5-2019

Elucidating the Mechanism of Action of Experimental Compound SW33 in Toxoplasma gondii

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Honors Program Senior Thesis
University of Nebraska at Omaha

Submitted by:

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April 2018

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Abstract

In recent years, antimicrobial drug resistance has become widespread and thus triggered an ever-growing need for the development of new, efficacious drug treatments. As an antimicrobial drug is developed, its mechanism of action is often identified before it becomes a potential candidate for clinical use. One potential method for identifying mechanism of action is chemical mutagenesis, in which induction of drug-resistant populations is followed by whole-genome sequencing of several clonal isolates. The subsequent observation of identical point mutations in the same gene across multiple drug-resistant populations can indicate a likely molecular target. However, this technique lacks the capacity to facilitate speedy and robust elucidation of the mechanism of action of experimental compounds, indicating a need for improved methods to enhance the efficiency of high-throughput drug target identification. In this work, a novel technique utilizing double chemical mutagenesis was developed in order to quickly induce the high levels of resistance indicative of specific resistance in the apicomplexan parasite *Toxoplasma gondii.* This technique is being used to identify drug targets in experimental compound, SW33, that has shown efficacy against *T. gondii.*

Introduction

The microbial infections caused by bacteria, viruses, and parasites are a leading cause of death worldwide [1,2]. Although there are currently a wide variety of treatments for microbial infections, the widespread use of antimicrobial drugs has led to unprecedented rates of resistance [3]. In fact, the World Health Organization has reported that combating drug resistance is one of the most important aspects of maintaining global public health [3]. During the drug development process, experimental compounds are first tested to *in vitro* then *in vivo* to determine their capability of fighting infections. The compounds that show efficacy in these conditions have the potential for clinical use. However, before they can move to pre-clinical trials, the way they function inside the cell, termed mechanism of action (MOA), is usually determined. It is important to elucidate the MOA as it can demonstrate that an antimicrobial drug is nontoxic; ideal drugs will target pathogens while leaving human cells unharmed. Additionally, elucidating the MOA can provide insight into the best best way to modify compounds to improve efficacy and ways to optimize combinational drug therapy.

The MOA of many antimicrobials is through the binding of a specific protein, termed the drug target, which renders the protein inactive. One of the ways that microbes develop resistance to drugs is through the development of mutations in the drug target which prevent the antimicrobial compound from binding. One method to elucidate MOA is through the utilization of this natural process. In the process of chemical mutagenesis, a chemical mutagen is applied to a large number of microbes to induce a specific number of mutations. An experimental compound is then applied to the mutant microbes at various concentrations and resistant populations are clonally isolated. Next generation

sequencing (NGS) is then performed and mutations in the same gene across several resistant microbes indicates a potential drug target. The mutant allele is then transfected into wild-type microbes and, if resistance is observed, it is likely the drug target has been identified. Further tests can then be performed to verify the correct identification of the drug target, including *in silico* drug-binding assays and cocrystallization of the protein with bound drug.

A current problem with chemical mutagenesis is that often times only low levels of resistance is observed. This can potentially lead to the false-identification of a drug target because, during the acquisition of antimicrobial resistance, low levels of resistance can be indicative of general fitness advantages while high resistance is generally associated with mutations in the drug target. Thus, when attempting to identify mutations in the gene encoding the drug target (as needed for MOA identification), it is ideal to demonstrate high levels of resistance. In order to do this, a novel method of double chemical mutagenesis was developed.

