-L-Alanine (4 mM). To do this, cells were cultured for 4–6-hour intervals at 37 degrees Celsius with a 5% $CO_2$ atmosphere immediately after thawing, and they were then spun at 200g for 10 minutes. Next, the media was aspirated to remove any potential contaminating DMSO. This process continued for 2 days; following this, the media was changed every two days following the spin-down procedure. After a week, a stable culture had developed. This culture was maintained on a 4-day pass schedule by taking an aliquot of cell solution and adding it to fresh medium. Cells were kept between 100,000 cells/mL and 1,000,000 cells/mL throughout the entire culture, for going under or over these constraints causes severe cell death or mutation. HL-60 can be differentiated into neutrophils through two different ways: the use of *trans*-retinoic acid and the usage of DMSO. Our lab selected the usage of DMSO, due to its creation of more transcriptomic regular neutrophils. To culture neutrophils, 1.3% DMSO was added into non-tissue treated T-75 cell culture flasks at 400,000 cells/mL and placed into a typical incubator.

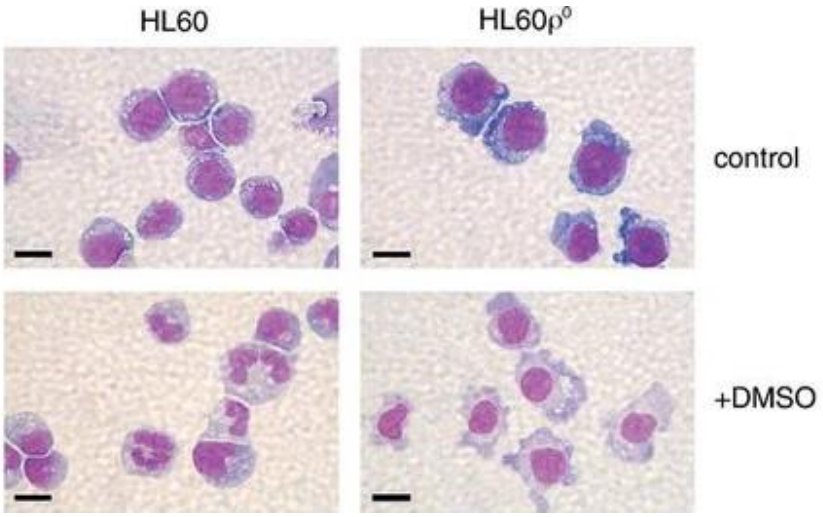



Figure 16. A modified Wright-Giemsa stain illustrating the ability of DMSO to differentiate HL-60 into a neutrophil-like morphology.

**Extraction of HL-60 RNA:**

HL-60 were cultured according to normal standards. Two different experiments were conducted: one with only undifferentiated cells and one with a differentiated group. The first experiment utilized undifferentiated cells with 50 uM C-01, 50 uM of C-01-59, and 100 ng/mL LPS. The purpose of this experiment was to see how these compounds affected the ability of HL-60 to differentiate on their own; in other words, we sought to observe whether our drug causes the creation of more neutrophils within the body. RNA was extracted at 2, 4, 8, and 24 hours using the New England Biotech Monarch Total RNA extraction kit. The extraction took place according to their procedure. RNA was stored at -20 ℃ for 2-3 days after which qPCR was performed. The second experiment utilized three different groups of cells. One group was undifferentiated and thus was simply cultured for 4 days in normal media, and another group was differentiated with 1.3% DMSO for 4 days. At the time of the experiment, the DMSO was

removed via a 200g spin for 10 minutes followed by aspiration and resuspension in fresh media. The last group consisted of cells with 50 uM of C-01 for 4 days. RNA was extracted with New England Biotech Monarch Total RNA extraction kits, and samples were stored for 2 days at -20 ℃.

**cDNA Synthesis from HL-60 RNA:**

cDNA was synthesized from RNA extracted from HL-60. RNA was assessed for quality via a Nanodrop, after which dsDNase buffer, dsDNase, and water were added to a final volume of 10 uL. These samples were warmed to 37 ℃ for two minutes and then put back on ice. DNA polymerase was added along with master mix. This was placed in the thermocycler for 10 minutes at 25 ℃, 15 minutes at 50 ℃, and the reaction terminated by heating the samples to 85 ℃ for 5 minutes. cDNA was stored at -20 ℃ for long term storage.

**qPCR Analysis of HL-60 Transcriptome:**

Primers were reconstituted to a final concentration of 100 uM and added to the master mix according to the New England Biolab specifications. Primer—master mix solution—was added to the tubes at a volume of 10.5 uL. Genetic material was added at a volume of 2 uL, and nuclease free water was added so the final volume in the tube reached 20 uL. After this, the tubes were gently centrifuged and placed in QuantStudio 3 for RTqPCR analysis. Cycle temperatures and times were used based on the master mix selection.

## Results:

### Superoxide Dismutase Assay:

In the superoxide dismutase assay, there was no discernable change in superoxide production after an *in* vivo C-01 treatment (Fig. 17). Our PMA controls functioned as expected, alongside the solvent controls. The results were found to be statistically significant ($p < 0.05$).

