BASE EXCISION REPAIR APURINIC/APYRIMIDINIC ENDONUCLEASES IN

APICOMPLEXAN PARASITE *TOXOPLASMA GONDII*

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ABSTRACT

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APICOMPLEXAN PARASITE *TOXOPLASMA GONDII*

Toxoplasma gondii is an obligate intracellular parasite of the phylum
Apicomplexa. *Toxoplasma* infection is a serious threat to immunocompromised
individuals such as AIDS patients and organ transplant recipients. Side effects
associated with current drug treatment calls for identification of new drug targets.
DNA repair is essential for cell viability and proliferation. In addition to reactive
oxygen species produced as a byproduct of their own metabolism, intracellular
parasites also have to manage oxidative stress generated as a defense
mechanism by the host immune response. Most of the oxidative DNA damage is
repaired through the base excision repair (BER) pathway, of which, the apurinic
/apyrimidinic (AP) endonucleases are the rate limiting enzymes. *Toxoplasma*
possesses two different AP endonucleases. The first, TgAPE, is a magnesium-
dependent homologue of the human APE1 (hAPE1), but considerably divergent
from hAPE1. The second, TgAPN, is a magnesium-independent homologue of
yeast (*Saccharomyces cerevisiae)* APN1 and is not present in mammals. We
have expressed and purified recombinant versions of TgAPE and TgAPN in *E.
coli* and shown AP endonuclease activity. Our data shows that TgAPN is the
more abundant AP endonuclease and confers protection against a DNA
damaging agent when over-expressed in *Toxoplasma* tachyzoites. We also
generated TgAPN knockdown *Toxoplasma* tachyzoites to establish that TgAPN is

important for parasite protection against DNA damage. We have also identified

pharmacological inhibitors of TgAPN in a high-throughput screen. The lead

compound inhibits *Toxoplasma* replication at concentrations that do not have

overt toxicity to the host cells. The importance of TgAPN in parasite physiology

and the fact that humans lack APN1 makes TgAPN a promising candidate for

drug development to treat toxoplasmosis.

William J Sullivan Jr. Ph.D. - Chairman

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List of Abbreviations

6TX	6-thioxanthine
AIDS	acquired immune deficiency syndrome
ATc	anhydrotetracycline
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3 related
BER	base excision repair
β-Me	beta-mercaptoethanol
BRCA	breast cancer susceptibility protein
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
cDNA	complementary deoxyribonucleic acid
DAPI	4',6'-diamino-2-phenylindole
DD	destabilization domain
DHFR	dihydrofolic acid reductase
DHFR-TS	dihydrofolic acid reductase-thymidylate synthase
DHPS	dihydropteroate synthase
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dRP	deoxyribophosphodiesterase
ECL	enhanced luminol-based chemiluminescent
EDTA	Ethylenediaminetetraacetic acid

EST	expressed sequence tag
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FEN1	Flap endonuclease 1
gDNA	genomic DNA
HAART	highly active anti-retroviral therapy
HFF	human foreskin fibroblast
hN	host cell nucleus
HRP	horseradish peroxidase
HXGPR	hypoxanthine-xanthine-guanine phosphoribosyltransferase
IFA	immunofluorescence assay
IMPDH	inosine-monophosphate dehydrogenase
kDa	kiloDalton
KO	knockout
MPA	mycophenolic acid
mRNA	messenger ribonucleic acid
NER	nucleotide excise repair
NHEJ	nonhomologous end-joining
NK	natural killer
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PFA	paraformaldehyde
PYR	pyrimethamine

RNA	ribonucleic acid
TgN	*Toxoplasma* nuclei
UTR	untranslated region
WT	wild-type

CHAPTER 1: INTRODUCTION

1. *Toxoplasma* overview

A. Apicomplexan parasites

Toxoplasma gondii is an obligate intracellular protozoan parasite of the phylum Apicomplexa. Phylum Apicomplexa is composed of almost 5,000 different species of obligate intracellular parasites [1]. The defining characteristic of an apicomplexan parasite is the collection of specialized organelles (micronemes, rhoptries and polar rings) at the apical end that help the parasites invade the host cell. Apicomplexa infect a wide range of cells from warm blooded animals ranging from birds to mammals [1].

The complex life cycle of apicomplexan parasites can be divided into three general stages: sporogony, merogony and gametogony, which can slightly differ among the species. The life cycle stages can also be distinguished between the sexual and asexual stages. In monoxenous species all stages occur in the same host animal, while in heteroxenous species they occur in different hosts animals [1].

Apicomplexa are important human and livestock pathogens that lead to serious economic burden in agriculture and the healthcare system [2]. The three most important pathogenistic apicomplexans to humans are: *Plasmodium, Toxoplasma* and *Cryptosporidium*. While *Plasmodium* accounts for the highest mortality rates for any apicomplexan parasite in humans, *Toxoplasma* emerged as a serious opportunistic pathogen during the AIDS epidemic [3].

B. *Toxoplasma* as a model apicomplexan

Other than being an important pathogen, *Toxoplasma gondii* is considered a model organism for the study of apicomplexan parasite biology. Unlike other members of the phylum Apicomplexa, such as *Plasmodium* and *Cryptosporidium, Toxoplasma* is more amenable to genetic manipulation in the laboratory. Cell biology studies are easily performed in *Toxoplasma* through transient and stable transfection. *Toxoplasma* can infect a wide variety of host cells and is comparatively easy to culture *in vitro* [4]. The *Toxoplasma* genome has been sequenced to >12X coverage and is an invaluable resource for the study of the parasite. The genomic information is made available at http://www.toxoDB.org. Therapeutic agents used against *Toxoplasma* are in some cases successful against *Plasmodium* [5]. Therefore, identifying new drug targets and therapeutic agents against *Toxoplasma* may be useful against other apicomplexan parasites.

C. Life cycle

Toxoplasma has a complex life cycle that involves multiple hosts throughout its different stages of development. Toxoplasma is a heteroxenous apicomplexan. The life cycle consists of a sexual phase that only occurs in the cat intestine, which is the definitive host. *Toxoplasma* must infect a cat to undergo a complete life cycle. The cats become infected with the parasite by eating prey that has cysts in its tissue. In the cat intestine, the parasite reproduces sexually culminating in the production of oocysts that are shed with

the cat feces. The oocysts are very hardy and can survive for several months, remaining capable of infecting a new host when ingested or inhaled [6].

Intermediate hosts such as humans and livestock become infected when the oocysts or bradyzoites are ingested. Once ingested, the bradyzoites inside the tissue cyst or the sporozoites inside the oocyst penetrate the epithelial cells of the intestines and infect different cells and tissues. The parasite has a predilection for heart, muscle and brain tissue, where it causes tissue damage and form cysts. The tissue cysts can survive in the host tissue indefinitely and sometimes for the duration of the host's life [6].

There are three stages of *Toxoplasma*: the tachyzoites (rapidly growing stage), the bradyzoites (slow growing stage in tissue cysts), and the sporozoites (in oocysts). These stages are linked in a complex life cycle:

Tachyzoites. The tachyzoite is crescent shaped and is the stage that was first identified in the North African rodent *gundi* [6]. This stage has also been called a trophozoite, the proliferative form, the feeding form, and the endozoite. It divides into two by a specialized process called endodyogeny, which is similar to binary fission. The tachyzoites are responsible for the acute phase of *Toxoplasma* infection.