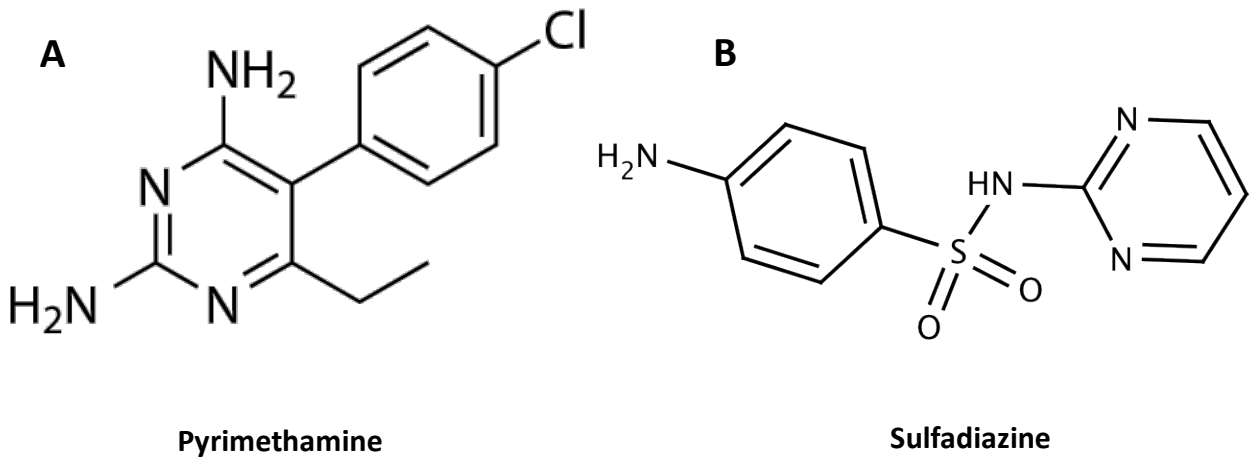
In the process of double mutagenesis, drug-targeted organisms are chemically mutagenized, selected for under drug pressure, then mutagenized a second time and selected for under still higher concentrations of experimental compound. This process is designed to take advantage of a trait in observed in microbes with high-antimicrobial resistance: a single mutation in the drug target may only induce low levels of resistance where subsequent mutations may confer high levels of resistance. Thus, a double mutagenesis is specifically designed to induce and select for single mutations in the drug-targeted gene with the first chemical mutagenesis then induce subsequent mutations in the gene with the second chemical mutagenesis.

In this work, the ubiquitous parasite *Toxoplasma gondii* was utilized. *T. gondii* makes an ideal target for drug development because it infects nearly one third of the world's population, causes devastation to pregnant woman and the immunocompromised, and there is currently no cure for the chronic stage [4,5,6]. Additionally, the current treatments for toxoplasmosis, pyrimethamine and sulfadiazine (Figure 1), are toxic to the host and, thus, the development of tolerable treatments is imperative [7]. Furthermore, the genetic similarities between *T. gondii* and the causative agent of malaria, *Plasmodium falciparum,* result in drugs effective against one of these pathogenic parasites often being effective against the other [8,9].

To test the efficacy of double mutagenesis, we utilized the folate-pathway-inhibitor pyrimethamine as it has a known drug target. Thus, after performing double mutagenesis, we were able to examine the accuracy of double mutagenesis by comparing the experimentally identified drug target to the known target. We then used mutagenesis to determine the drug target of the antioxoplasma compound, SW33. Following four separate mutagenesis experiments, we observed that double mutagenesis was unable to effectively confer resistance to SW33 in *T. gondii.* These results potentially suggest the drug target of SW33 is unable to function when mutations are introduced. This could indicate a drug target that is encoded in the apicoplast genome, as single mutations in these genes result in nonviable parasites [10]. Additionally, SW33 may function as an ionophore as previous works have demonstrated that mutagenesis is an ineffective tool for identifying their MOA [10]. Future experiments will need to be performed in order to test for the possibility of an apicoplast drug target and ionophore functions. Because apicioplasts are unique to apicomplexans, the potential for SW33 to target this plastid

would make it a promising drug for clinical use as it would be nontoxic to humans, thus giving it an advantage over current treatments.

Figure 1



Pyrimethamine **Sulfadiazine**

Figure 1: Structures of Pyrimethamine and Sulfadiazine
The current treatments for toxoplasmosis, sulfadiazine and pyrimethamine, both have affinity for human proteins and are therefore toxic. A) Pyrimethamine is the current treatment for acute toxoplasmosis. It functions by targeting DHFR which inhibits the folate pathway and pyrimethamine synthesis. B) Sulfadiazine also targets the folate pathway.

