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Auranofin Targets Thioredoxin Reductases in Trichomonas vaginalis

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AURANOFIN TARGETS THIOREDOXIN REDUCTASES IN TRICHOMONAS VAGINALIS

by

Jose Jauregui

A Thesis Submitted to the
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2017
AURANOFIN TARGETS THIOREDOXIN REDUCTASES IN TRICHOMONAS VAGINALIS

by

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I would like to give special thanks to Dr. Wrischnik. Never would I have imagined our relationship would become what it has when I first met her in high school, or that she would become such a constant throughout both my undergraduate and graduate careers. My experience at Pacific was marked by numerous mentors whose influence I’ve found indelible, and I am glad to say that she was one of them. Her guidance, wisdom, and patience were invaluable in completing this thesis, as were our various non-science related conversations, and for that, I thank her. I would also like to thank Drs. Vierra and Land, not only for being my committee members, but also for being great professors that taught some of the more memorable courses I took at Pacific, as well as for providing invaluable resources from their labs, without which I would have not been able to complete my research. I would like to thank Dr. Weiser, both for always finding humor in my jokes, and for supplying important reagents needed for my experiments. I also want to thank Dr. Lars Eckmann, our collaborator, without whom my project wouldn’t have been possible. And finally, I would like to thank Ryan Hekman. He was indispensable in carrying out all of the mass spectrometry analysis, and in helping to generate most of the graphs found within this thesis.
Auranofin Targets Thioredoxin Reductases in Trichomonas Vaginalis

Abstract

by Jose Jauregui

University of the Pacific
2017

*Trichomonas vaginalis* is an anaerobic, parasitic protozoan, responsible for trichomoniasis, the world’s most common, non-viral sexually transmitted infection. Lacking many of the defenses present in other organisms to combat oxidative stress, *Trichomonas vaginalis* relies extensively on the thioredoxin system—NADPH, thioredoxin reductase, and thioredoxin—as a means to protect against exposure to excess oxygen. Current trichomoniasis treatment relies exclusively on the 5-nitroimidazole drugs, but fear of drug-resistant strains and allergic reactions to 5-nitroimidazole treatment necessitate the discovery of a new treatment method for trichomoniasis.

Previous research has shown that auranofin, an FDA-approved drug, was effective at inhibiting activity of one of *Trichomonas vaginalis*’ isoforms of thioredoxin reductase (of which the organism has five total). Our research showed that only two of the isoforms were transcribed and expressed at high levels, and that both of these isoforms were susceptible to auranofin treatment. Not only that, these two isoforms were also shown to be susceptible to various auranofin analogs, having comparable or lower IC$_{50}$ values. Further tests on these analogs might show that they are actually better treatment
candidates if they exhibit less symptoms than auranofin. Experiments examining how mRNA and protein levels were modulated in response to two different concentrations of auranofin treatment showed that while some isoforms show increased levels, no one isoform experienced any drastic changes. Together, this data suggests that further studies should focus on these two most highly expressed isoforms of thioredoxin reductase.
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Chapter 1: Introduction

Trichomoniasis is the most common, non-viral sexually transmitted infection (STI) worldwide (Secor et al., 2014). Globally, there an estimated 248 million new cases each year (World Health Organization, 2011). In the United States, there are an estimated 3.7 million infected individuals, with around 1.1 million new infections each year, arguably making trichomoniasis the nation’s most prevalent parasitic infection (Satterwhite et al., 2013). Despite this, research concerning the infection and new treatment methods haven’t received the same level of priority as those of other STIs (Secor et al., 2014). Trichomoniasis’ status as a neglected infection can be attributed to two main reasons: 1) there is a lack in the understanding of the infection’s full impact on public health (Secor et al., 2014), and 2) the infection has the highest incidence among minority and low-income populations (Van der Pol, 2007).

Trichomoniasis is indiscriminate in affecting both sexes, although the majority of infected individuals are asymptomatic (Satterwhite et al., 2013). Symptoms typically manifest with greater occurrence in women, and can also be more severe (Secor et al., 2014). When present in women, symptoms typically manifest as swelling of the vagina, cervix, and urethra, vaginal discharge, and what’s known as strawberry cervix—small lesions on the genital epithelium (Wolner-Hanssen et al., 1989). These symptoms are typically mild to moderate in severity, however (Swygard, 2004). More adverse side effects of infection may be exhibited, particularly in pregnant women; trichomoniasis infection during pregnancy has been linked to amniotic sac rupture and stillborn birth
Conversely, the impact trichomoniasis has on men tends to be far less severe. The frequency of symptomatic infections in men is far lower, and most incidents of infection are believed to resolve themselves without the need for treatment (Weston and Nicol, 1963). In the few men that are symptomatic, the most common symptom is the swelling of the urethra, and again, this symptom is typically mild to moderate in severity (Krieger et al., 1993). Of course, being asymptomatic doesn’t negate the dangers of the infection. Asymptomatic men are still fully capable of transmitting trichomoniasis, and because they show no symptoms, are most likely unaware of the risk they pose to their sexual partners.

Common to both sexes is the dangerous link between trichomoniasis and HIV (Allsworth et al., 2009). Infection has been connected with making afflicted individuals more susceptible to other STIs, most notably, HIV; infected persons are both more likely to acquire and transmit HIV (Hughes et al., 2012). Individuals with both trichomoniasis and HIV have also been seen to be more resistant to trichomoniasis drug treatment, necessitating a longer treatment period (Kissinger et al., 2008). Because of the connection between trichomoniasis and HIV, it has been proposed that a more cost-effective means of reducing HIV transmission might lie through programs aimed at stemming and controlling trichomoniasis infections (Price et al., 2006), since trichomoniasis is much cheaper to treat than HIV (Owusu-Edusei et al., 2013).

Trichomoniasis is caused by infection with the protozoan parasite *Trichomonas vaginalis* (*T. vaginalis*) (Petrin et al., 1998). *T. vaginalis* is microaerophilic, meaning that
it requires the presence of some oxygen to survive, but high concentrations of oxygen are
damaging and toxic (Petrin et al., 1998). In fact, it has been shown that the organism
grows most optimally when exposed to concentrations of oxygen around .25µM (Paget
and Lloyd, 1990). *T. vaginalis* is also an amitochondriate, so while lacking mitochondria,
it possesses unique mitochondria-derived organelles called hydrogensomes that facilitate
energy production in an environment with limited oxygen levels (Dyall and Johnson,
2000). Despite being microaerophilic, the organism is still exposed to oxygen in the
natural environment, and so must have means of dealing with the resulting oxidative
stress (Coombs et al., 2004). Regularly, *T. vaginalis* is exposed to oxygen concentrations
of about 60µM, much higher than those needed for optimal growth, and also much more
harmful (Wagner and Levin, 1978).

A cell’s extracellular environment is oxygen rich, so secreted proteins will contain
many stabilizing disulfides (Arnér and Holmgren, 2000). When looking at the
intracellular environment, there is much less free oxygen present so proteins will instead
contain free sulfhydryl groups, reflecting this reducing environment (Gilbert, 1990).
Many proteins contain -SH groups that serve structural and catalytic roles, and the
unwanted oxidation of these important groups usually results in altered protein function
(Holmgren, 1985). It is because of this that cells maintain a reduced intracellular
environment. *T. vaginalis*, in particular, has hydrogenosomal-specific proteins that are
deactivated by oxygen (Coombs et al., 2004). Malfunctioning energy generating
organelles would be detrimental to the organism. Cells themselves also readily use thiol-
disulfide exchange reactions in protein pathways to control protein function because of
how fast and easily reversible they are (Arnér and Holmgren, 2000). Because excess
oxygen has the capacity to hinder energy production in *T. vaginalis* and disrupt the organism’s protein pathways, ways to combat possible oxidative stress are required.

Many other eukaryotes have molecules such as glutathione, glutaredoxins, and catalase to serve as redox buffers and antioxidants to protect against the oxidative stress and reactive oxygen species (ROS) that proceed exposure to high levels of oxygen (Ellis et al., 1994). *T. vaginalis*, however, lacks these protective molecules (Ellis et al., 1994). What the organism does possess, that is also found in most other organisms, is the thioredoxin system (Holmgren, 1985). Lacking other conventional means of protection, *T. vaginalis* relies almost exclusively on the thioredoxin system to protect against oxidative stress.

The thioredoxin system consists of NADPH, thioredoxin (Trx), and thioredoxin reductase (TrxR) (Arnér and Holmgren, 2000). NADPH gives an electron to TrxR, which is then able to reduce Trx, making it active and capable of reducing downstream targets (Figure 1). Trx is a dithiol-disulfide oxidoreductase, meaning that it specifically targets disulfides, reducing them into two free thiol groups (Holmgren, 1995). When looking at proteins specifically, the rate at which Trx targets proteins is much higher than glutaredoxin (a member of the very similar glutaredoxin system), which is why in many organisms the Trx system is the main way in which reducing conditions are maintained within cells (Holmgren, 1985).
The Trx system, acting through Trx, serves many roles. As stated above, thiol-disulfide exchange reactions are used in many signal transduction pathways. Many organisms have thus co-opted Trx and integrated it into various pathways within cells that serve crucial functions beyond just relieving oxidative stress (Table 1) (Arnér and Holmgren, 2000). One of the more notable functions of Trx outside of combatting oxidative stress is its ability to reduce ribonucleotide reductase, a critical enzyme involved in DNA synthesis (Holmgren, 1989). However, despite being dubbed a “moonlighting protein,” the Trx protein in *T. vaginalis*, a unicellular eukaryotic organism, doesn’t possess the many robust functions of Trx found in a more complex, multicellular organisms (Jeffery, 1999). The best characterized Trx is that of *E. coli*, and
when the Trxs of other organisms are compared to it, a sequence similarity ranging from 27-69% is observed (Eklund et al., 1991). It can be assumed that the different functions across organisms are tied to these sequence differences.