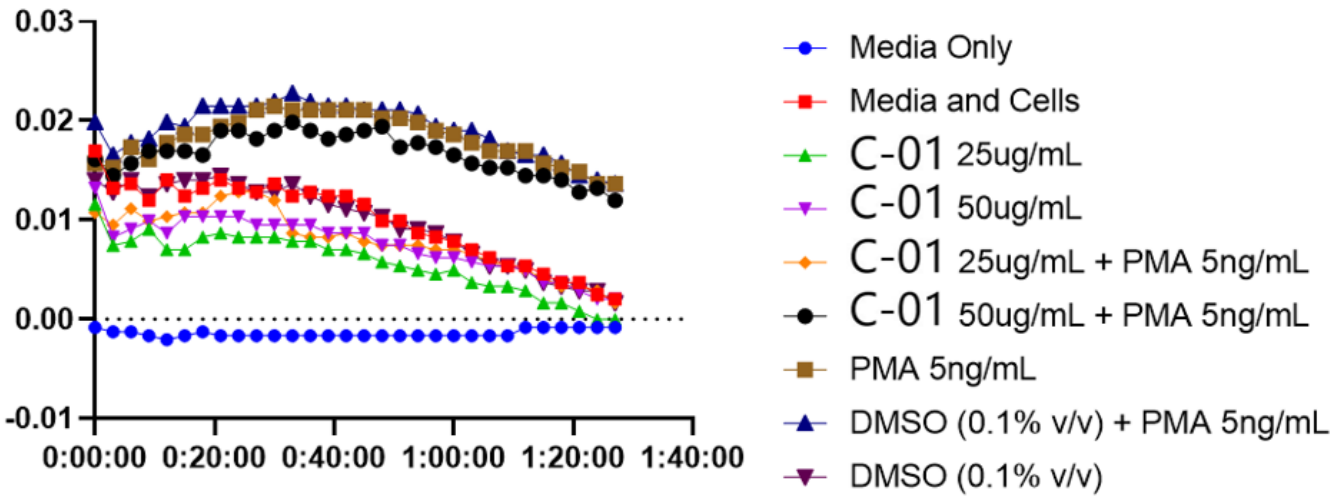



Figure 17. A Superoxide dismutase assay performed on eosinophils taken from a mouse (n = 3) and treated *ex vivo* with C-01. Readings were taken in real time on a Synergy HT plate reader at 550 nm and normalized to a cytochrome c control. The y-axis denotes nanomoles of superoxide and the x-axis is time after addition of PMA.

In our Luciferase ATP assay, we observed a serially diluted decrease in ATP concentration as the cells are serially diluted. We also observed a decrease in cellular ATP

concentration from an *in vivo* C-01 treatment (Fig. 18). Results were found to be statistically significant ($p < 0.01$).

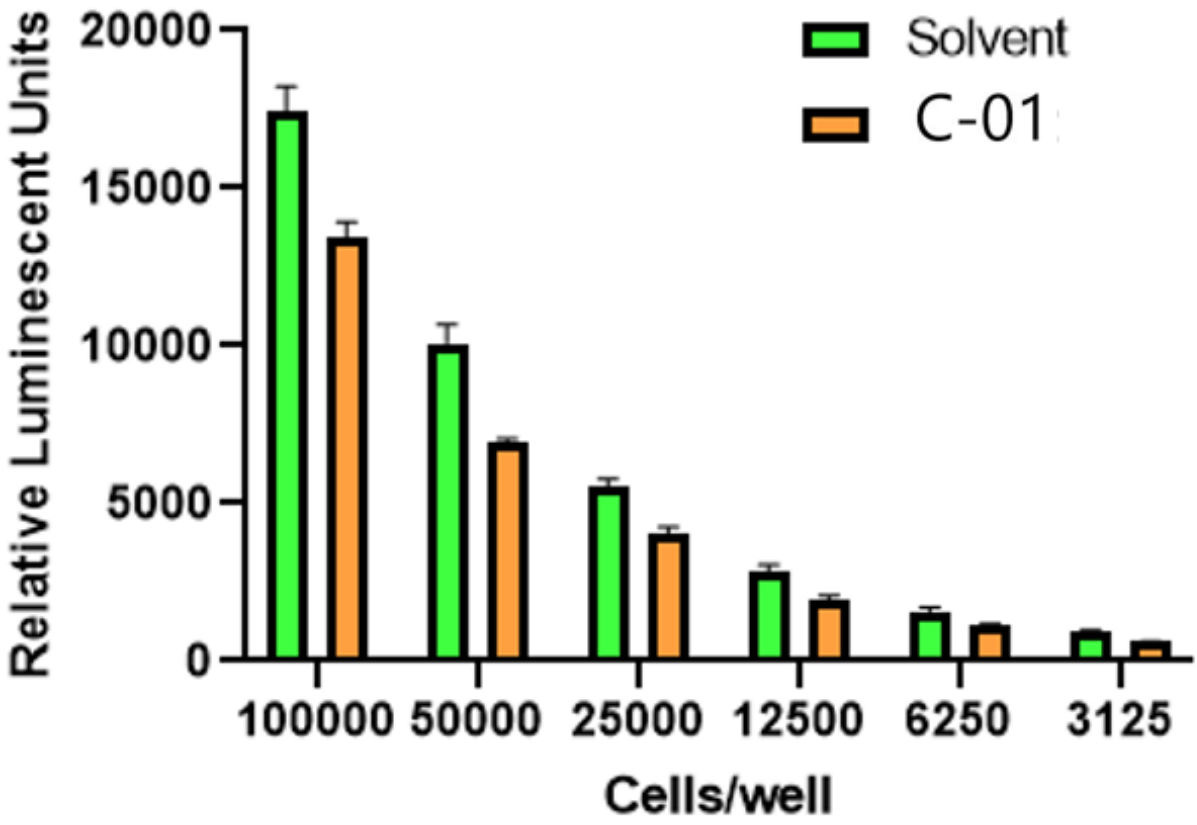



Figure 18. The luminosity of a Luciferase ATP assay from splenocytes taken from *in vivo* treatment of C-01 from mice (n = 3). Reading was taken on a Synergy HT plate reader.