Bradyzoite and tissue cysts. The bradyzoite is the encysted form of the parasite in the host tissues. Bradyzoites are also called cystozoites. The cyst wall is destroyed by pepsin or trypsin, but the cystic organisms are resistant to digestion by gastric juices (pepsin and HCl). In contrast, tachyzoites are not resistant to digestive enzymes. Bradyzoites invade the host intestinal cells,

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converting into tachyzoites, and ultimately infecting other tissues in the host's body. Tissue cysts are important in the life cycle of *Toxoplasma* because carnivorous hosts can become infected by ingesting infected meat [7]. The bradyzoites are slow growing and are responsible for chronic/latent toxoplasmosis.

Enteroepithelial, asexual and sexual stages. The cat intestine is the only environment that *Toxoplasma* sexual reproduction takes place. Also differentiation into micro and macrogametes, fertilization, up to formation of oocysts occurs. Asexual development of *Toxoplasma* can also occur in the enterocytes of the cat and are designated as the asexual enteroepithelial stages. The enteroepithelial stages are distinguished morphologically from tachyzoites and bradyzoites, which also occur in cat intestine. Fewer than 30% of cats shed oocysts after ingesting *Toxoplasma* cysts in prey tissue [7].

Felids

Livestock and rodents

Fecal oocysts

Tissue cysts

Tachyzoites

Humans
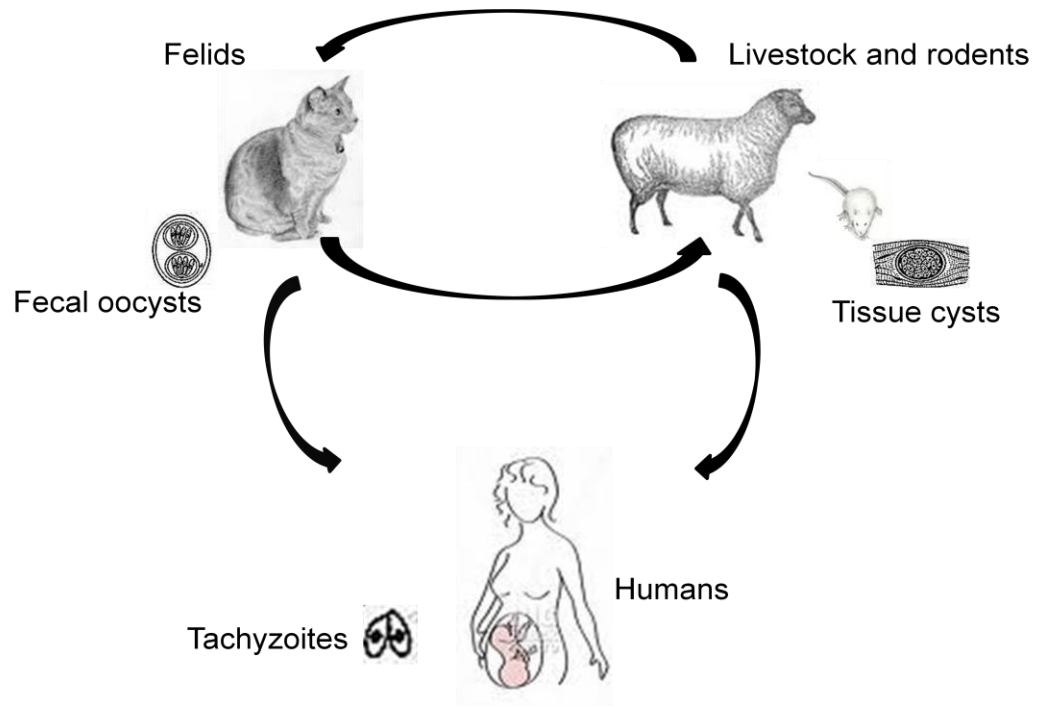
Figure 1: Life cycle and transmission of *Toxoplasma.* There are three stages: tachyzoites (rapidly replicating form), bradyzoites (slow replicating form in tissue cysts) and sporozoites in oocysts shed by the cat. The definitive host of *Toxoplasma* is the cat (or other felids), and it is within the cat's gut that the sexual cycle of the parasites takes place, resulting in the formation of oocysts. An infected cat will shed highly infectious oocysts into the environment. Livestock consume the oocysts that become tachyzoites and ultimately bradyzoites in animal tissue. If humans consume oocysts from the environment or tissue cysts from undercooked meat, they become infected. Tachyzoites are the active form of the parasite responsible for tissue destruction and disease symptoms. Tachyzoites can cross the placenta and cause congenital infection of the fetus if a woman becomes infected for the first time during pregnancy.

D. Transmission

Vertical transmission

Congenital. Congenital *Toxoplasma* infection in a human child was initially described by Wolf, Cowen, and Page (1939) and later found to occur in many species of animals, particularly sheep, goats, and rodents [86]. Congenital toxoplasmosis occurs when the mother becomes infected with *Toxoplasma* for the first time during pregnancy and the tachyzoites cross the placental barrier. Congenital infections can be repeated in some strains of mice with infected mice producing congenitally infected offspring for several generations [8].

Carnivorism. Congenital transmission occurs too rarely to explain widespread infection in man and animals worldwide. It has been suggested that most transmissions might occur through the ingestion of undercooked meat [9]. Evidence to support this idea was obtained by demonstrating that *Toxoplasma* bradyzoites are resistant to proteolytic enzymes. The tissue cyst wall is immediately dissolved by such enzymes, but the released bradyzoites survive to penetrate the intestinal epithelial cells of the host. Epidemiological evidence indicates toxoplasmosis is common in humans in some localities where raw meat is routinely eaten [9]. Populations that eat undercooked meat are also at risk of infection.

Fecal-oral transmission. *Toxoplasma* oocysts shed by felids can also cause *Toxoplasma* infection if ingested or inhaled. In approximately 3 days from being passed in the stool, the oocysts mature and become infective to other

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animals [10]. A risk factor for toxoplasmosis is contact with oocysts from cat feces contaminating water, soil and the food supply.

Regular contact with cat feces such as in cat litter is a predisposing factor for infection to humans in their living environment [10].

E. Epidemiology

Toxoplasma is a parasite with a broad spectrum of hosts ranging from birds to mammals. The definitive host of the parasite is the cat, which sheds oocysts that infect other animals including humans. The estimated worldwide seroprevalance for *Toxoplasma* in domestic cats is 30–40% [11]. Numerous farm animals are also afflicted with toxoplasmosis. Perhaps the most striking example is that of sheep farming, where economic losses from sheep abortions due to congenital toxoplasmosis are significant [12]. Since sheep are not carnivorous, they most likely acquire the infection through oocysts in their feed [86]. The marine environment has generally been considered too harsh for the survival of *Toxoplasma*, but marine mammals are unexpectedly susceptible to infection [13]. Among the affected marine mammals are seals, sea lions, and dolphins. The spread of toxoplasmosis into the marine population is alarming as it threatens animals that are already endangered and at risk of extinction [14].

Toxoplasma is found in humans, worldwide, under a variety of climates and socio-economic circumstances. The seroprevalence of *Toxoplasma* in the United States is as high as 40% [9]. The emergence of the AIDS epidemic has made *Toxoplasma* infection a more serious threat to global health. Death as a result of toxoplasmosis in AIDS patients ranges from 10% in the United States

and 30% in Europe [15]. *Toxoplasma* can also cross the placental barrier and cause congenital toxoplasmosis, which might lead to blindness, hydrocephalus, and mental retardation. The *Toxoplasma* infection can also cause spontaneous abortion. Four hundred to four thousand infants are affected by congenital toxoplasmosis in the United States each year at a cost of $7.7 billion [16].