Table 1. Summary of the roles of Trx in various organisms (from Arnér and Holmgren, 2000).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Role of thioredoxin</th>
<th>Comments and references</th>
</tr>
</thead>
<tbody>
<tr>
<td>All organisms (7)</td>
<td>DNA synthesis</td>
<td>Thioredoxin is a hydrogen donor for ribonucleotide reductase [4]</td>
</tr>
<tr>
<td></td>
<td>Protein disulfide reduction</td>
<td>Thioredoxin is a key player in keeping intracellular protein disulfides generally reduced [70,71]</td>
</tr>
<tr>
<td>Many organisms</td>
<td>Reduction of $\text{H}_2\text{O}_2$</td>
<td>Many peroxiredoxins, catalyzing reduction of $\text{H}_2\text{O}_2$ and thereby preventing oxidative stress and apoptosis induction, require reduction by thioredoxin [22,25,72]</td>
</tr>
<tr>
<td></td>
<td>Protein repair by methionine sulfoxide reduction</td>
<td>Thioredoxin is hydrogen donor for methionine sulfoxide reductases [73,74]</td>
</tr>
<tr>
<td>E. coli phages (T7, T1, M13)</td>
<td>Submit of T7 DNA polymerase</td>
<td>Increases processivity, specific for thioredoxin-(SH)$_2$ but not dependent on oxidoreductase activity [8]</td>
</tr>
<tr>
<td></td>
<td>Participates in filamentous plaque assembly</td>
<td>Thioredoxin is the only host E. coli protein required for plaque assembly and export [9,75]</td>
</tr>
<tr>
<td>Bacteria and yeast</td>
<td>Hydrogen donor for 3'-phosphoadenosylsulfate (PAPS) reductase</td>
<td>Assimilation of sulfur by sulfate to sulfite reduction [76,77]</td>
</tr>
<tr>
<td>Plants</td>
<td>Regulation of chloroplast photosynthetic enzymes</td>
<td>Photosynthesis regulation by light via ferredoxin [78]</td>
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<tr>
<td>Mammals</td>
<td>Redox regulation of transcription factors, e.g. Nf-κB, AP-1</td>
<td>Different transcription factors are either activated or inhibited by Trx [14]</td>
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<tr>
<td></td>
<td>Regulation of apoptosis</td>
<td>Thioredoxin-(SH)$_2$, but not thioredoxin-$\beta$, makes a complex with ASK1 and prevents downstream signaling for apoptosis [10]</td>
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<td>Immunomodulation</td>
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<td>Intra- and extracellular synthesis of thioredoxin from cytoplasmic and extracellular sources assist implantation and establishment of pregnancy [54,79,80]</td>
<td></td>
</tr>
<tr>
<td>Birth</td>
<td></td>
<td>Protection from hyperoxia at birth by induction of thioredoxin [81]</td>
</tr>
<tr>
<td>CNS</td>
<td></td>
<td>Thioredoxin secreted from glial cells promotes neuronal survival at ischemia/reperfusion [82]</td>
</tr>
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</table>

Trx, through its oxidoreductase activity, plays two main roles: 1) it serves as an electron carrier in signal transduction 2) and it protects the inactivation and aggregation of cytosolic proteins by reducing intra- or inter-molecular disulfides that arise as a consequence of oxidative stress (Arnér and Holmgren, 2000). Again, because *T. vaginalis* lacks the complexity present in multicellular organisms, it’s the second role of
Trx—and by extension, the Trx system—that’s of greater importance to the organism. Indeed, previous studies have demonstrated that exposure to oxidative stress results in increased levels of Trx and TrxR in *T. vaginalis*, highlighting the Trx system’s importance in helping the organism overcome the dangers of oxidative stress (Coombs et al., 2004).

Although the Trx system is *T. vaginalis’* main source of antioxidant activity, the system doesn’t act alone, particularly when dealing with reactive oxygen species (ROS) (Nakamura et al., 1997). Within the organism, free oxygen is dealt with directly by NADH oxidase and NADPH oxidase (Coombs et al., 2004). NADH oxidase converts free oxygen to water, and NADPH oxidase converts it to hydrogen peroxide. Additionally present is superoxide dismutase, whose function of eliminating superoxide radicals also results in the production of hydrogen peroxide (Petrin et al., 1998). While water is benign, hydrogen peroxide is a ROS and poses a threat to the cell. A family of peroxidases is found within *T. vaginalis* that reduces and neutralizes hydrogen peroxide. These peroxidases are themselves reduced and activated by Trx, and are thus called peroxiredoxins (TrxPs) (Hofmann et al., 2002). Just like the Trx system, these TrxPs are thought to be fairly ubiquitous among eukaryotes, highlighting both their importance and that of the Trx system (Hofmann et al., 2002).

Earlier, the ubiquity of the Trx system was mentioned, and while ubiquitous across a wide range of organisms, differences are present. The majority of differences concern TrxR. Most of the non-mammalian organisms that possess a Trx system, such as plants, bacteria, yeast, and *T. vaginalis*, have multiple isoforms of TrxR (Arnér and Holmgren, 2000). *T. vaginalis* contains five different isoforms (Figure 2), though the
reason for this many is yet to be elucidated (Mentel et al., 2008). This is in contrast to humans, who are known to have just one cytoplasmic TrxR, as well as a TrxR specific to mitochondria (Spyrou et al., 1997). The other main difference between the TrxRs of different organisms is that they are divided into two groups (Hirt et al., 2002). Less complex organisms, of which *T. vaginalis* is included, contain low molecular weight TrxRs (~33 kDa); and more complex organisms contain high molecular weight TrxRs (~56-65 kDa) (Williams et al., 2000).
Concerning trichomoniasis, current treatment relies almost exclusively on the 5-nitroimidazole drugs metronidazole and tinidazole (Leitsch et al., 2012). Metronidazole is the main treatment method, with tinidazole usually reserved only for metronidazole-
resistant strains (Seña et al., 2014). The nitroimidazoles are selectively toxic to microaerophiles and anaerobes (Leitsch et al., 2010). This is because the nitroimidazoles are actually prodrugs, and must be reduced in order to exert toxicity (Edwards, 1993). Reduction can still occur in aerobic organisms, but the abundance of oxygen quickly leads to the reoxidation of the drug, a process termed a “futile cycle” (Lloyd and Pedersen, 1985). This is good news for the *T. vaginalis*’s host, humans, since we are aerobic organisms, and so our cells are resistant to the drugs effects.

One main concern with the use of the nitroimidazoles is resistance. The first metronidazole-resistant strains were isolated in 1959, very soon after the drug was first introduced (Watt and Jennison, 1960). What’s unclear, however, is if these strains were naturally resistant to the drug or if their resistance arose through selection via drug treatment (Secor et al., 2014). A disparity exists when looking at cases of resistance on a global scale. In the U.S., only 5% of cases have exhibited metronidazole resistance (Schwebke and Barrientes, 2006). This is in contrast to 17% of cases in Papua New Guinea (Upcroft et al., 2009). Most metronidazole-resistant strains have been susceptible to tinidazole (Sobel et al., 2001), though some have been shown to be resistant to both drugs, making cross-resistance a great concern (Goldman et al., 2009).

Other pressing concerns also exist regarding metronidazole treatment. It has been noted that individuals also infected with HIV are more resistant to standard metronidazole treatment (Kissinger et al., 2008). Additionally, a small percentage of people are allergic to nitroimidazoles, although this can usually be circumnavigated through the use of incremental dosages (Helms et al., 2008). Those people that suffer severe anaphylaxis upon exposure to the drug should not be treated with nitroimidazoles,
however (Asensio Sánchez et al., 2008). But due to nitroimidazoles being the only drugs approved for trichomoniasis treatment in the U.S., these individuals have no other course of treatment (Secor et al., 2014). It is because of this that new methods to treat trichomoniasis are needed.

Auranofin is an FDA-approved gold(I) compound (Debnath et al., 2012). It has been on the market for 30 years to treat severe cases of rheumatoid arthritis (Casini and Messori, 2011). Despite it’s long history of clinical use, however, it’s precise mechanism of action is still not fully known (Casini and Messori, 2011). As such, studies involving the drug have mainly helped to elucidate it’s effects, showcasing its antirheumatic and antiproliferative functions (Mirabelli et al., 1985).

Many tests looking at auranofin’s effects have focused on specific proteins, leading to TrxR being a proposed target (Angelucci et al., 2009). It is believed that the drug works by targeting the TrxR enzyme’s active site, rendering it unable to reduce Trx, shutting down the Trx system (Ortego et al., 2014). A recent study was conducted looking at auranofin’s potential efficacy in treating amoebiasis (Debnath et al., 2012), an intestinal disease caused by the organism Entamoeba histolytica (E. histolytica), a protozoan parasite very similar to T. vaginalis (World Health Organization, 1998). E. histolytica is also an anaerobe that relies solely upon the Trx system to guard against oxidative stress (Arias et al., 2007). Results showed auranofin’s effectiveness at targeting TrxR, killing the organism (Debnath et al., 2012).

All of this helps to make auranofin a strong candidate to treat trichomoniasis. Already having FDA approval means that the drug can easily be repurposed to treat trichomoniasis (Ashburn and Thor, 2004), and having been shown to be safe for humans,
the development timeline can be expedited, saving money and effort that would have otherwise gone to prove auranofin was not a risk (Ashburn and Thor, 2004).

The main draw of the drug, though, is that it targets TrxR. Most importantly, auranofin has been shown to be most effective against low molecular weight TrxRs, like the ones found in *T. vaginalis* (Tejman-Yarden et al., 2013). And although the drug does effect high molecular weight TrxRs, like the one found in humans, auranofin’s potency for these is greatly reduced (Gromer et al., 1998). This limits the risk of damaging cross-reactivity between auranofin and our TrxR if auranofin is taken to treat trichomoniasis.