We see a lower fluorescent intensity in C-01 treated cells when compared to solvent treated cells in our Resazurin Reduction Assay (Fig. 19). Typically, this is associated with a higher cellular concentration of oxidizing agents, which are typically used in the synthesis of

ATP and reactive species within a cell. Results were found to be statistically significant ($p < 0.05$).

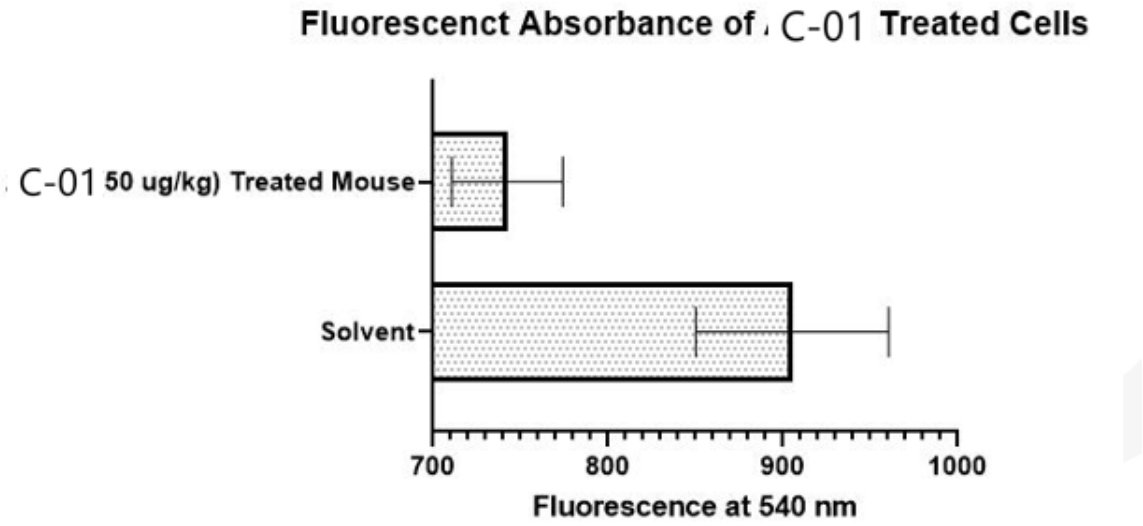



Figure 19. The fluorescent absorbance of C-01 treated cells taken at excitation 480 nm, emission 540 nm of *in vivo* 50 mg/kg dosage C-01 treated splenocytes. Cells were plated at 1,000,000 cells/mL at a volume of 100 uL. Splenocytes were removed from three mice and pooled together.

The results of the C166 transcriptional analysis illustrate no significant change in CXC11 from C-01 treatment (Fig. 20).