F. *Toxoplasma* as an opportunistic pathogen

Toxoplasma has emerged as a serious threat in immunocompromised individuals such as AIDS patients, cancer chemotherapy recipients, and organ transplant recipients. The reactivation of a previously acquired and chronic infection is the main cause of toxoplasmic encephalitis in AIDS patients, and it has been closely related to the severity and progression of immunosuppression. Immunocompetent persons with a primary infection are usually asymptomatic, but latent infection can persist for life [17]. In immunosuppressed patients, such as patients with AIDS, the parasite can reactivate and cause disease when the CD4 lymphocyte count drops below 100 cells/µL. Patients with AIDS and CD4 counts <100 cells/µL who are seropositive for *Toxoplasma* have a 30% probability of developing reactivated toxoplasmosis if they are not receiving effective prophylactic treatment; the most common site of reactivation is the central nervous system [18].

In transplant recipients, this risk of reactivation also exists for those who are seropositive for *Toxoplasma*. The infection is closely related to the degree and duration of immunodeficiency and, therefore, markedly differs according to the type of transplantation. At the chronic stage of toxoplasmosis, tissue cysts

are formed in the neural and muscular tissues, including the brain, eyes, skeletal muscles and cardiac muscles, but may also develop in visceral organs, including the lungs, liver and kidneys which can be transplanted [19]. Most cases of toxoplasmosis in heart and heart–lung transplantation have been reported in seronegative recipients receiving a heart transplant from a seropositive donor. Clinical symptoms usually occur in the first 3 months after transplantation and consist of febrile myocarditis, encephalitis or pneumonitis [20]. Thus, toxoplasmosis represents a major infectious complication in transplant recipients that can be prevented. This can be achieved by serological screening of the organ donor and recipient, screening for toxoplasmosis after the transplant, and treatment with sulfadiazine and pyrimethamine of the organ recipient [21].

G. Types of *Toxoplasma* infection

Congenital Toxoplasmosis

Toxoplasma can cross the placental barrier of an infected pregnant mother and infect the fetus, resulting congenital toxoplasmosis. Congenital toxoplasmosis can lead to encephalitis, hydrocephalus with intracranial calcification, chorioretinitis with scarring and loss of vision, hepatitis, and lymphadenopathy [22]. Children who survive to birth may subsequently develop toxoplasmosis-associated injuries, including deafness, microcephaly, and mental deficiencies [23].

Early treatment may prevent the further progress of the infectious process and the development of disabilities in children [24]. If *Toxoplasma* is detected in the amniotic fluid and if ultrasound examination is normal, a prescription of

pyrimethamine and sulfonamides, together with folic acid, can be administered

[25]. In the case of cerebral microcalcifications or hydrocephaly diagnosed by

ultrasound, a termination of pregnancy may be proposed to the parents. After

birth, the infected child is treated with pyrimethamine and sulfonamides for 12

months. Spiramycin, which does not cross the placental barrier, is used in

attempt to minimize transmission to the fetus during pregnancy. Pyrimethamine,

spiramycin and sulfonamides are well tolerated in neonates and they are non-

teratogenic, although sulfonamides might result in kernicterus (bilirubin cerebral

toxicity) in the neonates [26].

Cerebral toxoplasmosis

Cerebral toxoplasmosis is one of the most common opportunistic

neurological infections in AIDS patients, and it is typically observed in the later

stages of human immunodeficiency virus (HIV) infection. The infection may

involve the brain diffusely or form discrete abscesses. Clinical manifestations

include seizures, headache, focal neurologic deficits, and intercranial

hypertension [27]. Highly active antiretroviral therapy (HAART) has decreased

the incidence of cerebral toxoplasmosis by boosting the host's immunity.

Currently, the prevalence of *Toxoplasma* induced focal brain disorders still

accounts for a considerable proportion of mortality and morbidity in AIDS

patients, especially in developing countries [28]. Cerebral toxoplasmosis requires

long-term treatment with pyrimethamine-sulfadiazine or a pyrimethamine-

clindamycin regime.

Ocular Toxoplasmosis

Ocular toxoplasmosis is one of the most frequent causes of posterior uveitis. An ocular reactivation of the disease can occur when the cysts are present within the retina and the patient becomes immunocompromised. Epidemiological data suggests that most cases of ocular toxoplasmosis result from reactivation of ocular toxoplasmosis rather than a new infection. Symptoms vary, but generally consist of unilateral floaters or blurred vision when the disease becomes active. Inactive disease rarely causes visual symptoms unless scarring is near the central retina or macula. The slit-lamp examination reveals the presence of a granulomatous inflammation, a mild to moderate anterior chamber inflammation [30]. Fundoscopy reveals the presence of a yellow focus of retinochoroiditis [29]. Severe cases of ocular toxoplasmosis might result in visual impairment and blindness. Pyrimethamine-sulfadiazine regime as well as trimethoprim-sulfamethoxazole regime is used for the treatment of ocular toxoplasmosis.

Cutaneous Toxoplasmosis

A less common manifestation of *Toxoplasma* infection is the cutaneous form that shows up as lesions on the patient's skin. Skin involvement in toxoplasmosis is rare even in severely immunosuppressed individuals. Cutaneous toxoplasmosis is diagnosed by a biopsy of the skin lesion to reveal tachyzoites [31].

H. Diagnosis

Diagnosis of *Toxoplasma* infection occurs mainly through serological testing, which is also important in determining the prevalence of the infection in a population. The Sabin–Feldman test, which is among the most widely used, makes use of *Toxoplasma* tachyzoites derived from live culture, which are incubated with test serum and complement, followed by addition of a Sabin–Feldman dye. If the test serum comes from an individual infected with *Toxoplasma*, then the specific antibodies in the serum will recognize and bind to the parasite and fix the complement, resulting in lysis of the tachyzoites, which will appear as unstained when examined under a microscope [32]. Enzyme linked immunosorbent assay (ELISA) tests are also a reliable method to detect *Toxoplasma*-specific antibodies in humans. Another less commonly used method is the use of polymerase chain reaction (PCR), which detects *Toxoplasma*-specific DNA sequences [33].

I. Treatment and prevention

There are a variety of steps that can be taken to avoid becoming infected with *Toxoplasma*. Animal vaccines have successfully been used in the sheep industry to reduce *Toxoplasma*-induced abortion.

In humans, limiting contact with infective bradyzoites and oocysts is the most effective way to prevent infection. Good hygiene e.g. hand washing after soil contact, washing fruits and vegetables that are eaten raw, freezing meat at -12°C for 24 hours and/or cooking meat until an internal temperature of 66°C is reached, and not drinking untreated water, help prevent infection. Serological

screening during pregnancies and organ transplants can be administered to prevent spread of the parasites.