As stated above, *T. vaginalis* lacks other conventional means to combat and deal with oxidative stress, and so must rely extensively on the Trx system. By targeting TrxR and rendering it unable to reduce Trx, auranofin is depriving the organism of its sole means of preventing oxidative damage. As a microaerophilic organism, this would prove catastrophic for *T. vaginalis*. What’s more, auranofin has also been reported to target TrxPs (Cox et al., 2008). This would be doubly damaging for the organism, as auranofin could potentially strip it of another key enzyme it needs to protect against oxidative attack.

As with any gold drug, there are side effects linked with auranofin usage, stemming from the toxicity of the gold compounds (Taylor et al., 2012). The most common are diarrhea (experienced in around 50% of patients) and a rash (experienced in around 25% of patients) (Debnath et al., 2012). By and large, however, the most pressing concern surrounding auranofin is its immunosuppressive activity (Kean and Kean, 2008). As stated above, it’s used only to treat severe cases of rheumatoid arthritis, in large part due to its effect on the immune system. The silver lining is that, at minimum, auranofin
treatment for rheumatoid arthritis lasts 6 months but can go up to 1 year (Debnath et al., 2012). Typical trichomoniasis treatment, on the other hand, can either be one-time or two weeks-long (Smith, 2016). As such, gold toxicity would be of much less concern in the short-term treatment of trichomoniasis.

Previous work with auranofin and *T. vaginalis* focused on isoform 474 (Hopper et al., 2016). Here it was shown that the drug inhibited the activity of TrxR 474 in a concentration dependent manner. What’s more, auranofin was also shown to suppress the growth of *T. vaginalis*, stressing its effect on the organism.

This study hoped to expand on the findings of Hopper et al. (2016), and had three main aims; for the first, activity assays were conducted against the different isoforms of TrxR in *T. vaginalis* using auranofin and various analogs of auranofin to determine if any are more effective at targeting the enzyme and would thus make better drug candidates; for the second, mRNA levels of the different isoforms of TrxR were examined when cells were treated with and without drug to see if the organism might respond to drug exposure by upregulating certain isoforms; and for the third, we took the mRNA analysis further, so whole-proteome analysis using mass spectrometry was conducted to see how the organism responds to drug exposure.
Chapter 2: Materials and Methods

Transformation for Cell Culture/Proliferation

Our collaborator provided plasmids of all five TrxR protein isoforms in the pET-15b vector (Novagen). Transformations into Invitrogen TOP10F’ cells were carried out for each of the five isoforms. Labeled tubes were placed on ice. To these tubes, 15µL of competent cells were added, followed by .5µL of DNA. This mixture was incubated on ice for 20 minutes, and then heatshocked in a 42°C water bath for 30 seconds. The tubes were then placed back on ice and 100µL of lysogeny broth (LB) was added. Following this, the tubes were incubated at 37°C in a 225 rpm shaking incubator for 45 minutes. 50µL of the mixtures were then plated on LB plates that had 35µL of ampicillin (100µg/mL) added to them. Plates were incubated overnight in 37°C incubator and then stored in the fridge at 4°C.

Miniprep

Overnight culture. Picked three different colonies from each of the plates of the five isoforms that were transformed into TOP10F’ cells. The colonies were placed into culture tubes with 2mL of LB and ampicillin (100µg/mL). The tubes were incubated overnight in a 225 rpm shaking incubator at 37°C.
**10 minute miniprep.** The 10 Minute Mini Prep Method from Biotechniques was used to isolate construct DNA.

**Restriction Double Digest of pET-15b Construct**

The pET-15b bacterial expression vector was double digested with NdeI and BamHI-HF to make sure that correct versions all five isoforms were present in the vector. The total volume of the digest mixture was 20µL, and included 2µL of NEB CutSmart buffer, 0.5µL NdeI, 0.5µL BamHI-HF, 3µL of plasmid DNA, and 14µL of autoclaved H₂O. The plasmid DNA was added last. The samples were incubated overnight at 37°C and then run on a 0.8% agarose gel to visualize the five isoforms. 3µL of 10x loading dye was added to each sample, and 10µL of this mixture was loaded. 10µL of a 1,000 base pair ladder was also loaded.

**Transformation and Pilot Induction**

**Transformation for protein expression.** Plasmid DNA was added to New England Biolabs (NEB) competent cells for protein expression. Labeled tubes were placed on ice. To these, 20µL of competent cells were added, followed by 0.5µL of DNA. The mixture was incubated on ice for 20 minutes and then heatshocked for 45 minutes at 42°C. The tubes were then placed back on ice and 500µL of super optimal broth with catabolite repression (SOC) outgrowth media was added. Following this, the tubes were incubated in a 225 rpm shaking incubator at 37°C for 45 minutes. 10mL of LB and ampicillin (100µg/mL) was made in a 50mL conical, and to this, 500µL of the incubated mixture was added. The LB mixture was then grown overnight in the 225 rpm shaking incubator at 37°C.
**Pilot induction.** 1mL of the overnight culture was added to 9mL of LB and ampicillin (100µg/mL) in an 50mL conical. This culture was placed in a 225 rpm shaking incubator at 37°C until the correct optical density (OD) was reached. The OD was measured at 600nm, and the desired reading was to fall between 0.4-0.8, as this represented midlog growth phase. Once the correct OD was reached, 10µL of isopropyl β-D-1-thiogalactopyranoside (IPTG) (1mM final concentration) was added to the 10mL culture to induce protein expression. The cultures were grown for 24 hours. 1.5mL samples were taken at 4 and 24 hours to determine which time point resulted better expression yields. The samples were spun down in a microfuge at 16,000xg for 1 minute. The supernatants were discarded and the pellets stored at -80°C.

**Western Blot Analysis**

100µL of SDS loading buffer (800µL of 2X SDS gel loading dye and 200µL of 1M dithiothreitol (DTT)) were added to the pellets obtained from the pilot inductions. Mixture was pipetted up and down to resuspend cells. Once resuspended, tubes were placed on a heat block >95°C for 5 minutes. Tubes were quickly spun down to pellet any remaining debris. 20µL of sample was loaded into the wells of a hand-poured SDS-PAGE gel. 7.5µL of protein ladder was also loaded. The gel was run in BioRad 1X TGS Running buffer at 125V. The samples were then transferred from the gel to Whatman PROTRAN BA 83 Nitrocellulose membrane at 100V for 1 hour and 15 minutes in BioRad 1X TG Transfer buffer (+ methanol). The membrane was incubated overnight in 25mL of 5% block (2.5g block + 50mL 1X TBS-T). Following this, the membrane was incubated with 20µL of mouse monoclonal anti-His primary antibody (1:500 dilution in 10 mL of 1% block (2mL of 5% block in 8mL of 1X TBS-T); Santa Cruz Biotechnology)
on a shaker at room temperature for 1 hour. The membrane was then washed 3x for 10 minutes with 1X TBS-T (50mL 10X TBS + 450mL H₂O + 500µL Tween-20) on a shaker at room temperature. After the washes, the membrane was incubated with 2µL of anti-mouse horse radish peroxidase (HRP)-linked secondary antibody (1:5000 dilution in 10mL of 1X TBS-T) for 1 hour on a shaker at room temperature. The membrane was washed 3x as mentioned above. 3mL of HRP substrate (Thermo Fisher Scientific (Thermo)) was added to the membrane and spread across its surface. The membrane was visualized using a BioRad Molecular Imager ChemiDoc™ XRS+.

**Protein Purification**

**Large-scale induction.** Transformations into BL21 (DE3) cells were carried out as mentioned above. The only difference is that the transformed cells were added to 15mL of LB and ampicillin (100µg/mL) to grow up overnight, instead of 10mL. The 15mL of overnight culture was added to 250mL of LB and ampicillin (100µg/mL) in a 500mL Erlenmeyer flask. This mixture was incubated in a 225 rpm shaking incubator at 37°C until the correct OD was reached. 250µL of IPTG (1mM final concentration) were then added to induce protein expression. Cultures were incubated for the optimal time based off the results from the pilot inductions above. The contents of the flask were transferred to a 250mL bottle that was spun at 5,000xg for 10 minutes in a Sorvall® Super T21 at 4°C. The supernatant was removed and cell pellet was stored at -80°C.

Before cells were spun down in the Sorvall, a 1.5mL sample of the culture was taken to tests for induction before proceeding to the purification using the Western blot protocol mentioned above.
Protein purification under native conditions. Before starting the purification protocol, a 500mL stock of Buffer F (50mM Tris-HCl (pH 8.0), 0.3M NaCl, 10mM β-mercaptoethanol, 10% glycerol, 10mM imidazole) was made. The Tris-HCl, NaCl, and glycerol were first added to 425mL of H₂O, autoclaved, and then the remaining components were added.

16mL of Lysis buffer (20mL Buffer F + 100µL of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (0.2mg/mL) + 200µL lysozyme (100mg/mL)) was added to the cell pellet from the large-scale induction and the bottle was placed on ice. The mixture was pipetted up and down using a serological pipette until the pellet was resuspended. The contents were then transferred to a 50mL conical. The conical was placed in an ice bucket horizontally, and the ice bucket was placed on a shaker at high speed for 30 minutes. The mixture was then sonicated on ice with 6x 10 second bursts, with a 20 second wait between each burst. Following sonication, the mixture was spun at 10,000xg for 20 minutes in a Sorvall at 4°C. The supernatant (lysate) was transferred to a new 50mL conical.