Materials and Methods

Parasites and media

T. gondii parasites were maintained through serial passage in human foreskin fibroblasts (HFF) using DMEM media supplemented with 10% bovine calf serum as previously described [11]. All parasite strains are derived from Type 1 RH *T. gondii* strain isolated from a patient in 1939 [12]. Conditioned media was generated by infecting 175 cm^2 flasks confluent with HFF with 7mL of media confluent with RH parasites.

Plaque Assays

Plaque assays were performed as previously described [13]. Briefly, 10 flasks containing confluent HFF cells infected with *T. gondii* were cultured in 25 cm^2 flasks. Nine of the flasks were treated with varying concentrations of N-ethyl-N-nitrosurea (ENU) (1.25 mM, 2.5 mM, 5 mM), Ethylmethane sulfonate (EMS) (8mM, 10mM, 12mM), and a combination of both EMS and ENU, respectively (6mM/3mM, 4mM/2mM, 2mM/1mM) for four hours while the 10nth flask acted as an untreated control. Media was removed and the monolayer was rinsed 3x with 1x PBS. The monolayer was then scraped, homogenized, and syringe filtered. 1000 parasites from each flask were transferred to new flasks. After 1 week, plaques were counted and percent death was determined.

Double Chemical Mutagenesis

Chemical mutagenesis was performed using both pyrimethamine and SW33, separately, as previously described [13]. Briefly, a mixture of EMS (5mM) and ENU (3.5mM) were added to 11 175 cm^2 flasks confluent with RH strain parasites. Following 4 hours of

incubation, the media was removed and the monolayer was rinsed 3x with 1x PBS. The monolayer was then scraped, homogenized, syringe filtered, and passed to 175 cm^2 flasks confluent with HFF. After 24 hours, the flasks were then treated with 3.2μM, 4.26μM, 6.39μM 10.65μM, and 21.3μM of experimental compound SW33 or pyrimethamine. Following four days of treatment, the monolayers were homogenized, syringe filtered, and passed to 25 cm^2 flasks where treatment was continued for 10 days. Parasites viable at the highest SW33 concentration were subjected to a second round of EMS and ENU before selection under .26μM, 6.39μM 10.65μM, 21.3μM, 31.95μM SW33.

Clonal Isolation

Resistant parasites were clonally isolated using serial dilution in 96-well plates containing conditioned DMEM media. Singular plaques were identified through fluorescence microscopy. Following five days of plaque formation, contents of single-plaque wells were transferred to 25 cm^2 flasks confluent with HFF.

Half-maximal inhibitory concentration (IC_{50}) analysis

96-well plates confluent with HFF were infected with 2,000 parasites per well. Serial dilutions of SW33 were administered two days after infection. IC_{50} concentrations were determined to be the concentrations of SW33 that reduced fluorescence by 50% using flasks containing 1% DMSO as control. The IC_{50} of SW33 was determined to be 2.13 μM in previously unpublished works.

Results

Pyrimethamine double-mutagenesis optimization

To test the efficacy of double mutagenesis, pyrimethamine was utilized as the experimentally found drug target can be compared to the known drug target. Following the first mutagenesis experiment, the most drug-resistant mutant was three times more resistant (4.5 μM) than WT (1.5 μM). After a second round of mutagenesis, the most resistant mutant exhibited eight times more resistance than WT (figure 2). As DHFR is the known target of pyrimethamine, the *DHFR* gene was PCR amplified in the resistant mutant in order to compare the experimentally found drug target to the known drug target. Interestingly, the sequenced *DHFR* showed no mutations.