The current drug treatment of pyrimethamine and sulfadiazine has been in use since the 1950s. These two drugs target the folic acid synthesis pathway by inhibiting dihydrofolic acid reductase (DHFR) and dihydropteroate synthase (DHPS), respectively [34]. Spiramycin is used almost exclusively for cases of toxoplasmosis during pregnancy as it does not cross the placental barrier. Clindamycin or atovaquone are used in patients with sulfonamide allergies. The current drugs available for treatment of toxoplasmosis are limited as they cause severe side effects, such as skin rash and epidermal necrosis from sulfonamide toxicity. Pyrimethamine causes bone marrow suppression and megaloblastic anemia, which can be life-threatening [35]. The existing drug treatments are also limited to targeting the tachyzoite stage, as they do not kill bradyzoites protected by the cyst wall.

J. Immune response

In immunocompetent people, infection with *Toxoplasma* results in the development of protective immune response against the disease. As a result of the robust immune response, there have been several attempts to develop effective vaccines to combat the disease [36]. The importance of the immune system in protecting the host against *Toxoplasma* infection was dramatically emphasized with the emergence of AIDS in the 1980s. During the earliest stages of infection, *Toxoplasma* is able to induce the nonspecific activation of macrophages, NK cells, and other cells such as fibroblasts, epithelial, or

endothelial cells. This initial response peaks at the end of the first week and then slowly reduces until absent by the end of the second week. The activation of macrophages by the cytokine interferon gamma (IFN-γ) in the presence of co-signals, such as tumor necrosis factor-α (TNF-α), is necessary to induce the cytotoxic activity of the macrophages against *Toxoplasma*. IFN-γ activates the macrophages by increasing oxidative metabolism, releasing hydrogen peroxide, which damages DNA, lipids and proteins in cells [38]. Nitric oxide (NO) produced by macrophages activated by IFN-γ also restricts parasite growth during the chronic phase. IFN-γ also increases the activity of indoleamine 2, 3-dioxygenase, resulting in the breakdown of tryptophan, an amino acid required for growth of the parasite [37]. *Toxoplasma* cysts are protected from the immune system and can remain in host tissue for life. The antibodies in the humoral immune response and the complement system play a minor but important role in limiting the acute infection [39]. *Toxoplasma* has evolved the ability to prevent the translocation of STAT1 and NF-κβ to the nucleus, which prevents macrophages from producing IL12 and TNF-α, thereby enabling the parasite to subvert the host immune system [40].

K. *Toxoplasma* **adaptations to the host cell environment**

Toxoplasma is well adapted to invade and survive in the host cell. Like other apicomplexan parasites, *Toxoplasma* has a highly specialized protein secretion mechanism at its apical end that facilitates host cell invasion. During invasion, the contents of three separate secretory systems are discharged sequentially to allow adherence and consequently invasion of the cell. The

parasite secretes microneme and rhoptry proteins to form a parasitophorous vacuole membrane (PVM). The parasitophorous vacuole (PV) acts as a protective environment for the parasites in the host cell by avoiding endosome fusion [41]. The PVM also enables the parasites to obtain nutrients and other requirements such as amino acids and nucleotides from the host cell [42]. The PVM occupies the perinuclear region of the host cell, recruits host mitochondria and traffics along the host cell microtubules. Simple sugars, amino acids, nucleobases and cofactors are able to go through the PVM pores for use by the parasites [42]. Metabolites from the parasite can also pass through the PVM into the host cell cytoplasm. *Toxoplasma* is also able to take up lipids such as cholesterol through an intricate salvage pathway [43].

Toxoplasma also prevents apoptosis of the host cell. Apoptosis is a common strategy that cells infected with viruses and bacteria employ to minimize the spread of the infection. *Toxoplasma* has developed the ability to inhibit apoptosis of the host cell [44]. Live *Toxoplasma* can prevent apoptosis in cultured cells exposed to apoptotic inducers including ultraviolet rays and irradiation [45].

Reactive oxygen species (ROS) are a constant stress for parasites in the intracellular environment, especially in immune effector cells such as macrophages and neutrophils during the oxidative burst [47]. *Toxoplasma* has an advanced antioxidant system made up of superoxide dismutase, catalase, glutathione peroxidase and peroxiredoxins, which serve to protect the parasite against reactive oxygen species [46].

2. DNA damage and repair

A. Importance of DNA integrity and health implications

Maintenance of DNA integrity is essential for all cells and life forms. It is the prime objective of all organisms to pass their genetic material intact and unmutated to ensure survival and continuity of the species. However, DNA is constantly exposed to numerous endogenous and exogenous insults that cause damage to its stability as well as integrity. To counter this threat, cells have evolved intricate defense mechanisms that protect the DNA from damage and also repair any damage that occurs. Failure to protect and repair damaged DNA may result in deleterious mutations, disease, and cell death. The human genome experiences tens of thousands of DNA lesions each day [47]. These lesions are a serious threat to the normal function of each cell by causing problems in replication and transcription. If the DNA lesion is not properly repaired, mutations may result, compromising genomic stability and threatening cell viability. Improved understanding of DNA repair mechanisms is an important element in understanding the nature of many diseases, including cancer.

Paradoxically, DNA damage and mutations can actually be beneficial in cells and therapeutics. DNA damage can be used in the treatment of cancer by using DNA damaging drugs and radiation to target the cancer cells. The production of diverse antibodies and antibody classes are dependent on mutations resulting from reduced polymerase fidelity and weaker DNA damage response. Impaired DNA damage response will result in an impaired immune system by limiting antibody diversity [48].

DNA damage plays an important role in the life cycle of parasites and the progression of infectious disease. Mutations in a pathogen's genome facilitate the acquisition of traits that may limit the effectiveness of the host immune system and drug therapies. The integration of viral DNA into the host genome ensures its continued presence in the life of the host and, in some cases, even in host progeny. In addition, certain pathogens use DNA damage response mechanisms to promote virulence. For example, African trypanosomes evade host immune surveillance by using homologous recombination and mutations to periodically alter their protective variant-surface-glycoprotein coat [49]. DNA damage and repair has thus been proposed to be an attractive target in the treatment of infectious disease.

B. Sources of DNA damage

The sources of DNA damage can generally be divided into exogenous and endogenous causes. Exogenous causes include a wide range of insults such as mutagenic chemicals in the environment as well as physical damage from ionizing radiation. Exogenous damage was once thought to be responsible for the majority of DNA damage in cells, but recent studies have shown that most DNA damage actually occurs endogenously, as a result of normal cellular metabolic activity [50].

Abasic or apurinic/apyrimidinic (AP) sites are common DNA lesions that can be caused by both exogenous and endogenous insults. AP sites can be produced by spontaneous hydrolysis, alkylation-induced hydrolysis, or glycosylase-catalyzed base-excision repair. DNA bases are susceptible to

spontaneous hydrolytic deamination. 100 to 500 cytosines are spontaneously deaminated to uracil every day in human cells. If not immediately corrected, such changes can lead to transition mutations. Uracil glycosylases rapidly remove uracil from DNA, leaving an abasic site. The glycosidic bond between bases and deoxyribose in DNA is labile under certain conditions, such as heating, alkylation of bases, or the action of N-glycosylases. Cleavage of the glycosidic bond in DNA also leads to an abasic site [51].

Produced as a result of cellular metabolism as well as other biochemical reactions and external factors, reactive oxygen species (ROS) are perhaps the most abundant genotoxic element in the cell. ROS include superoxide, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. ROS cause damage by oxidizing DNA bases and sugars, which might lead to strand breakage. The damaged bases are removed by specific glycosylases such as Ogg1, leaving an abasic site [51].