4mL of 50% Ni-NTA slurry (1mL/4mL lysate) were added to a 50mL conical and spun at 2,500RPM for 2 minutes in a Sorvall at 4°C. The supernatant was removed and an equal amount of Buffer F was added. The mixture was resuspended and the spin in the Sorvall was repeated. The supernatant was removed and the lysate was added. The conical was left on a nutator in a 4°C fridge overnight so that the 6xHIS-tag on the induced protein would properly bind to the Ni-NTA agarose beads. After incubating, the mixture was spun down at 5,000xg for 2 minutes in a Sorvall at 4°C. The supernatant was moved to a new conical. The beads were then washed 3x by adding 10mL of Buffer
F and placing the conical on a nutator in a 4°C fridge for 10 minutes. In between the washes, the mixture was spun down at 5,000xg for 2 minutes in a Sorvall at 4°C, and the supernatant was removed. After the third wash, the Buffer F-Ni-NTA mixture was loaded slowly into a column with the bottom outlet capped so as to not create any air bubbles. This was done in a 4°C fridge, which is where the elutions took place. The bottom outlet was then opened and the flow through was used to pack the beads. Once the beads were packed, a step gradient of increasing concentrations of elution buffers was used to elute the protein through gravity filtration. In total, six elution buffers of different concentrations of imidazole were used: 50mM, 100mM, 150mM, 200mM, 250mM, and 300mM. 2mL of each buffer were used to elute protein, except the beads were washed twice with the 300mM buffer, so 4mL total were used for the highest concentration buffer. 50µL samples of the lysate, washes, and elutions were taken to analyze using Coomassie stain. Samples were all stored at -80°C.

**Coomassie stain.** 50µL of SDS loading buffer were added to the 50µL samples collected from the purification above. An SDS-PAGE gel was loaded and run as stated above. The gel was then removed and enough InstantBlue dye was added to completely cover the gel. The gel and dye were incubated for 1 hour on a shaker at room temperature. The dye was removed and deionized water was used to destain the gel. After destaining, the gel was visualized using the BioRad Molecular Imager ChemiDoc™ XRS+ to determine which concentration of imidazole eluted the greatest amount of protein.
Protein Quantification

**Bradford assay.** To quantitate the concentration of protein eluted from the native purifications, a Bradford assay was conducted. Stock bovine serum albumin (BSA) at a concentration of 10mg/mL was diluted 1:10 to a final concentration of 1mg/mL. This was used to make standards of 2µg/mL, 4µg/mL, 6µg/mL, 8µg/mL, and 10µg/mL. Enough H₂O was added to bring the total volume to 800µL. A tube with no added BSA had 800µL of H₂O added to serve as a blank. To separate tubes, 5µL of eluted protein were added, and again, were mixed with enough H₂O to bring the total volume to 800µL. All of the tubes had 200µL of Bradford Dye Reagent (BioRad) added to them (final concentration 1mL), and were incubated for at least 5 minutes but no greater than 1 hour at room temperature. After incubating, the absorbance for the standards and samples was measured at 595nM. Using Excel, the values for the standards were used to generate a standard curve. The equation of the curve was used to determine the concentrations of the samples.

**Coomassie stain for protein quantitation.** To ensure the results of the Bradford were accurate, a Coomassie stain was performed. 30µL of BSA (1µg/µL) and 30µL of SDS loading buffer were mixed to create a BSA standard solution with a concentration of 0.5µg/µL. 8µL (4µg), 12µL (6µg), 16µL (8µg), and 20µL (10µg) were loaded into the wells of a hand-poured SDS-PAGE gel to serve as protein concentration comparisons for the eluted proteins from the native purification. 2 and 4µL of eluted protein were also loaded. The same Coomassie protocol was followed as listed above. Once destained, the bands of the standards were compared to the bands of eluted protein to estimate the concentration of the eluted protein.
TrxR Drug Treatment Assay

Purified TrxR was used to determine the affects of different concentrations of auranofin and auranofin analogs on the enzyme’s activity. The total reaction volume was 100µL. The reaction was carried out in a 100µL cuvette that was heated to 37°C in a Thermo Scientific NanoDrop 2000c Spectrophotometer. 13.3µL of 15mM Ellman’s Reagent (DTNB) (final concentration of 2mM) was added to a microfuge tube, followed by a variable amount of 0.1M KPO₄ buffer warmed to 37°C in a heat block. (The amount was variable because it depended on the concentration of the purified TrxR isoform. The isoforms purified had different concentrations, and so different amounts of buffer were needed to bring the total volume of the reaction up to 100µL). The two reagents were mixed. Then, a 100nM enzyme mix of TrxR and Trx proteins was added to the tube and mixed as well. The final thing mixed into the tube was 1µL of either DMSO or drug. This mixture was then transferred to the cuvette and incubated for 1-2 minutes in the NanoDrop. The NanoDrop was blanked, and 10µL of 2mM NADPH (final concentration of .2mM) was added to the cuvette and pipetted up and down several times. The cuvette was placed back into the NanoDrop and the activity was measured every 30 seconds for 3 minutes at 412nm.

Eight concentrations of drug were tested: .1nM, 1nM, 10nM, 50nM, 100nM, 250nM, 500nM, and 1µM. The drugs were solubilized in DMSO, and so DMSO was used as the negative control. Treatments for auranofin were carried out in duplicate, and those for the analogs in triplicate. All of the reagents except the buffer and drug/DMSO were kept on ice. The buffer was heated to 37°C and the drug/DMSO were kept at room temperature.
IC<sub>50</sub> Calculation for Auranofin and Auranofin Analogs

The absorbance data obtained from the drug treatment assays was transferred to Excel and used to calculate the nmols of NADPH consumed per minute, a measure of the TrxR protein enzymatic activity. To account for any variability and human error, these values were normalized to the values obtained from that trial’s DMSO treatment. So the final data were presented as percent activity as compared to DMSO, with DMSO treatment corresponding to 100% activity. The values were input into GraphPad Prism software, and IC<sub>50</sub> curves and values were generated and calculated, respectively.

qPCR Analysis of Isoform Levels Post-Drug Treatment

Drug treatment. An 8µL sample was taken from a 10mL tube containing T. vaginalis G3 cells and placed onto a hemocytometer to determine what volume contained 2x10<sup>6</sup> cells. This volume was added to three separate tubes with enough media present so that the total volume would be 10mL. One tube had 5µL of DMSO added, another had 2.5µL of a 1mM stock of auranofin added (0.25µM final concentration), and the third had 5µL of the 1mM auranofin stock added (0.5µM final concentration). The cultures were grown overnight at 37°C. A hemocytometer was once again used to ensure that the drug treatments worked on the cells. The cells were spun for 5 minutes at 1,000xg and had the supernatant removed, followed by a wash with 10mL of PBS. Another 5-minute spin at 1,000xg was performed. The supernatant was removed, and the pellet stored at -80°C. The drug treatments were completed in triplicate.

RNA isolation. RNA was isolated from G3 cells treated with DMSO, 0.25µM auranofin, and 0.5µM auranofin using the TRIzol® Reagent RNA Isolation Procedure.
from invitrogen. Once isolated, RNA concentrations were determined by using a Thermo Scientific NanoDrop 2000c Spectrophotometer, and the samples were stored at -80°C.

**Reverse transcription.** RNA isolated from G3 cells that underwent the three different treatment conditions mentioned above was reverse transcribed. First, the RNA concentrations calculated were used to determine what volume contained 1µg of RNA. Because, the concentrations were so high, they were first diluted 1:10, and the concentrations of these dilutions were used to determine what volume contained 1µg of RNA. Enough RNase-free H₂O was added to bring the total volume up to 8µL. To this tube, 1µL of 10X DNase I Buffer and 1µL of DNase I were added, and the mixture was incubated at room temperature for 15 minutes. 1µL of 25mM EDTA was added, and the the tube was incubated on a 65°C heat block for 10 minutes, followed by an incubation on ice for 5 minutes. 1µL of oligo DT (0.5µg/µL) and 1µL of 10mM dNTPs were added. This was followed by another incubation cycle of 65°C for 10 minutes, and ice for 5 minutes. Next, 4µL of 5X Superscript Buffer, 1µL of 0.1M DTT, 1µL of RNase Out, and either 1µL of Superscript or H₂O were added. (One of the treatment sets was reverse transcribed in duplicate without the addition of reverse transcriptase to serve as a negative control). The tubes were placed in a PCR machine programmed for 50°C for 1 hour, 70°C for 15 minutes, and 10°C overnight. 1µL of RNase H was added, followed by a 37°C incubation for 20 minutes. The mixture was then diluted 1:10 for subsequent qPCR analysis.

**qPCR analysis.** The reverse transcribed cDNA of the G3 cells exposed to the three treatment methods from above was used to analyze levels of TrxR isoform levels. The total reaction volume was 50µL. To PCR tubes, 25µL of iTaq 2X, 1µL of forward
primers, 1µL of reverse primers, 13µL of RNase-free H₂O, and 10µL of cDNA were mixed together on ice. Actin was used as the reference gene, since its levels are known to not be affected by auranofin treatment. The samples were loaded into an MJ Research DNA Engine Opticon™ 2 qPCR machine, and the levels of the TrxR isoforms were analyzed across the three treatment methods and the triplicate treatments.

The data was transferred to excel, where values for average Cₜ, ΔCₜ, ΔΔCₜ, and fold change were calculated.

**Proteomic Analysis**

**Drug treatment.** *T. vaginalis* G3 cells were treated under the same conditions as described above, except treatments were performed in duplicate, not triplicate.

**In-solution digest.** 175µL of Lysis Buffer (6.4M urea, 200mM NH₄HCO₃, pH 7.5) was used to resuspend pellet. Mixture was transferred to a microfuge tube and cells were lysed by incubating for 1 hour at room temperature. Following the incubation, cells were quickly sonicated (3-5 short bursts) and spun at 16,000xg for 10 minutes. Supernatant was collected into two microfuge tubes—100µL to continue with the protocol, and the rest for quantification of protein using a Bradford assay as mentioned above. 0.5µL of 1M DTT (5mM final concentration) was added to the tube, and the mixture was incubated for 1 hour at room temperature (can be done with cell lysis step, above). Next, 15µL of iodoacetamide (IAA) (15mM final concentration) were added, and the mixture was incubated for 30 minutes at room temperature in the dark. Following that, the urea was diluted out with the addition of 3 volumes (300µL) of 50mM NH₄HCO₃ (1:4 dilution). Using the concentration amount obtained from the Bradford, enough Trypsin was added to produce a sample:Trypsin ration of 10:1. Sample
was incubated with Trypsin overnight at 37°C. After the incubation, 1-part Sample Buffer (2% trifluoroacetic acid (TFA) in 20% acetonitrile(ACN)) was added to 3-parts of the overnight sample and Trypsin digest.