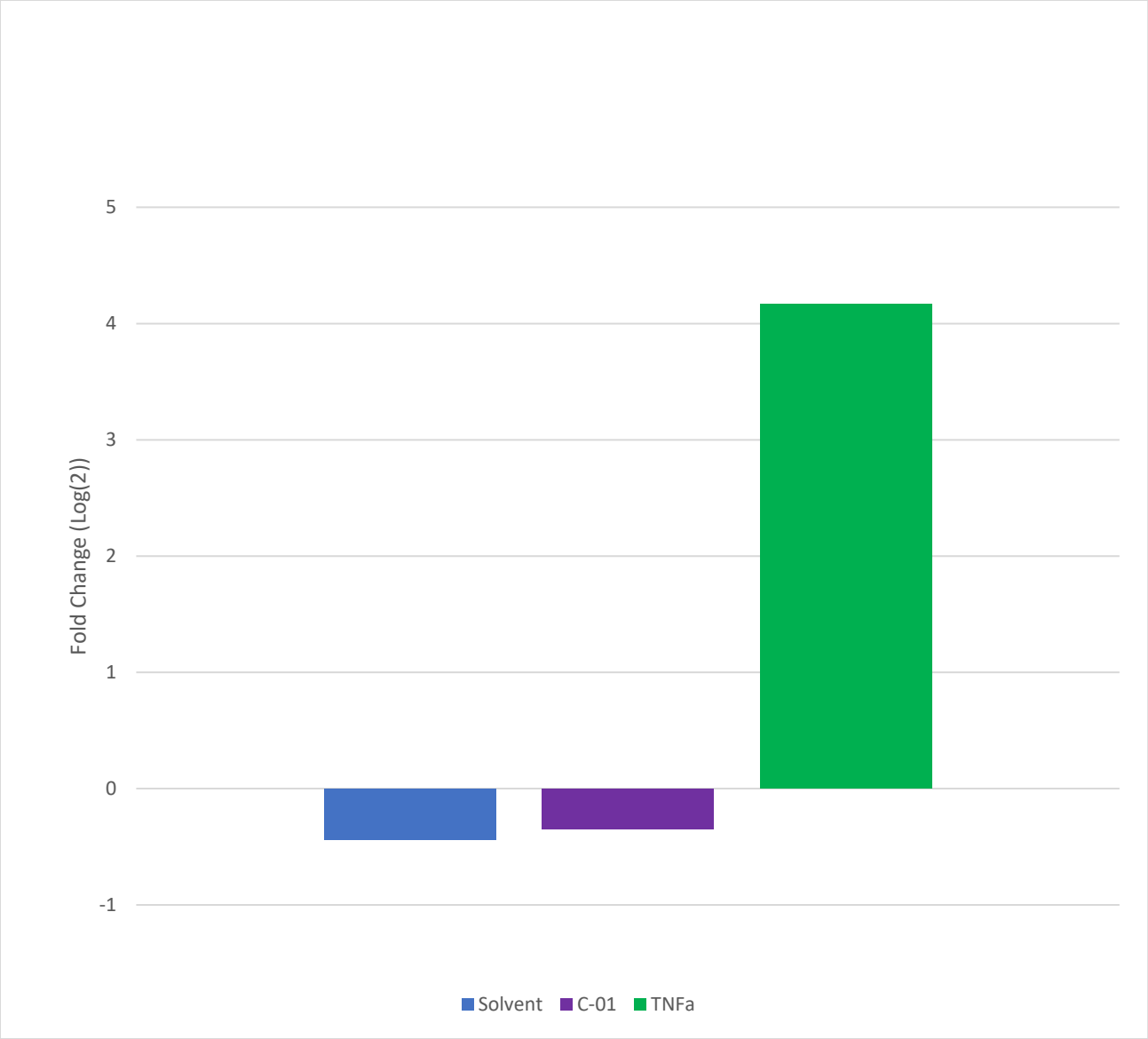



Figure 20. A qPCR gene expression plot of C166 endothelial cells treated with C-01.

Treatment with C-01 increases the fluorescent intensity of cells incubated with fluorescent *E. coli* bioparticles (Fig. 22). A dosage response is also observed with increased treatment. We observed that keeping the plate at 4 ℃ lowered the fluorescent intensity. Images

were also taken on the plate after reading (Fig. 21). Fluorescence was not only increased in C-01 and LPS treatment, but also more localized, meaning that it was closer to the center of the cell. Data was found to be statistically significant ($p < 0.05$).