DNA damage can also occur as a result of alkylation of the bases. The most common DNA alkylation agent is S-adenosylmethionine (SAM) [52]. It is a reactive methyl group donor contributing to physiological enzymatic DNA methylation, which plays a role in regulation of gene expression. Aberrant methylation by SAM can result in alkyl damage in bases. Some alkylation damage to bases, such as guanine methylation, is highly mutagenic. In most cases, the damaged base is recognized and removed by a specific glycosylase leaving an abasic site [51].

There are less characterized sources of DNA damage that still play an important role in the stability of the genome. Such damage includes the products of lipid peroxidation, which are highly reactive and cause DNA lesions [88]. Several oestrogen metabolites can cause DNA damage directly or indirectly through redox cycling processes that generate reactive radical species [87]. Spontaneous DNA strand breakage is also a source of DNA damage that is ultimately cytotoxic if not repaired [51].

C. Cell response to DNA damage

In the event of DNA damage, a cell has a wide range of responses depending on the type and extent of damage. These DNA damage response reactions include: (i) removal of DNA damage and repair of the DNA, (ii) activation of a DNA damage checkpoint, which arrests cell cycle progression so as to allow for repair and prevent the transmission of damaged or incompletely replicated chromosomes, (iii) a transcriptional response, which causes changes in gene expression that may be beneficial to the cell, (iv) DNA damage bypass polymerases, and (v) apoptosis, which eliminates heavily damaged or seriously deregulated cells [53].

Severely damaged DNA that cannot be repaired usually triggers an apoptotic response. Apoptosis induced by DNA damage is indistinguishable from genetically programmed cell death that occurs during development of a variety of tissues. The severe damage induces the activation of caspases to initiate apoptosis. However, in extreme cases of DNA damage, cells may undergo a necrotic death [53].

The detection and subsequent removal of DNA damage is not completely efficient. Some DNA lesions are persistent and may not be adequately repaired. DNA replication, transcription or recombination machineries may encounter sites of DNA damage before they are repaired. DNA damage tolerance allows cells to overcome the potentially lethal effects of blocked replication and transcription until a time when the damage can be removed. Higher eukaryotes have multiple specialized polymerases that have low fidelity and can bypass damage sites on DNA. These low fidelity polymerases are utilized in the diversification of antibodies in the immune system [54].

DNA damage activates cell cycle checkpoints to prevent progression of the damage and minimize mutations. DNA damage checkpoints are biochemical pathways that delay or arrest cell cycle progression in response to DNA damage. The DNA damage checkpoints employ damage sensor proteins, such as ATM (Ataxia telangiectasia mutated), ATR (Ataxia telangiectasia related), the Rad17-RFC complex, and the 9-1-1 complex, to detect DNA damage and initiate signal transduction cascades to repair it [55]. DNA damage checkpoints also facilitate the transcription of genes that help remedy the damage [55].

Cells respond to DNA insults by trying to repair the damage and maintain the integrity of the genome. There are numerous DNA repair pathways that work independently as well as in concert to maintain DNA integrity. The pathway to repair DNA is mainly dictated by the type of damage incurred. The most common DNA repair pathways include nucleotide excision repair (NER), homologous recombination (HR), non-homologous end joining repair (NHEJ), mismatch

repair, and base excision repair (BER). As it is central to my thesis, BER will be described in detail.

D. Base excision repair

Base excision repair (BER) is the primary DNA repair pathway that corrects base lesions that arise due to oxidative, alkylation, deamination, and depurinatiation/depyrimidination damage. The first step in BER is the recognition of a damaged base by a DNA glycosylase (Figure 2). After recognition of the damaged base by the appropriate DNA glycosylase, this glycosylase catalyzes the cleavage of an N-glycosidic bond, effectively removing the damaged base and creating an apurinic /apyrimidinic site (AP site). Following the removal of the damaged base, AP endonucleases cleave the phosphate backbone 5' of the abasic site. DNA lyase activity (present in some glycosylases) can also cleave the phosphate backbone 3' to the abasic site. The newly created nick is processed by the AP endonuclease, creating a single nucleotide gap in the DNA. Importantly, the gap contains a 3'-hydroxyl and a 5'-phosphate, substrates compatible with the downstream enzymatic reactions in BER. A DNA polymerase fills in the gap with the correct nucleotide. Finally, a DNA ligase completes the repair process and restores the integrity of the helix by sealing the nick (Figure 2).

Glycosylases recognize specific damaged bases and excise them from the genome, effectively initiating BER. There are numerous DNA glycosylases that are specific for different types of base damage; to date, there are 11 characterized mammalian glycosylases. DNA glycosylases fall into two major

classes that differ with respect to catalytic mechanism. Monofunctional

glycosylases catalyze the single-step displacement of the damaged base.

Bifunctional DNA glycosylases also excise the lesion base as well as the DNA

backbone on the 3′ side of the AP site followed in some cases by backbone

severance on the 5′ side as well [56].

BER remedies DNA damage through two general pathways: short-patch

and long-patch. The short-patch BER pathway leads to repair of a single

nucleotide while the long-patch BER pathway produces a repair tract of at least

two nucleotides. How the cell decides to repair damage through short-patch or

long-patch BER is poorly understood. One hypothesis suggests that the switch

from short-patch to long-patch BER depends on the relative ATP concentration

near the AP site [57]. It has been shown that long-patch BER occurs more

frequently at low ATP concentrations, whereas short-patch BER appears to be

the preferred mechanism with elevated concentrations of ATP. Another

hypothesis is that BER proceeds through the short-patch pathway if the

deoxyribophosphate (dRP) can be efficiently removed by

deoxyribophosphodiesterase (dRP) lyase activity of POLβ. However, if the dRP

cannot be effectively removed, the BER pathway proceeds by the long-patch

mechanism, apparently to avoid generating a nick that is refractory to the action

of a DNA ligase [58].

XRCC1 is one of the first proteins recruited to the nick generated by the

action of a glycosylase and/or AP endonuclease in short-patch BER. XRCC1 has

no enzymatic activity but is considered an important scaffolding protein in BER.

23

Long-patch BER requires proliferating cell nuclear antigen (PCNA). Polymerase delta and polymerase epsilon are required for long-patch BER in a PCNA-dependent manner, displacing more than one base. The strand displacement activity of polymerase delta and epsilon produces a flapped substrate that is refractory to ligation. Flap endonuclease 1(FEN1) resolves the problem of an unligatable DNA junction by catalyzing the removal of the flap generated by polymerase activity [55].

The one-nucleotide short-patch BER pathway is completed by the action of DNA ligase III. Alternatively, DNA ligase I completes the ligation of the nick in long-patch BER.