Thermo Scientific Pierce® C-18 Spin Columns were prepped by adding 200µL of Activating Solution (50% methanol) to wet column walls and resin. The column was spun down at 1,500xg for 1 minute, and the flow-through was discarded. Activations step was repeated once more. Next, 200µL of Equilibration Solution/Wash Buffer (0.5% TFA in 5% ACN) was added, and the column was centrifuged at 1,500xg for 1 minute. The flow-through was discarded. This last step was repeated once more.

Sample was added to column, and the column was attached to a fresh collection tube. The column was centrifuged at 1,500xg for 1 minute, and the flow-through was saved. Column was washed by adding 200µL of Wash Buffer and spinning at 1,500xg for 1 minute. Flow-through was discarded. Wash step was repeated. Protein was eluted by adding 20µL of elution buffer (70% ACN) and spinning the column at 1,500xg for 1 min. Elution was repeated for a total volume of 40µL. The entire protocol starting at the equilibration step was repeated 3x, for a total eluted volume of 160µL. The ACN was lyophilized off using a Labconco FreeZone 12 Plus. Protein was then resuspended in high-performance liquid chromatography (HPLC) grade H₂O with 0.1% formic acid to get a final concentration of about 0.5-0.6µg/µL (usually between 30-50µL). The sample was then added to a mass spectrometry vial for analysis.

**Mass spectrometry analysis.** An Orbitrap Fusion™ Tribrid™ Mass Spectrometer from Thermo was used to analyze the proteomes of the G3 cells exposed to the three treatment methods listed above through tandem mass spectrometry. Peptides
were fragmented using three different methods: collision-induced dissociation (CID), higher-energy collisional dissociation (HCD), and electron-transfer dissociation (ETD).

Originally, to detect more peptides, the m/z range that the Orbitrap detects was divided into a low-mass group (m/z=300-600) and high-mass group (m/z=600-1,800). There wasn’t enough of one of the samples to do this dual range, however, and so the analysis focused on the low-mass range.

The data obtained form the Orbitrap was input into the proteomics software PEAKS to sequence the peptides and identify and quantitate the proteins. Data was normalized to actin. Data generated from PEAKS was used to graph whole-proteome changes in response to the different drug treatments. Aside from the quantities of peptide fragments that were present in the three fragmentation methods, PEAKS also took 3 Surveys to determine overall peptide quantities. These survey values were averaged to determine how specific protein levels changed in response to drug treatment, when compared to DMSO. Levels were normalized to DMSO and presented as a group profile ratio.
Chapter 3: Results

Pilot Inductions of TrxR Isoforms

There are five different isoforms of TrxR in *T. vaginalis* (Table 2)—for convenience, the isoforms will be referred to by the first three digits of their gene accession number (bolded).

Table 2. List of the five different TrxR isoforms present in *T. vaginalis*, as well as their molecular weight and gene length.

<table>
<thead>
<tr>
<th>TrxR</th>
<th>kD</th>
<th>bps</th>
</tr>
</thead>
<tbody>
<tr>
<td>TVAG_474980</td>
<td>32.4</td>
<td>915</td>
</tr>
<tr>
<td>TVAG_125360</td>
<td>33.1</td>
<td>936</td>
</tr>
<tr>
<td>TVAG_328010</td>
<td>38.1</td>
<td>1059</td>
</tr>
<tr>
<td>TVAG_281360</td>
<td>37.3</td>
<td>1041</td>
</tr>
<tr>
<td>TVAG_240530</td>
<td>36.1</td>
<td>1005</td>
</tr>
</tbody>
</table>

Our collaborator had all five isoforms cloned into the pET-15b vector. These five constructs were subsequently transformed into TOP10F’ cells to proliferate. Three colonies were picked for each construct (designated a, b, and c), and the DNA was miniprepped. The constructs were cut using NdeI and BamH1-HF. The digested DNA
was run out on a gel to confirm the presence of the isoform DNA within the plasmid constructs. All three colonies from the five isoforms showed bands of the expected size.

We chose to do pilot protein expression studies using the two colonies of each construct that appeared to contain the most DNA. Since that meant 10 colonies total, several rounds of Westerntons were performed to examine them all.

Figure 3 shows the results of the pilot inductions, with cell cultures collected at two time points following addition of IPTG: 4 and 24 hrs. A sample was also collected just prior to addition of IPTG to serve as a negative control. Looking at the blots, it is observed that for many of the colonies, the negative lane had the most prominent band. This is owed to the cell line used to express the isoforms, BL21 DE3. These cells result in leaky expression prior to IPTG addition. Before induction, colonies were grown up overnight. From these overnight colonies, 1mL was added to fresh media and grown up until the correct OD was reached. It was then that IPTG was added. The negative samples were taken from the overnight colonies, however. These cells had been slowly producing protein for over a day, resulting in the prominent band in the negative lanes.

Before using BL21 DE3 cells, BL21 cells were used, but it was found that many of the isoforms were inadequately expressed in this cell line. Before switching over to BL21 DE3 cells, however, isoform 125 had been expressed, and so this is what was used as a positive control.
Figure 3. Western blot analysis of pilot TrxR isoform expression in *E. coli* strain BL21 DE3. Anti-His antibody probe used. Cell cultures collected 4 and 24hrs after IPTG induction. Negative lanes contain samples collected prior to IPTG addition. Positive lanes contain a TrxR 125 that had been previously expressed.
Protein was present for all colonies tested, although TrxR was present in much greater abundance in isoforms 474 and 125 than in the other three. Looking at 474 first, colony “a” had better induction levels than colony “c”. While for isoform 125, both colonies yielded high protein levels. For both of these higher expressing isoforms, it appears that the 4hr induction resulted in maximum amount of protein than the 24hr induction. Looking at all five colonies, it seems as if the 4hr induction resulted in higher protein yields than the 24hr induction.

The other three isoforms had levels of TrxR much lower than those of 125 and 474, and so the pilot inductions had to be repeated. Repeatedly, though, proteins levels remained low. Culture conditions were altered to see if different temperatures might produce more optimal inducing conditions, but these attempts saw no change in levels produced. Cell lines were switched for a second time in an effort to produce different results. BL21 DE3 RIPL cells were used, leading to increases in the amounts of protein produced of isoforms 281 and 348. Isoform 240 never expressed adequate amounts of protein, preventing any further work.

**Large-Scale Purification of TrxR Isoforms**

Based off the data from the Westerns, isoforms 474 and 125 yielded the highest amounts of protein by a large margin, even after isoforms 348 and 281 were moved to the RIPL strain of cells. Because of this, and because we hadn’t been able to induce decent amounts of protein from the other three isoforms, we decided to proceed first with these two better-inducing isoforms.

BL21 DE3 cells were grown up in 250mL cultures for large-scale inductions in order to purify large amounts of protein for future drug assays. After inducing for 4hrs, the proteins were purified by gravity filtration using Ni-NTA agarose beads under native
conditions using a step gradient with increasing concentrations of imidazole to elute. Following that, SDS-PAGE gels were run, followed by Coomassie stains to ensure that the purification had worked properly and to determine which elution contained the highest amounts of protein (Figures 4 and 5).

Figure 4. Coomassie stain of TrxR 474 protein purification fractions. A step gradient of increasing concentrations of imidazole was used to elute the protein. Protein was eluted with 300mM of imidazole twice (lanes 9 and 10). Protein was purified from *E. coli* under native conditions.
Both purifications were successful. For 474, the greatest amount of protein was eluted with 100mM imidazole. For 125, the greatest amount of protein was eluted with 150mM imidazole.

Despite having shown good induction levels during the pilot inductions using the RIPL cells, the large-scale inductions of isoforms 348 and 281 struggled to yield similar results. Despite repeated attempts, large-scale inductions of 281 never resulted in enough protein to proceed with assays. For isoform 348, eventually enough protein was successfully induced large scale so that assay work could proceed. The protein first had to be concentrated, however.
**Quantifying purified TrxR.** A Bradford assay was performed to quantitate amount of protein eluted. Two elutions for each isoform were tested to see how comparable the concentrations were: 100mM and 150mM for 474; and 150mM and 200mM for 125. µM amounts were only calculated for the elutions with the highest concentrations. For 474, the 100mM elution had a concentration of .87 µg/µL, (27µM). For 125, the 150mM elution had a concentration of .75 µg/µL, (23µM). Once concentrated, the concentration of 348 was determined to be 2.22µg/µL (58.4µM).

Because the elutions didn’t look very clean, a Coomassie was also used to quantify the amounts of isoforms 474 and 125 (Figure 6). Different amounts of BSA standards (4, 6, 8, and 10µg) were used to estimate the amount of enzyme for 474 and 125. It should be noted that some of the prepared BSA solution was lost before all of the standards were pipetted into the wells. This is why the lane for the standard of 10µg contains less protein than that of the lane for 8µg. Two separate volumes of isoforms were loaded (2 and 4µL). The results helped to validate the results obtained from the Bradfords.
Drug Assays

We began enzymatic assays with the purified isoforms to see how auranofin affects the different isoforms and if analogs of the drug have a stronger inhibitory effect on the enzymes.

Auranofin assays. The purpose of testing auranofin analogs against the isoforms was to potentially find any drugs with similar or greater efficacy than auranofin that
could then be further tested to inspect if they result in less side effects. If so, that would make the analogs better drug candidates and further testing could proceed with them instead. Because of this, auranofin assays first needed to be conducted in order to have a baseline for which to compare the results of the analogue tests.