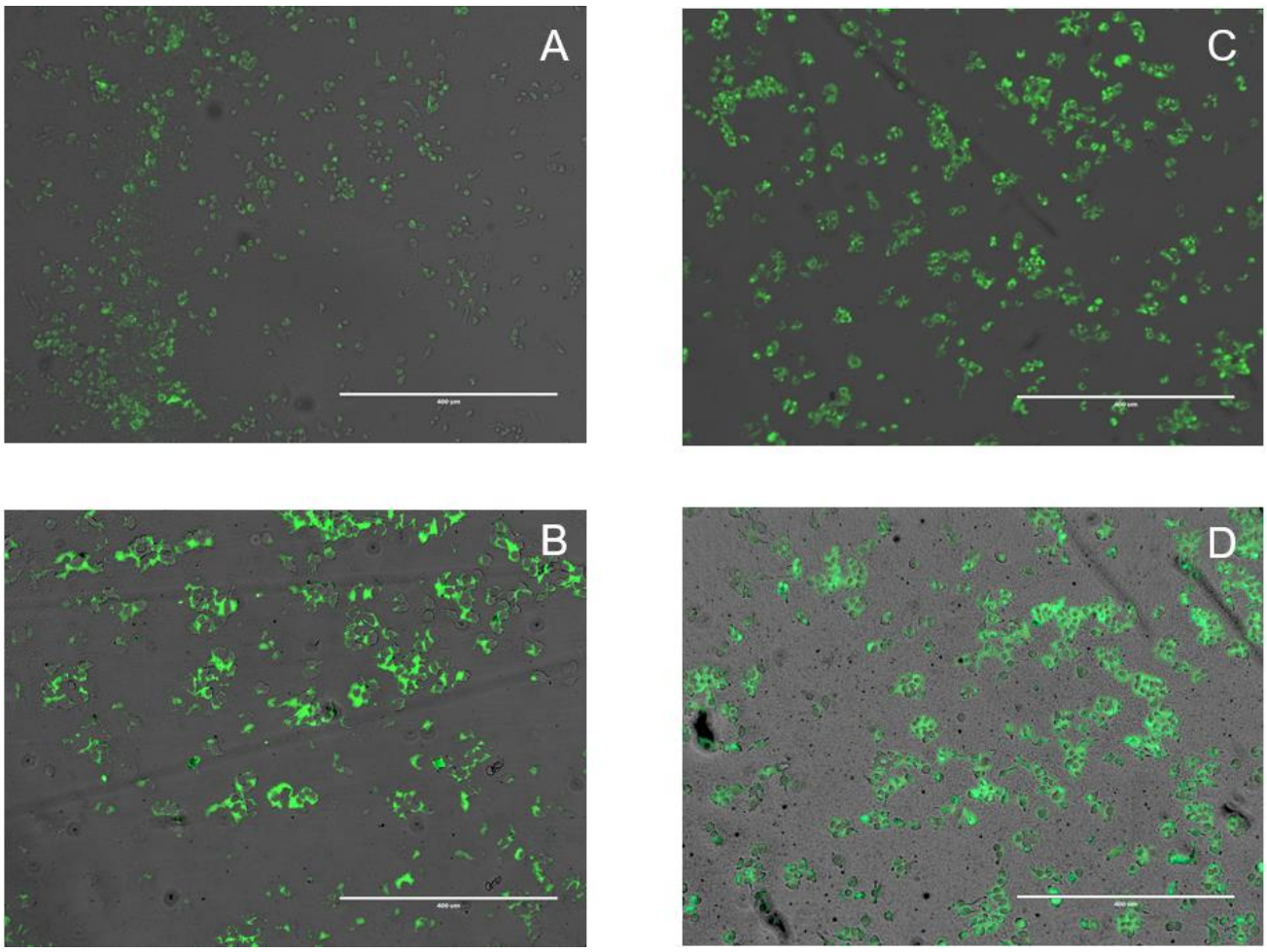



Figure 21. Fluorescent microscopy images taken with a GFP filter overlayed on a brightfield image. A) Cells containing only fluorescent *E. coli*. B) Cells placed in the refrigerator. C) C-01 treated cells. D) Cells treated with lipopolysaccharide.

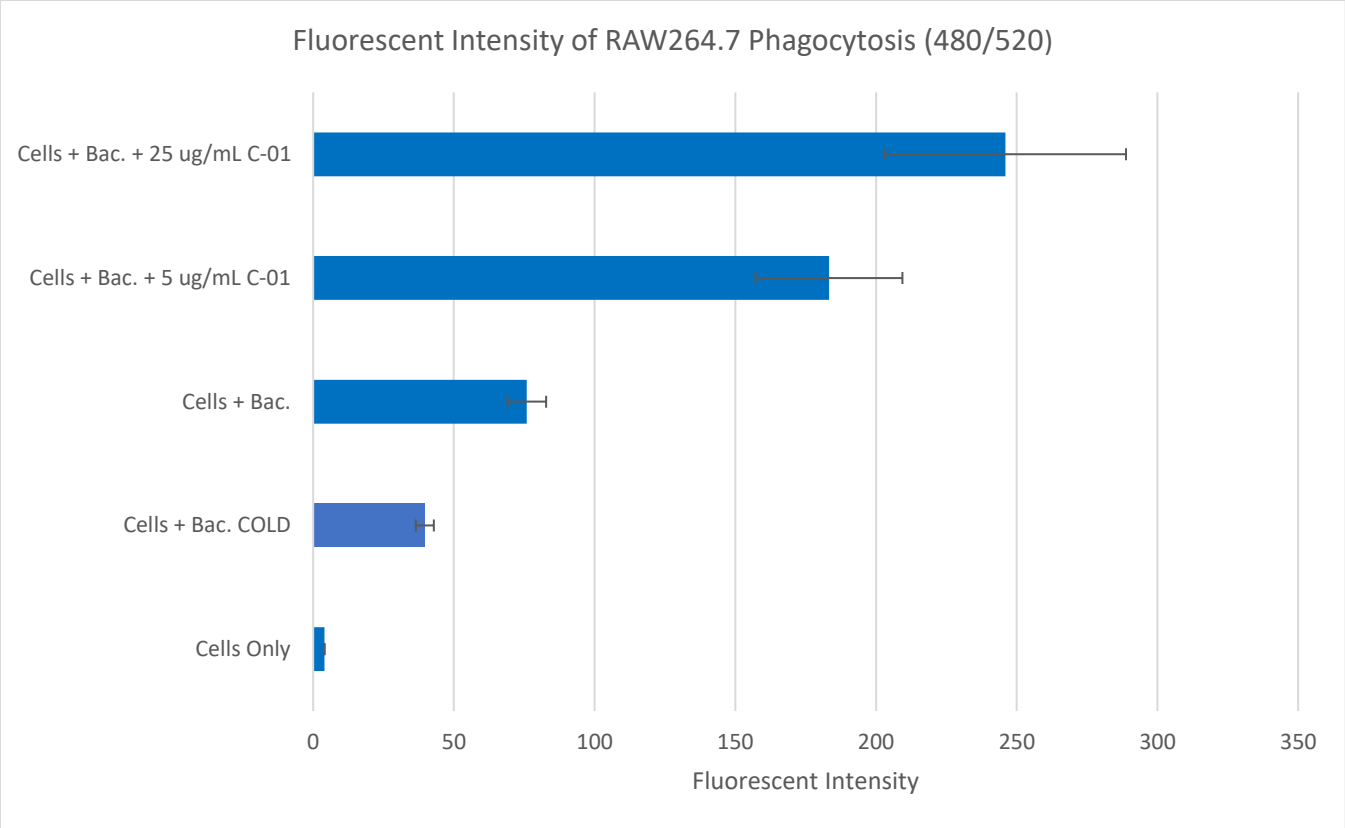
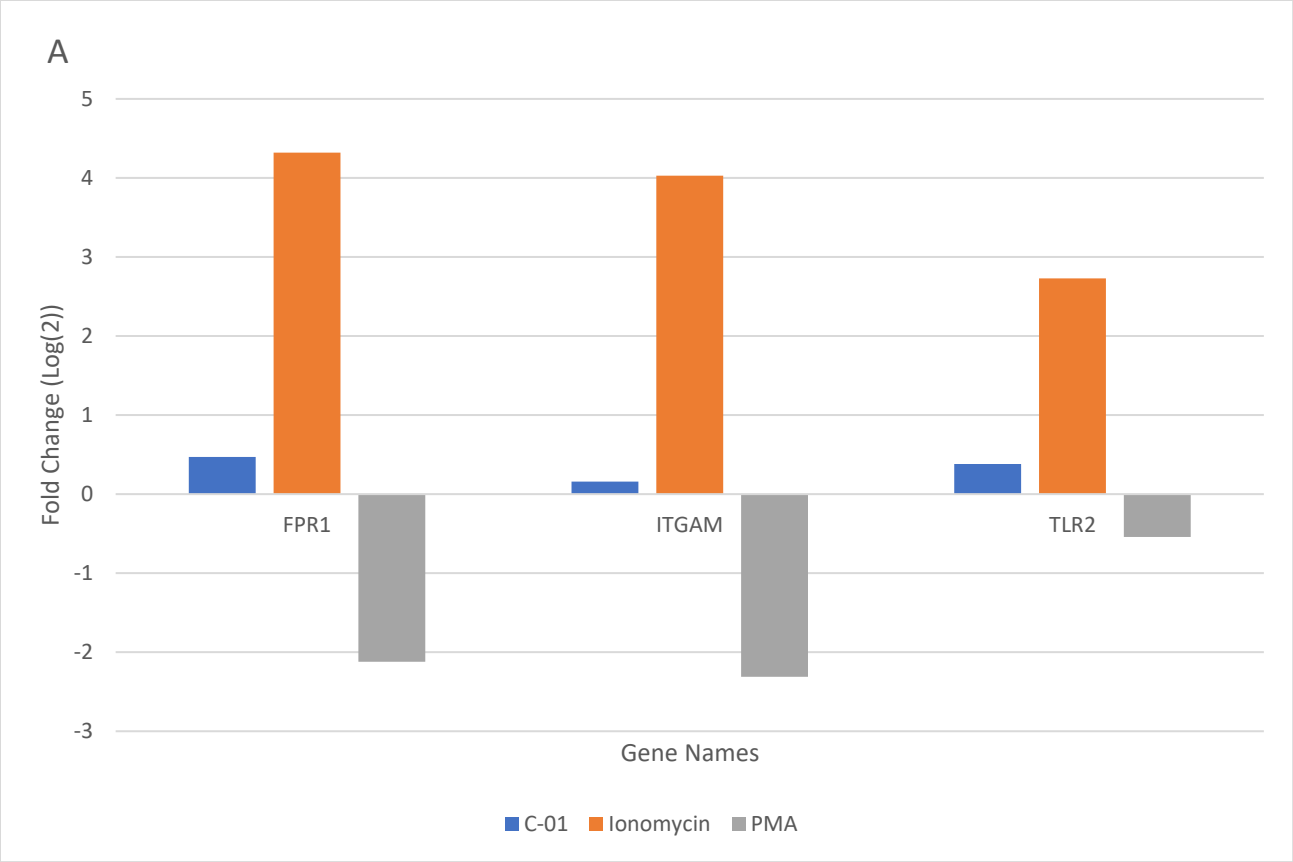