A C X T G G
T G T A C C

DNA glycosylase

A C T G G
T G T A C C

AP endonuclease

OH P
A C T G G
T G T A C C

Polymerase β

Polymerase β
Polymerase D

OH P
A C A T G G
T G T A C C

P
T
T
OH
A C A T G
G
T G T A C C

Ligase 3

Ligase 1

A C A T G G
T G T A C C
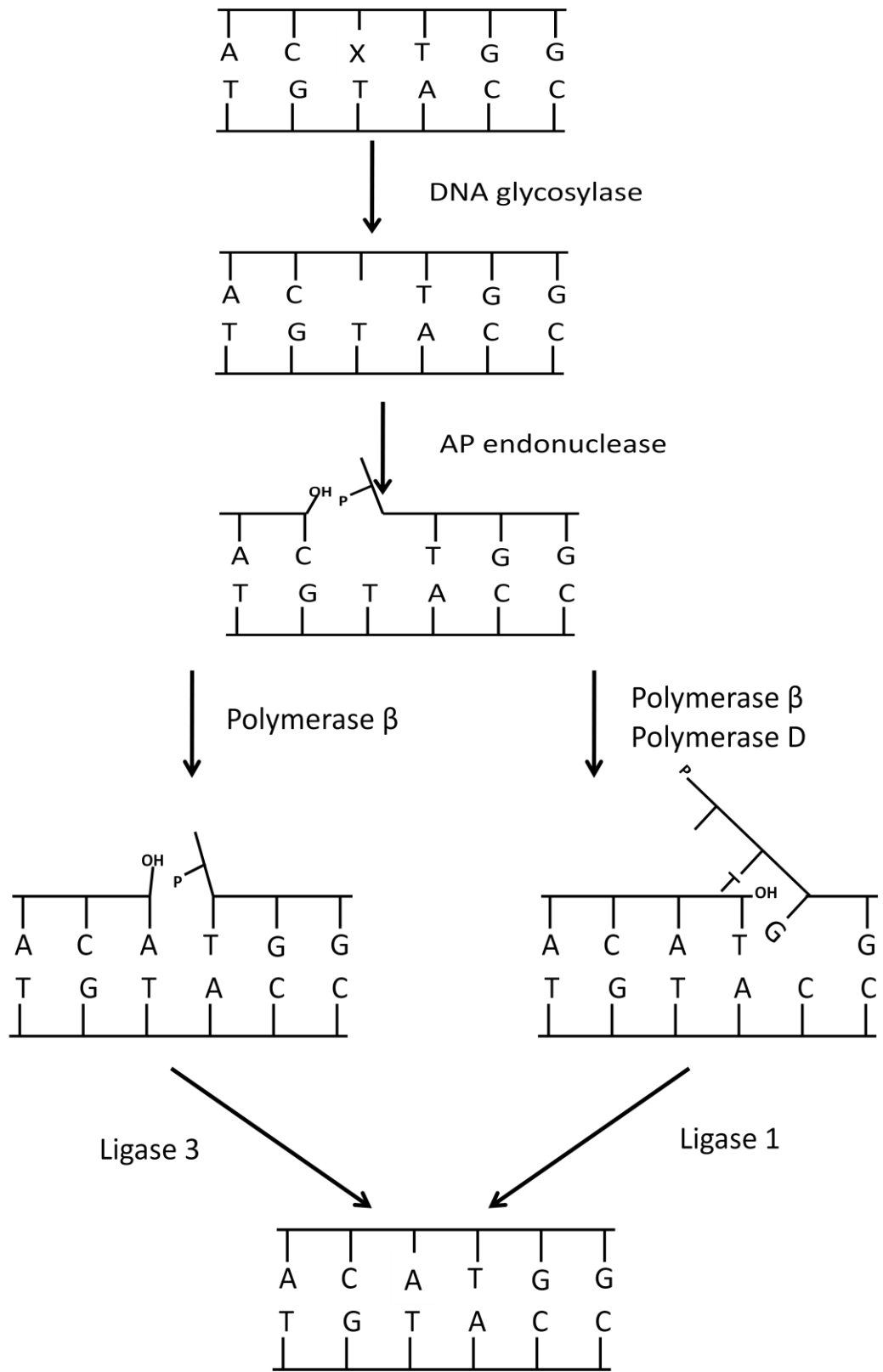
Figure 2: Representation of the base excision repair pathway. This figure shows the proteins and DNA interactions in both the short-patch (left branch) and long-patch (right branch) mammalian BER pathways. Damaged base (represented by X) is recognized and excised by a specific DNA glycosylase, generating an AP site. AP endonuclease, the rate limiting enzyme, then hydrolyzes the phosphodiester bond 5' to the AP site. The repair can at this point follow either short-patch or long-patch pathways. Finally, the resulting nucleotide gap is filled through the action of DNA polymerase and a ligase seals the repaired strand.

E. AP endonucleases

AP endonucleases are the rate limiting enzymes of the BER pathway. There are two main families of AP endonucleases based on their homology to *E. coli* endonucleases: exonuclease III (Exo III) and endonuclease IV (Endo IV) [59]. Structural studies of representative enzymes of the two families reveal similarities as well as differences in the mode of action on AP sites. The fact that these two decidedly different enzymes have evolved independently to process AP sites highlights the biological need for DNA repair.

Analysis of both the cleaved and uncleaved AP–DNA complexes of both Exo III and Endo IV reveals that they employ similar strategies to cleave the target phosphate bond. Despite different structural chemistries at their active sites, the proposed reaction mechanisms both involve enzyme-activated hydroxyl nucleophile attack on the target phosphorus atom [60].

Exonuclease III family

The Endonuclease III family consists of AP endonucleases that are small (30,000 to 40,000 Da) monomeric divalent-metal ion dependent enzymes. APE1 enzymes can be inactivated by metal-chelating agents such as ethylenediaminetetraacetic acid (EDTA). The structure of Exo III (*E. Coli* homologue) revealed the characteristic four-layered α,β-sandwich fold, which is conserved in the human homologue APE1. Conserved amino acids essential for nucleolytic activity are concentrated around the active site and divalent metal-ion (Mg^{2+}) binding site [60].

Crystal structures of human APE1 bound to synthetic abasic site-containing DNA, both with and without the divalent metal ion, show how APE1 recognizes abasic sites and cleaves the target bond. APE1 binds a flipped-out abasic nucleotide with residues penetrating both the DNA minor and major grooves. The enzyme-bound AP–DNA is bent at 35° and there is a large displacement, or kinking, of the DNA helical axis [60].

Endonuclease IV family

Endo IV family members are small (30,000 Da) Zn^{2+}-dependent endonucleases that, unlike the Mg^{2+}-dependent Exo III family of AP endonucleases, resist inactivation by EDTA. The structure reveal that Endo IV is a single domain αβ protein with secondary structure elements arranged as a β-barrel having eight parallel β-strands surrounded by eight peripheral α-helices. The structure is conserved in the *Saccharomyces cerevisiae* homologue, APN1. Endo IV enzymes have a trinuclear zinc cluster in their active sites. Two of the Zn atoms are bridged by a tightly bound water molecule, which is likely deprotonated in the enzyme active site, and acts as the nucleophile in the hydrolysis reaction. The third zinc atom plays a role in stabilizing the 3′ hydroxyl group produced in the reaction.

Crystal structure studies of Endo IV bound to a synthetic abasic site-containing DNA revealed the structural features that underlie AP site recognition and cleavage. In the Endo IV:DNA complex structure, the AP–DNA is not kinked but instead is bent by 90° with both the abasic site and its partner nucleotide flipped out of the DNA helix for cleavage [60].

F. DNA repair in parasites and *Toxoplasma*

In general, protozoan parasites are highly sensitive to hydrogen peroxide and other organic peroxides as well as drug-generated and phagocyte induced ROS [61]. Likewise, resistance to ROS has been linked to virulence of some species of parasites. Despite the effectiveness of ROS in killing protozoan parasites, little is known about the DNA repair and antioxidant mechanisms of parasites.

Parasites are especially vulnerable to DNA damage by the ROS from immune effector cells. Not only do parasites have to ensure the integrity of nuclear and mitochondrial DNA in a manner analogous to other eukaryotes, many parasites possess other extra-nuclear DNA, including extra-chromosomal DNA elements in the apicoplast of apicomplexans and the kinetoplast of kinetoplastids [62]. Thus, parasites would be more dependent on accurate DNA repair.