The assays were carried out small scale using five components (Table 3). The total volume of the assay was 100 µL. DMSO was used as a control because all of the drugs were suspended in it. The drug concentrations used were .1nM, 1nM, 10nM, 50nM, 100nM, 250nm, 500nM, and 1µM. 1µM was used as the highest concentration because previous work by Hopper et al. had shown that this concentration of auranofin was very potent at inhibiting enzymatic activity. .1nM was chosen as the lowest dosage because we wanted a concentration that correlated to negligible amount of drug. In this way, the eight drug concentrations spanned a comprehensive spectrum. And as such, each assay set consisted of testing the isoform against 9 compounds (DMSO plus the 8 concentrations). For auranofin, sets were carried out in duplicate.

Table 3. Components of the drug activity assay. Total volume is 100µL.

<table>
<thead>
<tr>
<th>DTNB</th>
<th>Enzyme Mix (100nM)</th>
<th>Drug/DMSO</th>
<th>NADPH (2mM)</th>
<th>Buffer (.1 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.3µL</td>
<td>2.5µL of Trx + TrxR (3.7µL 474, and 4.35µL 125)</td>
<td>1µL</td>
<td>10µL</td>
<td>69.5µL for 474 68.85µL for 125</td>
</tr>
</tbody>
</table>

DTNB gets reduced by Trx, producing 2 TNB molecules, which are yellow. As the reaction proceeds, more and more yellow is produced as more and more DTNB is reduced by Trx. The breakdown of DTNB into TNB, and subsequent yellow production,
results in a change in absorbance. This absorbance change is what was monitored as the reaction proceeded. Because NADPH is the initial electron source, and because its starting amount is known, it is possible to use the change in absorbance (production of TNB molecules) to calculate the amount of NADPH consumed. This amount is what was used to gauge how active the isoforms were. Since DMSO was the negative control, all values were normalized to it. This meant that in the end, data was in the form of %DMSO activity, with DMSO-treated isoforms having 100% activity. This data was entered into GraphPad Prism to generate IC\textsubscript{50} curves and to calculate IC\textsubscript{50} values for 474 and 125 (Figures 7 and 8). For TrxR 474, auranofin’s IC\textsubscript{50} = 255nM. For TrxR, auranofin’s IC\textsubscript{50} = 397nM. From this, we can see that auranofin is more effective against isoform 474, bringing its activity down to 50% at a lower concentration.

Comparing these results to those previously observed by Hopper et al. (2016), a difference in IC\textsubscript{50} values is seen. They reported a value far lower (IC\textsubscript{50}=80nM), than our calculated value. This difference might be the result of differences in assay conditions. Their assay was done on a larger scale (1mL total volume), and this might account for the different IC\textsubscript{50} values observed. Some variation on protein concentrations tested might also be the cause of the differing values.
Figure 7. IC$_{50}$ graph of auranofin’s effect on the activity of TrxR 474. IC$_{50}$=255nM.

Figure 8. IC$_{50}$ graph of auranofin’s effect on the activity of TrxR 125. IC$_{50}$=397nM.
IC\textsubscript{50} data for isoform 348 could not be calculated because the protein was found to have very little activity. Looking back at Figure 2, it can be seen that when examining the active sites of the five isoforms, only isoforms 474, 125, and 281 have the correct amino acid sequence (Mentel et al., 2008). This would account for the observed inactivity of isoform 348 in the assay, since it and isoform 240 both have non-active active sites. To confirm this inactivity, another round of large-scale inductions was performed and more of isoform 348 was purified. Again, however, no activity was observed, and so the analog assays were only carried out with isoforms 474 and 125.

**Analog Assays.** The analog assays were carried out in the same manner as the auranofin assays. The same eight concentrations of drug were tested, and DMSO was still used as a negative control. The only difference was that analog assays were carried out in triplicate, rather than in duplicate.

Two different analog libraries were screened against isoforms 474 and 125. From the first library, five drugs were tested. These were analog 1, analog 3, analog 4, analog 5, and analog 7. The second library consisted of 15 drugs (LEAU 1-15). Each of these drugs was tested against TrxR 474 at 1\(\mu\)M, since that was the highest concentration of auranofin used in the previous assays. Full-scale assays on isoforms 474 and 125 were conducted using only the analogs whose inhibition of enzymatic activity was either on par or greater than that of auranofin. There were only three analogs from this second library that met those criteria: LEAU 8, LEAU 9, and LEAU 14. So in total, 8 analogs were tested against the two isoforms (Figures 9-24).
Figure 9. IC$_{50}$ graph of analog 1’s effect on the activity of TrxR 474. IC$_{50}$=270nM.

Figure 10. IC$_{50}$ graph of analog 1’s effect on the activity of TrxR 125. IC$_{50}$=508nM.
Figure 11. IC$_{50}$ graph of analog 3’s effect on the activity of TrxR 474. IC$_{50}$=6.28µM.

Figure 12. IC$_{50}$ graph of analog 3’s effect on the activity of TrxR 125. IC$_{50}$=4.45µM.
Figure 13. IC$_{50}$ graph of analog 4’s effect on the activity of TrxR 474. IC$_{50}$ = 831nM.

Figure 14. IC$_{50}$ graph of analog 4’s effect on the activity of TrxR 125. IC$_{50}$ = 2.86μM.
Figure 15. IC₅₀ graph of analog 5’s effect on the activity of TrxR 474. IC₅₀ = 2.35µM.

Figure 16. IC₅₀ graph of analog 5’s effect on the activity of TrxR 125. IC₅₀ = 6.05µM.
Figure 17. IC₅₀ graph of analog 7’s effect on the activity of TrxR 474. IC₅₀=24.74µM.

Figure 18. IC₅₀ graph of analog 7’s effect on the activity of TrxR 125. IC₅₀=49.57µM.
Figure 19. IC$_{50}$ graph of analog LEAU 8’s effect on the activity of TrxR 474. IC$_{50}$=95nM.

Figure 20. IC$_{50}$ graph of analog LEAU 8’s effect on the activity of TrxR 125. IC$_{50}$=291nM.
Figure 21. IC$_{50}$ graph of analog LEAU 9’s effect on the activity of TrxR 474. IC$_{50}$=135nM.

Figure 22. IC$_{50}$ graph of analog LEAU 9’s effect on the activity of TrxR 125. IC$_{50}$=210nM.
Figure 23. IC$_{50}$ graph of analog LEAU 14’s effect on the activity of TrxR 474. IC$_{50}$=210nM.

Figure 24. IC$_{50}$ graph of analog LEAU 14’s effect on the activity of TrxR 125. IC$_{50}$=414nM.
A summary of the IC\textsubscript{50} values for auranofin and the various analogs in regards to isoforms 474 and 125 is provided in Table 4. Here we can see that certain drugs affected the isoforms differently, and inhibited the activity of one much more greatly than it did the other. As such, it’s best to discuss the effect the drugs had on the isoforms separately.

Looking at TrxR 474 first, we can see that the effects of analogs 1 and LEAU 14 were fairly comparable to those of auranofin. Analogues LEAU 8 and 9, however, were more effective at inhibiting the enzymes activity, with LEAU 8 being the most effective of the two.

When looking at TrxR 125, we see that the only analog that was comparable to the effects of auranofin was LEAU 14. And just like TrxR 474, 125’s activity was most inhibited by analogs LEAU 8 and 9. Unlike TrxR 474, however, 125 was most greatly inhibited by LEAU 9.

<table>
<thead>
<tr>
<th></th>
<th>AF</th>
<th>A1</th>
<th>A3</th>
<th>A4</th>
<th>A5</th>
<th>A7</th>
<th>LEAU 8</th>
<th>LEAU 9</th>
<th>LEAU 14</th>
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<tbody>
<tr>
<td>TrxR 474</td>
<td>255nM</td>
<td>270nM</td>
<td>6.28μM</td>
<td>831nM</td>
<td>2.35μM</td>
<td>24.74μM</td>
<td>95nM</td>
<td>135nM</td>
<td>210nM</td>
</tr>
<tr>
<td>TrxR 125</td>
<td>397nM</td>
<td>508nM</td>
<td>4.45μM</td>
<td>2.86μM</td>
<td>6.05μM</td>
<td>49.57μM</td>
<td>291nM</td>
<td>210nM</td>
<td>414nM</td>
</tr>
</tbody>
</table>

qPCR Analysis of Isoform Levels

There are five isoforms of TrxR present within \textit{T. vaginalis}, but that doesn’t mean that all of the isoforms are transcribed equally. Transcript levels might even be influenced by exposure to auranofin. The organism could possibly modulate transcription of certain isoforms to deal with the stress caused by drug treatment.
Knowing this would be beneficial to understanding ways in which *T. vaginalis* might respond to auranofin.

Three tubes of G3 cells were treated with either DMSO (negative control), .25µM auranofin, or .5µM auranofin. Cells were treated with a high and low concentration of the drug to see if it would respond differently to varying levels of stress. These drug treatments were carried out in triplicate. After treatment, total RNA was isolated from the cells. This RNA was subsequently reverse transcribed to produce cDNA. During the reverse transcription, one set of tubes didn’t have reverse transcriptase added, and this set served as the negative control. Once the cDNA was obtained, the real-time PCRs could be carried out. Before that, however, test PCRs were performed with the five different isoforms to determine what annealing temperature was best for them. A temperature gradient was tested ranging from 57.3°C-62.0°C. For all five isoforms, the lowest temperature proved optimal. Primers used are listed in Table 5.
The reference gene used was actin. Its Ct values were used to calculate \( \Delta C_T \) values. The \( \Delta C_T \) values of the DMSO treated cells were averaged across the triplicate treatments and used to compare basal mRNA levels of the five isoforms by looking at fold changes in expression (Figure 25). The isoform with the lowest basal levels (TrxR 281), was used as a reference to determine the levels of the other four isoforms. The data shows that isoforms 348, 281, and 240 all present in the lowest amounts. The levels of isoform 125 are around 80- and 70-fold higher than these three isoforms, while the levels of isoform 474 are over 240-fold higher.