Figure 22. A graphical representation of a phagocytosis assay performed on RAW264.7 murine macrophages taken on a Synergy HT plate reader at excitation 480, emission 520. Cells were incubated with bioparticles for 2 hours, and fluorescence was quenched with 0.4% Trypan Blue solution. Cells kept cold were kept at 4 degrees Celsius during the incubation time.

Finally, we performed our transcriptional analysis on HL-60 differentiated with both 1.3% DMSO and C-01 (Fig. 23). Statistics were not performed on this analysis as there was not replicates built into the experimental design. In the future, we plan to look at this data further.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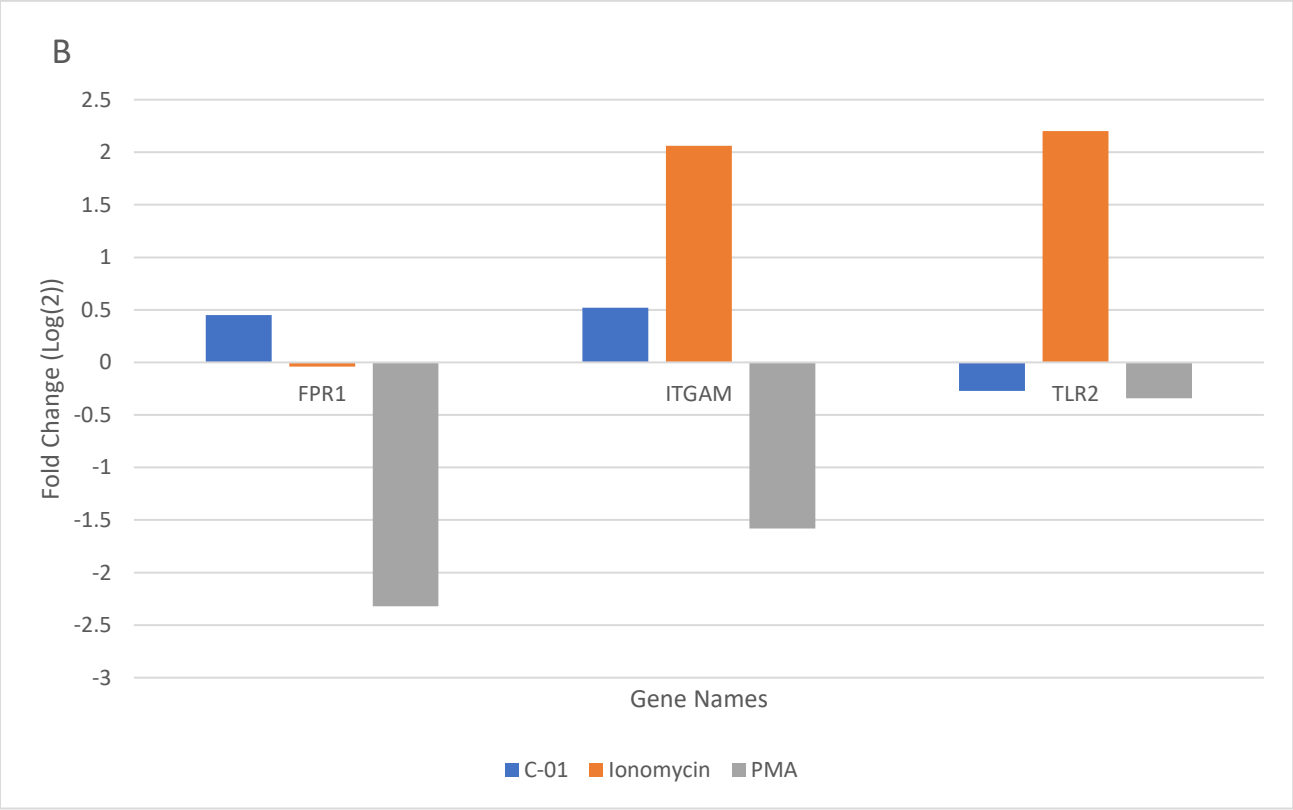
Gene Names
C-01
Ionomycin
PMA


B
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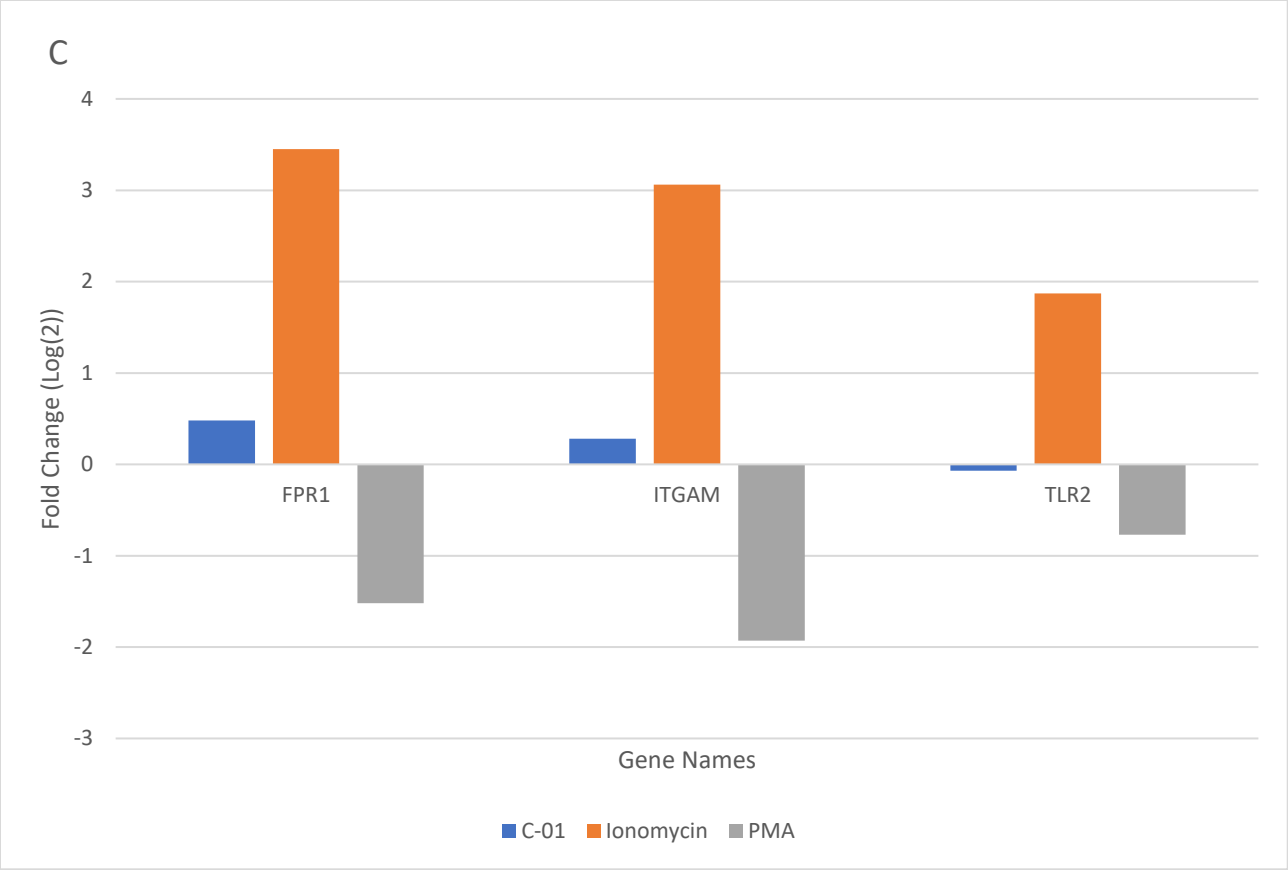


Figure 23. qPCR analysis performed on a series of HL-60 treated with C-01. A) Normal, undifferentiated HL-60. B) HL-60 differentiated with 1.3% DMSO for 4 days. C) HL-60 "differentiated" with C-01 for 4 days.