Figure 2

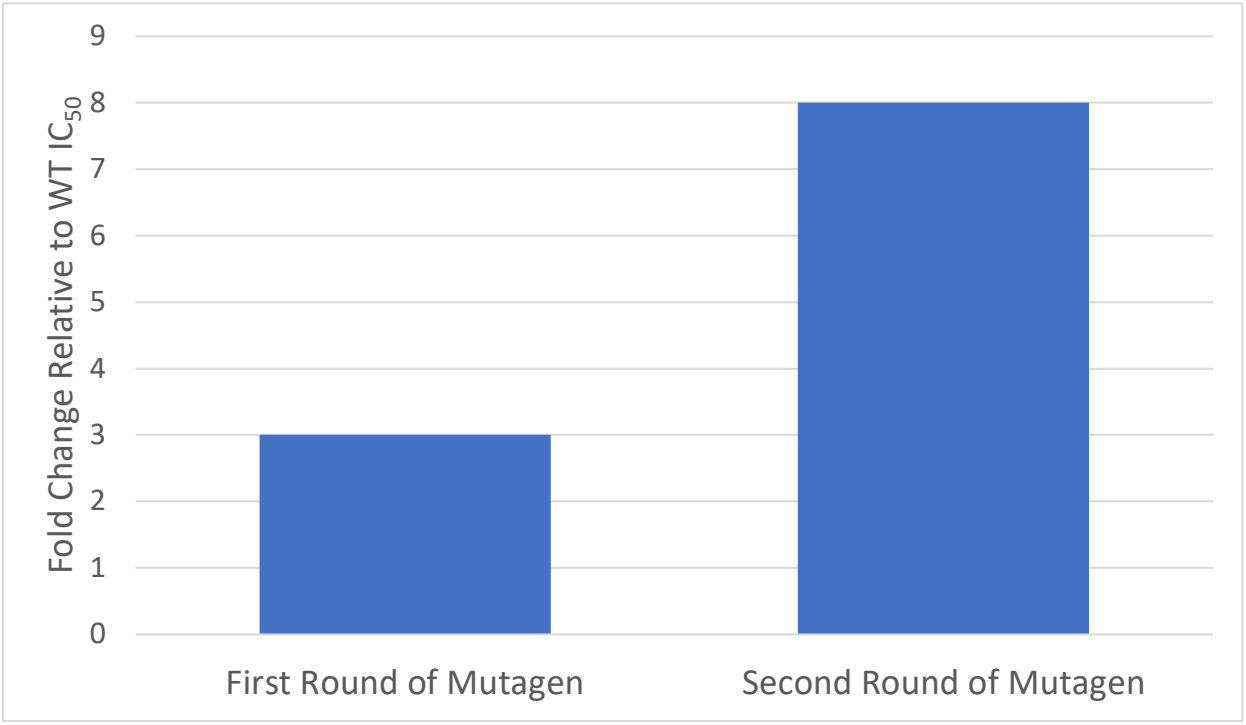


Figure 2: Observed Resistance of Parasites Before and After Double Mutagenesis
Double Mutagenesis introduced an 8-fold resistance to pyrimethamine compared to WT. However, Sanger Sequencing of the *DHFR* of the most resistant clone showed no mutations.