Table 5. Forward and reverse qPCR primers designed for the five TrxR isoforms.

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>TrxR 474</td>
<td>F: GCTACAGCCGTCTACCTTGG</td>
</tr>
<tr>
<td></td>
<td>R: ACGGCAGTGATCATGTGG</td>
</tr>
<tr>
<td>TrxR 125</td>
<td>F: TAAATTTAGTTCACAGAGAGACC</td>
</tr>
<tr>
<td></td>
<td>R: CTGACTTCTGTTGTGACAACAT</td>
</tr>
<tr>
<td>TrxR 348</td>
<td>F: ATGAAGTTCTTGGCGAAGATTTTC</td>
</tr>
<tr>
<td></td>
<td>R: TGCCCTGTAAACTTTGTCTGCG</td>
</tr>
<tr>
<td>TrxR 281</td>
<td>F: CAAGTCCTTGCAAAATCTACCG</td>
</tr>
<tr>
<td></td>
<td>R: GTTGCAAGGTCTGTCTCATG</td>
</tr>
<tr>
<td>TrxR 240</td>
<td>F: AAACACCTTCAAGTAACGGAATC</td>
</tr>
<tr>
<td></td>
<td>R: CCTGGCTTGTACAATCTCCAG</td>
</tr>
</tbody>
</table>
From the ΔC\textsubscript{T} values, average ΔΔC\textsubscript{T} values and fold-change amounts across the triplicate treatments were calculated for G3 cells treated with 0.25µM and .5µM auranofin. Figures 26-30 show how mRNA levels changed for the five TrxR isoforms in relation to DMSO across the two drug treatments.
Figure 26. Comparison of mRNA levels of TrxR isoform 474 in response to drug treatment. DMSO levels are considered basal, and so are given a value of 1. The values for the drug-treated cells are in relation to DMSO-treated cells. G3 cells treated with .25µM auranofin had a fold change of 1.073. G3 cells treated with .50µM auranofin had a fold change of 1.536.
Figure 27. Comparison of mRNA levels of TrxR isoform 125 in response to drug treatment. DMSO levels are considered basal, and so are given a value of 1. The values for the drug-treated cells are in relation to DMSO-treated cells. G3 cells treated with .25µM auranofin had a fold change of 1.644. G3 cells treated with .50µM auranofin had a fold change of 2.377.
Figure 28. Comparison of mRNA levels of TrxR isoform 348 in response to drug treatment. DMSO levels are considered basal, and so are given a value of 1. The values for the drug-treated cells are in relation to DMSO-treated cells. G3 cells treated with 0.25 µM auranofin had a fold change of 1.729. G3 cells treated with 0.50 µM auranofin had a fold change of 0.927.
Figure 29. Comparison of mRNA levels of TrxR isoform 281 in response to drug treatment. DMSO levels are considered basal, and so are given a value of 1. The values for the drug-treated cells are in relation to DMSO-treated cells. G3 cells treated with .25µM auranofin had a fold change of 1.429. G3 cells treated with .50µM auranofin had a fold change of 1.827.
From these figures (data summarized in Table 6), we can see that no general trend was shared by the five isoforms. There was no consistency in how mRNA levels changed in regards to the concentration of drug treatment, and the fold changes of the mRNA levels also show variance. Isoform 474, the isoform with the greatest amount of basal mRNA, had an almost negligible fold increase for the lower concentration drug treatment, and a little over a half-fold increase for the higher concentration treatment. This differs markedly from isoform 125, the isoform with the second highest levels of basal mRNA, and the isoform that experienced the highest fold change. The lower
concentration of drug resulted in a half-fold increase, while the higher concentration produced an almost 1.5-fold increase. Isoform 348 was the only one that experienced a fold decrease between the low and high concentration drug treatments. The final two isoforms, 281 and 240, shows similar trends of experiencing progressively increased fold increases with increased drug concentration. These values show that treatment with different auranofin doses affects the isoforms differently, in a manner independent of the proteins’ basal mRNA levels.

Table 6. Averages of ΔΔC_T and fold-change values of the five TrxR isoforms when treated with two concentrations of auranofin from qPCR results.

<table>
<thead>
<tr>
<th>Sample</th>
<th>ΔΔC_T</th>
<th>Fold-Change from DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>.25μM AF 474</td>
<td>-0.102</td>
<td>1.073</td>
</tr>
<tr>
<td>.50μM AF 474</td>
<td>-0.619</td>
<td>1.536</td>
</tr>
<tr>
<td>.25μM AF 125</td>
<td>-0.717</td>
<td>1.644</td>
</tr>
<tr>
<td>.50μM AF 125</td>
<td>-1.249</td>
<td>2.377</td>
</tr>
<tr>
<td>.25μM AF 348</td>
<td>-0.790</td>
<td>1.729</td>
</tr>
<tr>
<td>.50μM AF 348</td>
<td>0.109</td>
<td>0.927</td>
</tr>
<tr>
<td>.25μM AF 281</td>
<td>-0.515</td>
<td>1.429</td>
</tr>
<tr>
<td>.50μM AF 281</td>
<td>-0.870</td>
<td>1.827</td>
</tr>
<tr>
<td>.25μM AF 240</td>
<td>-0.640</td>
<td>1.559</td>
</tr>
<tr>
<td>.50μM AF 240</td>
<td>-0.671</td>
<td>1.592</td>
</tr>
</tbody>
</table>
**Proteome Analysis by Mass Spectrometry**

Changing mRNA levels can sometimes be indicative of changing protein levels, but that isn’t always the case, due to post-translational modifications. To further test how auranofin treatment might affect TrxR isoform levels, mass spectrometry was used to conduct a whole proteome analysis on treated and untreated cells. Again, G3 cells were treated with either DMSO, .25µM auranofin, or .5µM auranofin. This time, tests were carried out in duplicate. An in-solution digest was carried out to breakdown the proteins into peptides. Following this, the peptides were isolated and prepped for the Orbitrap Fusion™ Tribrid™ Mass Spectrometer where they were analyzed by tandem mass spectrometry. Three different fragmentation methods (CID, HCD, and ETD) were used to identify peptides. The proteomics software PEAKS was used to identify and quantitate proteins in our samples using the fragment data. Protein quantities were normalized to actin levels.

**Figure 31** represents the whole-proteome analysis that was conducted. Each blue dot represents one peptide, and the figure compares how abundance of peptides compared between both treatments and across the different treatment conditions. While the whole-proteome analysis provided expansive information, the data that was most relevant to us was how TrxR isoform levels responded to the different concentrations of drug. Next, we also looked at how other cellular redox proteins were affected as well. When examining the data, one can see how the abundance of various peptides changes depending on what the cells were treated with. When the DMSO-treated cells from treatments 1 and 2 are compared, the scatter plot is very linear. This is what we would hope and expect to see, because many of the same peptides should be present in untreated G3 cells in very similar abundance. The linearity disappears most dramatically when DMSO-treated cells are
compared to cells treated with .5\mu M auranofin. Here, the scatter plots are much more dispersed. This is due to changes in peptide abundance between the two treatment conditions, indicating that drug treatment does lead to a change in protein levels in \textit{T. vaginalis}, whereby some proteins experienced upregulation while others downregulation.

Figure 31. Whole proteome analysis of two treatments of G3 cells treated with DMSO, .25\mu M auranofin, or .5\mu M auranofin. “-1” and “-2” denote treatment 1 and treatment 2. Each blue dot is one peptide. Graph shows different comparisons based on peptide mass and abundance between both treatments and all three treatment conditions. A more linear scatter plot indicates greater similarity in peptide abundance between treatment conditions. A more disorganized scatter plot indicates decreased similarity in peptide abundance between treatment conditions, and thus a difference in protein levels.
Looking specifically at cellular redox proteins, group profile ratios were used to compare changes in protein levels in response to drug treatment. These ratios represent the abundance of the proteins under the two auranofin treatments, normalized to the abundance present in the DMSO treatment (the abundance assumed to represent basal levels). Starting with the five TrxR isoforms, only two of them, 474 and 125, were detected by the mass spectrometer. Figures 32 and 33 show the changes in abundance for these two isoforms across the two drug treatments when normalized to DMSO. From this data, we see that the isoforms differed in the way their protein levels responded to auranofin treatment. While both isoforms experienced a decrease in levels when treated with .25µM of the drug, it was only isoform 125 that experienced an increase in levels for both replicates conducted when treated with .5µM of the drug. It should also be noted that the higher concentration of drug resulted in a much larger fold increase in protein amount for both isoforms for treatment 1 when compared to treatment 2. This is likely due to the increased potency of the high concentration of auranofin at killing the cells. Because the cells treated at the higher concentration are under greater stress and many are dying, their intracellular protein levels might be expected to be in a greater state of flux. Since actin was used to normalize the data, it is very possible that at the high-concentration drug treatment, actin protein levels are not uniform across cells, resulting in the differences in fold change of protein levels observed.
Figure 32. Comparison of protein levels of TrxR isoform 474 across two separate drug treatments. Cells were treated with two concentrations of auranofin. DMSO levels are considered basal, and so are given a value of 1. The values for the drug-treated cells are in relation to DMSO-treated cells.
Besides the TrxR isoforms, we also looked at how various other cellular redox proteins (Trxs, TrxPs, alcohol dehydrogenases, and superoxide dismutases) responded to drug treatment (Table 7). For most of these proteins, the same trend seen for the isoforms, where the higher concentration of drug from the first treatment saw a higher fold change compared to the second, was observed. There was no clear consensus when looking at the Trxs. The TrxPs, however, both had the highest levels of protein in cells exposed to .5µM auranofin and the lowest in cells exposed to .25µM auranofin. The superoxide dismutases demonstrated a similar pattern, with one protein being
dramatically elevated when treated with .5µM of drug. For the dehydrogenases, on the other hand, drug treatment resulted in decreased levels across the board.