## Discussion:

Our initial hypothesis was that C-01 was causing a sort of reactive oxide release within the host's body, for this would be a typical anti-helminth response. However, in our superoxide dismutase assay (which quantifies the release of reactive oxygen species), we did not observe a marked increase or decrease in the amount released by the murine splenocytes (Fig. 17). We

come to this conclusion because the C-01 treated cells follow the same curve as the normal cells. This indicates to us that C-01 is not binding the any sort of immune cell and is not causing those cells to release reactive oxygen species. Furthermore, we used PMA and C-01 in conjunction to see if C-01 needs a secondary stimulation signal to release more reactive species (Fig. 17). This also did not affect the amount of reaction oxygen present; thus, we had to revisit our hypothesis and ask more basic questions.

Next, two assays were performed in tandem—the Luciferase ATP assay and the resazurin reduction assay. A Luciferase ATP assay allows us to quantify the amount of ATP present within a cell right after lysing. Our assay shows that C-01 treated cells had a lower luminosity than solvent treated cells (Fig. 18). This indicates that C-01 is causing the host to use up their ATP stock, as a lower luminosity means that there was a lower concentration of ATP present. Next, we performed a resazurin reduction assay which measures how reductive a cellular environment is. Cellular reductivity is important within a cell because the components that make a cell reductive are utilized in a series of important processes such as the synthesis of ATP or the creation of reactive species used to fight pathogens. We see a lower fluorescent intensity in C-01 treated cells when compared to solvent treated cells (Fig. 19). This results from C-01 causing the cellular environment to be less reductive (i.e., there are less precursors to the synthesis of reactive compounds). Together, the Luciferase ATP assay and the resazurin reduction assay suggest to us that C-01 is not causing the cells to directly release any compounds; rather, it is causing the cells to become “primed.” This means that they are ready to attack any foreign invaders.

Thus, we revised our hypothesis to state that C-01 is priming the immune system. To further answer this question, we looked at the transcriptome, specifically CXCl11, which is the

primary gene involved with the migration of immune cells from the circulatory system into the tissue of C166.We selected CXCL11 as our gene of interest as this would answer the question of whether C-01 is causing the migration of the immune system. In our qPCR analysis, we saw no significant change in gene expression from C-01 treatment (Fig. 20).

Next, we performed a phagocytosis assay using RAW264.7 macrophages, an immortalized murine cell line. We performed this assay because of the single cell transcriptomics data where we saw an increase in reticulocytes. An increase in this cell type is typically associated with a concept known as regenerative erythropoiesis, which is a key marker of an inflammatory response. With inflammation, we should also see an increase in phagocytosis. Therefore, we performed a typical phagocytosis assay, where we saw a higher fluorescent intensity from C-01 treatment (Fig. 22). This indicates an increase in phagocytosis, which is corroborated by our fluorescent microscopy (Fig. 21). We also see a dosage response, which is promising. The results of this phagocytosis assay corroborate our hypothesis that C-01 is causing an inflammatory-like immune response. To further our understanding of how C-01 was affecting the immune response, we looked at HL-60 a promyeloid cell line.

qPCR was performed on HL-60 looking at three target genes: TLR2, FPR1, and ITGAM. These genes all are key markers of neutrophil activation. However, with the three groups tested— undifferentiated, 1.3% DMSO differentiated, and 50 uM C-01 differentiated—there was no meaningful change in the genes analyzed (Fig. 23). Ionomycin functioned as intended, but C-01 saw no change.

## Conclusion and Future Directions:

In conclusion, there is a clear and pressing need for a novel treatment for Schistosomiasis, for praziquantel (the current treatment) has many unattractive properties and has become less effective over time. Our lab, in collaboration with others, has developed a novel anti-*Schistosomal* compound that has been illustrated to have an impressive ability to clear worms with a low cytotoxicity. However, before we can utilize this drug among humans, we must first understand its mechanism of action. From our data, we know that our compound is modulating how the immune system works. We see this in the increase in phagocytosis as well as the decrease in cellular reductivity and ATP.

In the future, we plan to perform single cell transcriptomics on HL-60 at different time points following C-01 treatment. We also would like to sequence the whole transcriptome of HL-60 using an Oxford MinION. Both of these experiments would enable us to further our understanding of how C-01 affects the immune system.

## Acknowledgements:

We would like to acknowledge our colleagues in the lab of Dr. Paul H. Davis. Specifically, we would like to thank Thomas Schulze and Andrew Neville for their guidance and leadership. We would also like to thank Sarah Alsuleiman for her assistance with PCR and PCR analysis. This project would not have the data we have today without the assistance of our collaborations in the labs of Dr. J. Vennerstrom and Dr. J Keiser. Finally, we would like to thank INBRE, FUSE, the UNO Honors Program, and the University of Nebraska at Omaha for their continued financial and moral support.

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