Figure 3

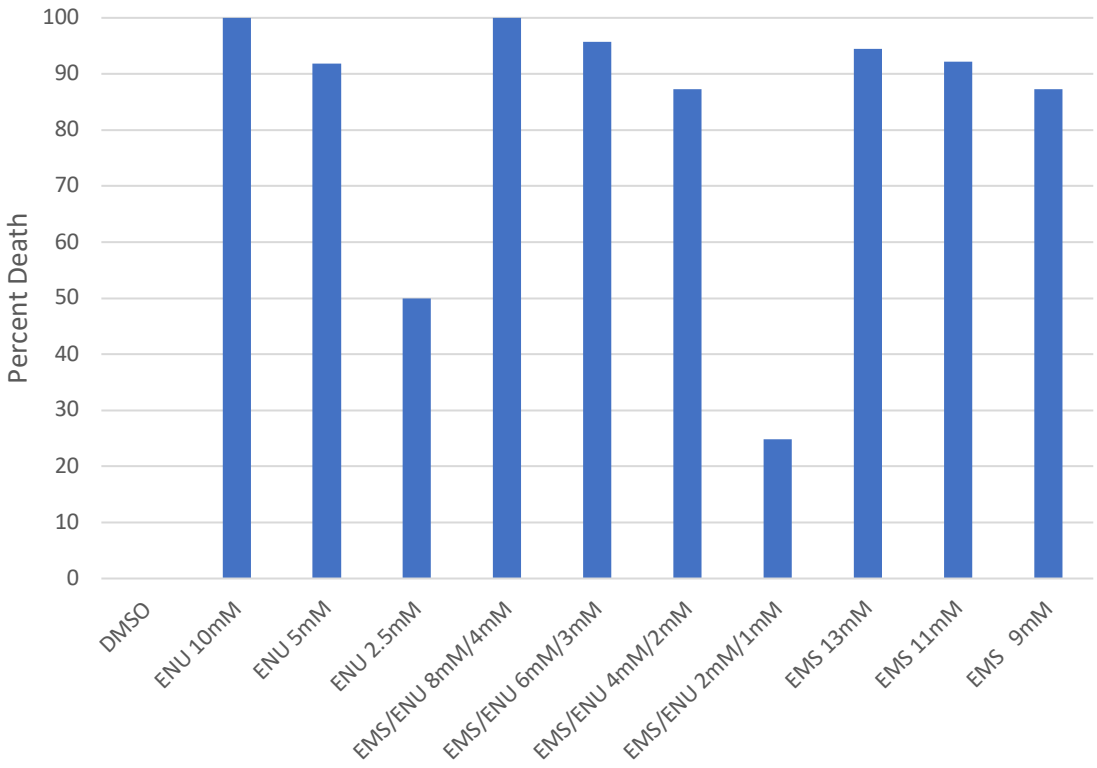


Figure 3: Percent death caused by EMS and ENU
Plaque assays were performed using various concentrations of EMS, ENU, and a combination of both EMS and ENU. A combination of EMS and ENU was chosen at 5mM/2.5mM, respectively, as the concentrations that yielded 90% death. For the mutagenesis using solely EMS, a concentration of 10mM was chosen.

SW33 double mutagenesis

ENU and EMS are often used in chemical mutagenesis due to their ability to induce a controlled amount of single nucleotide variances (SNVs)[13]. However, both mutagens have distinct mutagenic profiles [13]. Thus, EMS and ENU were administered concurrently, in order to induce a wide range of distinct mutations. Plaque assays using both ENU and EMS indicate that 5mM EMS with 2.5mM ENU induce 90% killing as determined using a best fit line (figure 3). This is the percent killing recommended to induce one SNV per million base pairs as previously demonstrated [13]. Double chemical mutagenesis was performed four separate times and drug selection above 4.26μM consistently killed all parasites. In previous mutagenesis experiments, resistant parasites were able to be maintained under constant drug selection above 50x IC_{50} [13]. Following mutagenesis, SW33 concentrations over 3.2 μM (1.5x IC_{50}) decimated the mutant parasite populations. This unique result indicates that resistance may be difficult or impossible to induce. A single flask of mutant parasites (population D) was able to be maintained at 4.26 μM (2x IC_{50}) after being treated for fourteen days with SW33, followed by removal of drug treatment for approximately thirty days, then resumed treatment with SW33.

After four double chemical mutageneses, there were four distinct parasite populations viable at 3.2μM SW33 and a single population viable at 4.2μM (population group D, Table 1). Clonal isolation was performed, and three clones were isolated from each population resulting in fifteen clonal isolates. Half-maximal inhibitory concentrations were derived for eight of the fifteen clonal isolates (table 1). Although only low levels of resistance was observed, we are currently isolating DNA and sending

samples to NGS. The next procedure will be to perform bioinformatics analysis on the raw NGS data.