The first line of defense the parasites utilize to protect their DNA is to neutralize the genotoxic elements before they damage the DNA. Parasites have evolved a complex network of antioxidants consisting of enzymatic and non-enzymatic components. Key among antioxidant enzymes are superoxide dismutases (SODs), catalases and peroxidases. One of the most ubiquitous non-enzyme antioxidants is the tripeptide glutathione (GSH). *Toxoplasma* also possesses effective antioxidant defenses, particularly to contend with ROS produced by macrophages and neutrophils. *Toxoplasma* antioxidants include three SODs and glutathione as well as thioredoxin-dependent peroxidases of the

peroxiredoxin family. *Toxoplasma* also possesses a cytoplasmic catalase that protects the parasite from hydrogen peroxide [63].

DNA repair mechanisms have been studied in kinetoplastids *Leishmania major* and *Trypanosoma cruzi* [71]. Over-expression of AP endonucleases (ExoIII homologue) in these parasites protects them from alkylating and oxidizing DNA damaging agents. AP endonucleases from *Leishmania* and *Trypanosoma* can complement exonuclease III deficiency in *E. coli.*

Among Apicomplexa, DNA repair has been studied in *Plasmodium falciparum. In vitro* experiments have shown that the malaria parasite is capable of repairing damaged bases through the base excision pathway. The experiments showed that malaria parasites contain glycosylase as well as AP endonuclease activities [65]. This data indicate that the BER pathway may be conserved in Apicomplexa. Further evidence of the BER pathway in Apicomplexa comes from the discovery of a FEN-1 homologue, PfFEN-1 in *P. falciparum*. FEN-1 is a key enzyme in long-patch BER in other eukaryotic cells [64].

There is virtually no characterization of DNA repair pathways in *Toxoplasma.* Reports are limited to the identification of DNA repair enzymes in *Toxoplasma*. TgDRE (Tg DNA repair enzyme), a homologue of DRT111 of *Arabidopsis thaliana,* and TgPREX (Plastid replication and Repair Enzyme Complex) are among the few identified [66]. TgDRE is capable of complementing an *Escherichia coli* mutant lacking ruvC endonuclease and recG helicase. TgPREX has been identified as a possible apicoplast DNA repair enzyme. No

enzymes of the BER pathway have been previously characterized in
Toxoplasma.

Damaged bases in *Toxoplasma* are likely to be repaired through BER as
suggested by studies of *Plasmodium*. Comparative genomics studies performed
in our laboratory have revealed that apicomplexan parasites possess both
exonuclease III and endonuclease IV homologues. *Toxoplasma*, for example*,*
has homologues to exonuclease III (TgAPE) and endonuclease IV (TgAPN).
TgAPE is a homologue of human APE1, but is considerably divergent. TgAPN is
a homologue of yeast (*S. cerevisiae*) APN1 and is not present in mammals.

3. Pharmacological inhibition of DNA repair enzymes

Inhibition of the DNA repair process as a therapeutic approach has been
explored in the treatment of cancer. Scientists have previously focused on
preventing cancer cells from repairing the damage to their DNA by
chemotherapeutic drugs and radiation [67]. A robust DNA repair mechanism has
been linked to increased resistance to cancer chemotherapeutic agents.

In 2009, BSI-201, or iniparib, became the first DNA repair inhibitor for
breast cancer to reach a phase III clinical trial [68]. Iniparib blocks a signaling
enzyme called poly(ADP – ribose) polymerase (PARP1). The PARP1 enzyme
recognizes and eliminates oxidized DNA bases, which would otherwise
accumulate and kill cells. Inhibition of Chk1 and Chk2 — protein kinases involved
in checkpoint control following DNA damage — sensitized brain cancer cells to
radiotherapy in cell culture [69]. Other DNA repair proteins being studied as drug

targets include ATM and ATR kinases, breast cancer susceptibility protein (BRCA) proteins, and DNA mismatch repair proteins [68].

In the BER pathway, inhibitors of glycosylases have been explored as cancer chemotherapy agents. Due to the multiple DNA glycosylases in each cell, it is difficult to disrupt BER by inhibiting glycosylases. As the rate limiting enzymes in BER, AP endonucleases are very attractive targets for disruption of the pathway. Indeed, targeting AP endonucleases has been studied in the treatment of cancer. There are currently three compounds known to inhibit the human AP endonuclease, APE1: methoxyamine (MX) and two compounds, 7-nitroindole-2-carboxylic acid (NCA) and Lucanthone [69].

Lucanthone, originally identified as a topoisomerase II inhibitor, is considered to be a direct inhibitor of APE1's DNA repair activity [69]. Lucanthone was extensively used to treat schistosomiasis and shown to be safe from a clinical standpoint [70]. Cancer cells treated with lucanthone exhibit a dose-dependent increase in AP sites due to inhibition of APE1 repair activity. Patients with brain metastasis treated in combination with radiation and lucanthone showed increased regression of the tumors with the combination as compared to radiation alone [70]. The detrimental impact of DNA inhibition on human cells needs to be weighed carefully before targeting this pathway in pathogenic protozoa such as *Toxoplasma* or *Plasmodium*. It is plausible that intracellular parasites, being insulted with ROS from the host, rely on DNA repair pathways much more than human cells, and therapeutic concentrations that inhibit parasites will be significantly lower. In addition, there appear to be nuances in the

parasite pathways that could be exploited for therapy. As we will show, the

Toxoplasma APE1 homologue is remarkably different than its human

counterpart, and some enzymes, such as TgAPN, are not present in mammals.

4. Summary and specific aims

As mentioned previously, current drug therapies for *Toxoplasma* infection are limited due to toxic side effects [35]. It is therefore necessary to identify new drug targets with an increased likelihood of selective toxicity towards the parasite.

DNA repair is likely to serve as a good drug target in *Toxoplasma*. As an obligate intracellular parasite, *Toxoplasma* is exposed to host cell metabolites such as ROS that induce DNA damage [61]. ROS are also produced by immune effector cells to kill the parasite. *Toxoplasma* is also susceptible to respiratory burst activity from monocytes responding to *Toxoplasma* invasion [47]. The rapid growth and replication of *Toxoplasma* can also lead to a high frequency of base mismatches during asexual reproduction. In other species, the majority of DNA oxidative damage is repaired through the base excision repair (BER) pathway, and apurinic/apyrimidinic (AP) endonucleases are the rate limiting enzymes in BER. *Toxoplasma* possesses two different AP endonucleases. We have cloned both AP endonucleases in *Toxoplasma* (termed TgAPN and TgAPE). TgAPE is a magnesium-dependent homologue of human APE1 (hAPE1), but TgAPE is considerably divergent from hAPE1. TgAPN is a magnesium-independent homologue of yeast APN1 endonuclease and is not present in mammals. The absence of APN in mammals makes TgAPN particularly attractive to investigate as a potential drug target. Preliminary data from Western blot analysis and real-time PCR suggests that TgAPN is more abundant than TgAPE in the parasite. Thus, our hypothesis is: TgAPN is the predominant AP endonuclease in

Toxoplasma that is critical for DNA repair and viability. Our research strategy to address this hypothesis focuses on the following aims.