If looked at broadly, the data does show two trends. The first, .25µM auranofin treatment led to decreased amounts of protein compared to DMSO for all of the proteins listed. Why the lower concentration of drug led to this drop in protein levels of these redox proteins remains uncertain. It might be because this dosage was less acute. Perhaps if cells were treated longer than 24 hours, similar results to the higher concentration would be observed. The second, compared to the .25µM treatment, most of the proteins saw an increase in protein levels during the .5µM treatment. Again, the reason why that was remains to be determined, and again, this general increase in levels from .25µM to .5µM treatment was not present when looking at changes between the DMSO- and .5µM drug-treated cells.
Table 7. Comparison of ratios of various cellular redox proteins among *T. vaginalis* cells treated with DMSO or different concentrations of auranofin. Data is from two replicates (T1 and T2).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Group Profile (Ratio) Average</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMSO</td>
<td>.25μM AF</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>T2</td>
</tr>
<tr>
<td>TrxR 474</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>TrxrR 125</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Trx</td>
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<td>1.00</td>
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<td>Trx</td>
<td>1.00</td>
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</tr>
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<td>TrxP</td>
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<td>Trx family protein</td>
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<tr>
<td>Trx family protein</td>
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<td></td>
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</tr>
<tr>
<td>Superoxide Dismutase</td>
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<td>1.00</td>
</tr>
<tr>
<td>Superoxide Dismutase</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>
Chapter 4: Discussion

Finding a new means to treat Trichomoniasis would afford comfort in knowing that possible metronidazole-resistant strains could still be treated. Not only that, but we would have a new drug to treat a highly prevalent sexually transmitted infection with dangerous ties to HIV. Auranofin, an FDA-approved oral drug currently used to treat severe cases of rheumatoid arthritis, appears to be a strong candidate to target *Trichomonas vaginalis*, killing the organism and treating trichomoniasis.

Our studies confirmed the effectiveness of auranofin, and several of its analogs, in targeting the two major TrxR isoforms in *T. vaginalis*, inhibiting both enzymes. Activity assays showed that the addition of drug decreased the rate at which DTNB is reduced in a dose-dependent manner, a process dependent on TrxR activity. The fact that analogs of auranofin were also shown to be effective at inhibiting enzymatic activity of these crucial proteins allows for an expansion of the candidate drug pool. Despite its FDA approval, several symptoms are linked to auranofin usage. Knowing that auranofin has analogs with similar or lower IC$_{50}$ values allows for more drugs to be used in clinical tests. It might turn out that these analogs don’t produce any of the symptoms associated with auranofin treatment. Or, if the symptoms do manifest, they might be less commonly seen, or less severe. Regardless, this would mean that the analogs are actually better drug candidates than auranofin, and future studies can proceed with them instead.

Our results showed that out of the analogs tested, both LEAU 8 and 9 had IC$_{50}$
values for TrxR isoforms 474 and 125 that were lower than those of auranofin. Two other analogs, analog 1 and LEAU 14, were also shown to have comparable IC\textsubscript{50} values to those of auranofin. Even though LEAU 8 and 9 performed the best, future studies should incorporate all four analogs. It might be revealed that symptoms might still be present, or be even more prevalent or severe, in the two best-performing analogs. And it could be that the two comparable analogs actually have no symptoms associated with usage, making them the much better choices for future studies.

Regardless of what future tests reveal, our results have shown the effectiveness of auranofin and its analogs of inhibiting the two major isoforms of TrxR in \textit{T. vaginalis}.

When searching for new methods to treat an STI, it’s always good to look towards the future. Resistance to metronidazole has already been observed, and it’s probable that \textit{T. vaginalis} might someday develop resistance to auranofin as well. Preemptively looking for ways in which resistance might develop could allow us to tailor current treatment methods to curtail that resistance from developing. Anticipating resistance could also allow us to focus on searching for or developing drugs that would be effective on these resistance strains.

\textit{T. vaginalis} contains five isoforms of TrxR, but it’s uncertain whether the isoforms are all expressed in equal amounts. It may be that only some of the isoforms are present in high amounts, or in the same cellular location, rather than all. The presence of five different isoforms could also be a defense mechanism. Under stress, the organism might upregulate the transcription or translation of certain isoforms. It would be beneficial to know this, as this could be a means by which \textit{T. vaginalis} might try to counteract drug treatment.
The data from the drug assays would also be more useful if we had a bigger picture to go off of. We know how effective auranofin is against the isoforms, and how it targets them. The results showed that it was more effective against isoform 474 than 125. Knowing how the transcript levels of these proteins are modulated in response to drug treatment would better allow us to contextualize the assay results. If drug treatment increases amounts of 125 but not 474, then auranofin being a more potent inhibitor of 474 won’t really matter because of the greater abundance of 125.

The results from the qPCR analysis showed that basal mRNA levels of the five isoforms aren’t affected very differently in response to drug treatment. If anything, the results showed that all five isoforms have a mild fold-increase in response to auranofin, but no one isoform experienced greatly elevated levels of transcription. What the qPCR results did show, though, is that basal mRNA levels of the five isoforms varied greatly. Isoforms 474 and 125 had much higher levels when compared to the three other isoforms, with the levels of isoform 474 being the highest of all, by a very large margin. When compared to how the inductions and purifications of the five isoforms turned, the basal mRNA data helps to reassure what was seen. The two isoforms with the highest basal levels were easily expressed and purified, while the three with the lowest basal levels were not. We can thus be confident that the drug assays were performed on the TrxR isoforms most important to the organism, and that data is not missing for any other isoform of apparent importance. We can put more trust in this belief, considering that mRNA levels saw no major change post-drug treatment.

This is only a partial story, however. Various post-translational modifications might result in protein levels of the five isoforms that differ from what was seen for the
mRNA levels. Just because substantial differences in mRNA levels weren’t observed, doesn’t mean that the same will hold true for protein levels, and so mass spectrometry was performed to examine how protein levels responded to drug treatment. Important to note is that the mass spectrometer only detected isoforms 474 and 125, validating the qPCR data. We saw that when treated with .25µM of auranofin, both isoforms saw decreases on their protein levels when compared to DMSO-treated cells. The exact reason for this decline is unknown. It might be due to .25µM being a low enough concentration that doesn’t outright kill the cells, but instead causes them to experience some stress. If *T. vaginalis* isn’t overtly stressed, but still under some duress, it could be that other proteins, ones not focused on here, were upregulated. They may not focus on combatting oxidative stress, but instead nutrient storage. Further studies would need to be conducted to delve into this drop in protein levels further.

Unfortunately, when looking at cells treated with .50µM of auranofin, there is too much of a difference between the results of the two replicate treatments conducted to have a clear understanding of how the levels of isoforms 474 and 125 are responding. The mass spectrometry analysis would need to be redone to get a clearer picture of how the higher concentration of drug is affecting isoform levels.

Looking to the future, various next steps can be taken to further the results observed in these experiments. The first could be to see how the four analogs of interest actually do when it comes to killing *T. vaginalis*. From our data, it was made evident that the drugs are effective at targeting the two major TrxR isoforms, but it still is yet to be determined how effective these drugs are at killing the actual organism, critical
information needed if these analogs are to be considered serious drug options for treatment.

The qPCR analysis of the basal mRNA levels can also be greatly expanded. Our collaborators' lab did similar tests on a different lab strain of *T. vaginalis*. Their data showed that for their strain, while isoforms 474 and 125 were still expressed at the highest levels, isoform 348 had much higher basal levels than strain G3. Considering that we were both looking at lab strains, and there are various clinical strains that we aren’t working with, it would be prudent to attempt to characterize basal mRNA levels of all known *T. vaginalis* strains, both lab and clinical. Perhaps all strains show the same pattern of expression as G3, with 474 being expressed at the highest amounts, followed next by 125, and then by the other three. And like observed by our collaborators, some differences might exist in actual expression levels, but the trend remains observed. Or it could be that some strains express the isoforms at completely different levels. Regardless, knowing would be useful when planning treatment for future patients.

The mass spectrometry analysis will also need to be conducted. Human error was very likely the cause of the inconsistent results seen for the higher concentration treatment of auranofin, and so those tests need to be redone to get a better understanding of how exposure to high levels of drug is affecting isoform levels.

And then of course, future studies can even attempt to focus on possible resistance to auranofin. A resistant cell line can be generated, and mass spectrometry analysis can be performed to examine what has changed between susceptible and resistant cells. Getting ahead of resistance might allow drugs to be generated that are able to kill
resistant cells before these cells even pop up, ensuring that no patients would need to go without treatment.
REFERENCES


selected anticancer gold compounds. Current Topics in Medicinal Chemistry 11,
2647–2660.

Coombs, G.H., Westrop, G.D., Suchan, P., Puzova, G., Hirt, R.P., Embley, T.M.,
Trichomonas vaginalis contains a Divergent Thioredoxin-linked Peroxiredoxin

Cotch, M.F., Pastorek, J.G., Nugent, R.P., Hillier, S.L., Gibbs, R.S., Martin, D.H.,
Trichomonas vaginalis associated with low birth weight and preterm delivery.
The Vaginal Infections and Prematurity Study Group. Sex Transm Dis 24, 353–
360.

reductase inhibitor auranofin triggers apoptosis through a Bax/Bak-dependent
process that involves peroxiredoxin 3 oxidation. Biochem. Pharmacol. 76, 1097–
1109.

Debnath, A., Parsonage, D., Andrade, R.M., He, C., Cobo, E.R., Hirata, K., Chen, S.,
drug screen for Entamoeba histolytica identifies a new lead and target. Nature
Medicine 18, 956–960.

Dyall, S.D., and Johnson, P.J. (2000). Origins of hydrogenosomes and mitochondria:


Inflammopharmacology 16, 112–125.