Figure 4

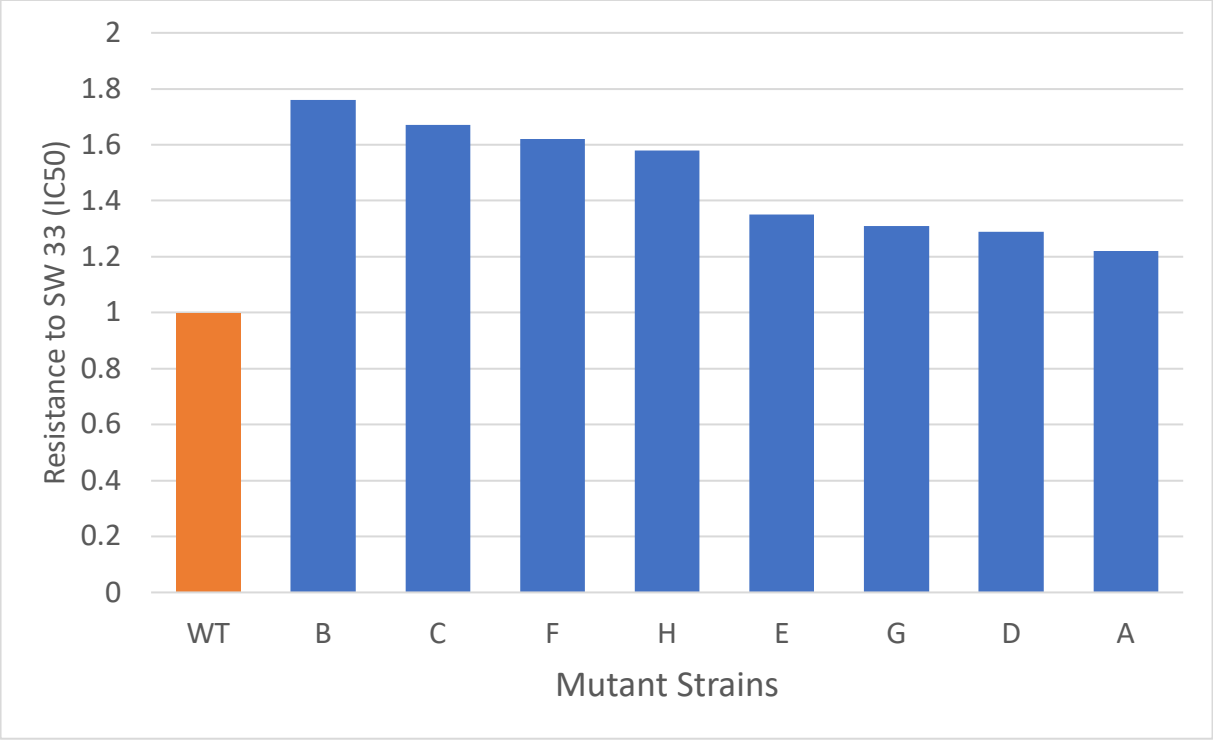


Figure 4: Induced Resistance to SW33
While mutagenesis was able to induce mutations that conferred resistance to SW33, only 1.76x resistance was observed. This unique result may be indicative of an apicoplast-gene target or ionophore activity.

Discussion

The pyrimethamine optimization yielded eight times more resistance than WT parasite but no mutations were found in the *DHFR*. While this result is interesting, limited funds hindered our ability to further examine the genome using NGS . However, future experiments may include the examination of these parasites, as their resistance to pyrimethamine through other means besides DHFR mutations may provide insight into the potential for the development of clinical resistance in a similar fashion. Despite an not finding the correct target, we believe double mutagenesis has viability for determining MOA as high resistance was induced and future double mutageneses will include the isolation of several clonal isolates with which to compare mutations. Sequencing multiple clonal isolates and comparing their mutations will greatly improve the likelihood of correctly identifying the drug target.

Given the lack of concurrent EMS and ENU applications in the literature, a concentration that resulted in 90% killing was used in order to induce one SNV per megabase (approximately 70 mutations per parasite). The results of the plaque assays indicate that 5mM EMS and 2.5mM ENU lead to 90% death, as determined using a best fit line (Figure 2). Because this concurrent treatment has never been administered, it is unclear whether these concentrations induce one SNV per megabase; further sequencing results are needed. There is a possibility that using both EMS and ENU simultaneously leads to deleterious outcomes not present when used separately. Current SW33 mutagenesis experiments are being performed with EMS (no ENU) in order to address this concern.

Despite four separate double mutagenesis experiments, the most resistant clonal isolate was just 1.76x more resistant than WT. Population D was able to remain viable at the highest SW33 treatment, 2x IC_{50} concentrations. Interestingly, isolates from population group D do not show the highest resistance. Low levels of resistance may be the result of the drug target being an essential gene that cannot be mutated. This is characteristic of genes encoding in the apicoplast as they are unable to be mutated and still produce viable parasites [14]. Thus, there is a possibility that the drug target is a protein synthesized in the apicoplast. This would be ideal as apicoplasts are unique to apicomplexans and therefore SW33 would likely be nontoxic to humans. If the drug target of SW33 is an essential gene whose mutations invariably cause parasite death, it is unlikely that mutagenesis will lead to the elucidation of the mechanism of action. The exception to this would be if mutations caused changes in gene expression, as upregulation of the drug target would result in observed resistance.

While these results are not ideal in terms of robustly elucidating mechanism of resistance, they may imply a potential difficulty or inability of the *T. gondii* to gain resistance to SW33. If true, this would make SW33 a strong candidate for clinical use. Further experiments should include the use of double mutagenesis on *Plasmodium falciparum* using SW33. Although double chemical mutagenesis is proving unsuccessful with SW33, it may still be a valuable lab technique. Mutagenesis can not only be used to find drug targets but can provide valuable information into the ease of which an organism may develop antimicrobial resistance to a specific drug. This information can then be used as a metric to determine the best drugs for clinical trials as the drugs which parasites

have the most difficulty building resistance to would potentially be the best drugs for clinical use.

References

1. The top 10 causes of death. (n.d.). Retrieved April 3, 2019 from http://www.who.int/mediacentre/factsheets/fs310/en/

2. FastStats. (2017, August 3). Retrieved April 3, 2019 from https://www.cdc.gov/nchs/fastats/leading-causes-of-death.htm

3. Wise, R., Hart, T., Cars, O., Streulens, M., Helmuth, R., Huovinen, P., & Sprenger, M. (1998). Antimicrobial resistance: Is a major threat to public health . *BMJ : British Medical Journal*, 317(7159), 609–610.

4. Weiss, L. M., & Dubey, J. P. (2009). Toxoplasmosis: a history of clinical observations. *International Journal for Parasitology*, 39(8), 895–901. doi: 10.1016/j.ijpara.2009.02.004

5. Dubey, J. (2009). History of the discovery of the life cycle of Toxoplasma. *International Journal for parasitology*, 39(8), 877-882. doi:10.1016/b978-012369542-0/50003-9

6. Lüder, C. G. K., & Rahman, T. (2017). Impact of the host on Toxoplasma stage differentiation. *Microbial Cell*, 4(7), 203–211. doi: 10.15698/mic2017.07.579

7. Reynolds, M. G., Oh, J., & Roos, D. S. (2001). In Vitro Generation of Novel Pyrimethamine Resistance Mutations in the Toxoplasma gondii Dihydrofolate Reductase. *Antimicrobial Agents and Chemotherapy*, 45(4), 1271-1277. doi:10.1128/aac.45.4.1271-1277.2001

8. Neville, A. J., Zach, S. J., Wang, X., Larson, J. J., Judge, A. K., Davis, L. A., Davis, P. H. (2015). Clinically Available Medicines Demonstrating Anti-Toxoplasma Activity. *Antimicrobial Agents and Chemotherapy*, 59(12), 7161–7169. doi:10.1128/AAC.02009-15

9. Kim, K., & Weiss, L. M. (2004). Toxoplasma gondii: the model apicomplexan. *International Journal for Parasitology*, 34(3), 423–432. doi:10.1016/j.ijpara.2003.12.009

10. Khan, A., & Grigg, M. E. (2017). *Toxoplasma gondii*: Laboratory Maintenance and Growth. *Current Protocols in Microbiology*. doi:10.1002/cpmc.26

11. Pfefferkorn ER, Pfefferkorn LC: *Toxoplasma gondii*: isolation and preliminary characterization of temperature-sensitive mutants. *Exp Parasitol* 1976, 39:365–376.

12. Coleman BI, Gubbels MJ. 2012. A genetic screen to isolate Toxoplasma gondii host-cell egress mutants. *J Vis Exp* doi:10.3791/3807.

13. Farrell, A., Coleman, B. I., Benenati, B., Brown, K. M., Blader, I. J., Marth, G. T., & Gubbels, M. (2014). Whole genome profiling of spontaneous and chemically induced mutations in Toxoplasma gondii. *BMC Genomics*, 15(1), 354. doi:10.1186/1471-2164-15-354

14. Amberg-Johnson, K., & Yeh, E. (2018). Host cell metabolism contributes to delayed-death kinetics of apicoplast inhibitors in *Toxoplasma gondii*. Antimicrobial Agents and Chemotherapy. doi:10.1128/aac.01646-18