AIM 1: Determine the role of TgAPN in the *Toxoplasma* response to DNA damage.

To address this aim, we generated a FLAG-tagged TgAPN over-expressor parasite. The parasites engineered to over-express TgAPN under a strong tubulin promoter were used to determine if there is increased protection against DNA-damaging agents such as methyl methanesulfonate (MMS) compared to wild-type parasites. Second, we created a TgAPN conditional knockout parasite that allows for the regulation of an ectopically expressed TgAPN using a tetracycline analogue. Finally, we generated a parasite with the endogenous TgAPN protein fused to a destabilization domain. Parasites expressing regulatable TgAPN protein were used to determine if loss of TgAPN makes the parasites more susceptible to DNA damaging agents.

AIM 2: Pharmacological inhibition of TgAPN

No inhibitors of APN1 have been identified to date. As our preliminary data suggest that TgAPN is more abundant in the parasite than TgAPE, and TgAPN is not present in mammals, we characterized TgAPN inhibitors obtained from a high-throughput screen against TgAPN enzyme activity. The most promising inhibitors from the high-throughput screen were tested against the parasite *in vitro* to determine inhibition of parasite growth. We also determined the IC50s of the compounds using standard parasite growth assays.

CHAPTER 2: MATERIALS AND METHODS

1. Parasite culture and manipulation

A. Parasite culture and tissue culture

Toxoplasma gondii tachyzoites were cultured in human foreskin fibroblasts (HFF) in Dulbecco Modified Eagle's Media (Gibco # 21013-024) supplemented with 1% heat inactivated fetal bovine serum (Gibco # 16000-044). Flasks were maintained in a humidified incubator at 37°C and 5% CO_2. Type I RH strain parasites were used for all experiments, and were obtained from the AIDS Research and Reference Program (Division of AIDS, NIAID, National Institutes of Health).

HFF host cells (American Tissue Culture Company) were grown to confluency before infection with parasites. Host cell media was comprised of Dulbecco Modified Eagle's Media (Gibco # 21013-024) and 10% heat inactivated fetal bovine serum (Gibco # 16000-044). All tissue culture was carried out under sterile conditions in a laminar flow hood. HFF cells are commonly used for the culture of *Toxoplasma* [76]. HFF cells offer several advantages in the culture of *Toxoplasma* such as: a large contiguous monolayer that allows for multiple parasite replication cycles, yielding large parasite titers. The HFF cells also have high contact inhibition that ensures a continuous monolayer can be prepared long before they need to be used. The confluent HFF cells are resistant to many metabolic inhibitors and facilitate drug selection and drug assays. As primary host cells, HFF cells provide a more clinically relevant model system than other cells.

Splitting HFF cells for subsequent passage was done using trypsin digestion. The HFF media was aspirated and the monolayer washed with 3 mls of PBS before adding of 3 mls of trypsin. The flask is incubated at 37°C for 1 minute to facilitate trypsin digestion. The flask was then agitated to ensure more complete dislodging from the flask surface. Trypsin activity was neutralized by addition of HFF media before being passed into new sterile flasks.

Parasite and host cell tissue culture were checked monthly for *Mycoplasma* contamination. *Mycoplasma* is a common tissue culture contaminant and can affect the outcome of experiments. *Mycoplasma* is a bacteria genus that lacks a cell wall and is resistant to many types of antibacterial agents. *Mycoplasma* can be free living or saprophytic in decaying matter. The extremely small size of the bacteria makes detection by light microscopy difficult. It has been estimated that at least 11 to 15% of U.S. laboratory cells cultures are contaminated with *Mycoplasma* [72]. Cultures were tested for the presence of *Mycoplasma* using the MycoAlert *Mycoplasma* Detection kit (Lonza # LT07-318). The MycoAlert kit detects *Mycoplasma* through activity of *Mycoplasma* enzymes. The viable *Mycoplasma* cells are lysed and the enzymes react with the MycoAlert substrate catalyzing the conversion of ADP to ATP. This increase in ATP can be detected using a bioluminescent reaction quantified using a luminometer. Although *Mycoplasma* was not detected in the cultures, contamination can be removed from tissue culture. Passing the parasites through a mouse will eliminate the *Mycoplasma* through the mouse immune system. *Toxoplasma*

cultures contaminated with *Mycoplasma* can also be treated with *Mycoplasma* Removal Agent (MP Biomedicals # 093050044), an inhibitor of bacterial gyrase.

B. Freezing and thawing

Parasites were frozen for storage in liquid nitrogen. To prepare a frozen stock of parasites, they were grown to the point where about 80% of the HFF cell monolayer was disrupted by lysis. The remaining infected cells were harvested by scraping with a sterile spatula (Fisher # 08-773-2). Free parasites and infected host cells from the flask were pelleted by centrifugation at 8,000 g at 4°C for 10 minutes. The pellet was resuspended in 1 ml FBS with 10% DMSO. The mixture was aliquated into (1 ml) cryovials (Cryotube # 368632) and frozen at -80°C in a Styrofoam container before being moved into the liquid nitrogen tank. Parasites are thawed by immediately placing the frozen cryovial in a 37°C water bath. Once thawed, the cryovials are cleaned with 70% ethanol to avoid contamination, and then inoculated onto a new HFF cell monolayer. The parasite media was changed 24 hours post-infection.

C. Purification of parasites from HFF cells

Purification of parasites helps remove host cell debris that might hinder downstream applications. To ensure safety and sterility, all steps involving parasites are carried out in a laminar flow hood.

The first step of parasite purification involves scraping the infected HFF cells with a spatula. The material was then placed into a syringe (Fisher # 14-840-52) attached to a 3.0 µM filter (Whatman # 110612). The media and parasites are forced through the filter into an awaiting collection tube by

compressing the plunger. *Toxoplasma* tachyzoites are ~2 x 6 microns and will pass through the filter while the majority of host cell debris remains behind [76]. The filtered parasites are then centrifuged at 8,000g for 10 minutes. Filter-purified parasite pellets are washed through resuspension in 15 ml sterile PBS for another round of centrifugation. The parasites are washed in PBS two more times before used in downstream applications.

D. *Toxoplasma* lysate preparation

Toxoplasma protein lysate was made in RIPA buffer (50 mM Tris; 150 mM NaCl; 1% NP40; 0.5% Na-deoxycholate) unless specified otherwise. Also included was the mammalian protease inhibitor cocktail (Sigma # p8340), used as per manufacturers suggestion (1:100). After the parasites are purified as described above, the pellet was resuspended in RIPA buffer containing protease inhibitors. The parasites are then sonicated three times on ice for 15 second bursts interspersed with 30 second recovery period on ice. The sonicated lysate was centrifuged at 13,000g for 10 minutes at 4°C to pellet cell debris, insoluble protein and DNA. The supernatant that contains soluble protein was collected using a pipette.

E. Quantification of parasites

Quantification of parasites was achieved using a hemocytometer (Hauser) (Figure 3). The parasites are first filter-purified as described above and 10 µl was loaded into each counting chamber. The hemocytometer was placed under a Leica DM IL inverted microscope and the parasite counted at (100X). Two independent counts are made for each sample i.e. the hemocytometer was

cleaned and fresh sample was loaded and counted. The average of each of the counts was used to calculate the number of parasites in the sample. The final average was multiplied by 25 because of the 25 squares of the grid. The result was multiplied by 10,000 because of the volume of the grid in the hemocytometer was approximately 1 microliter.
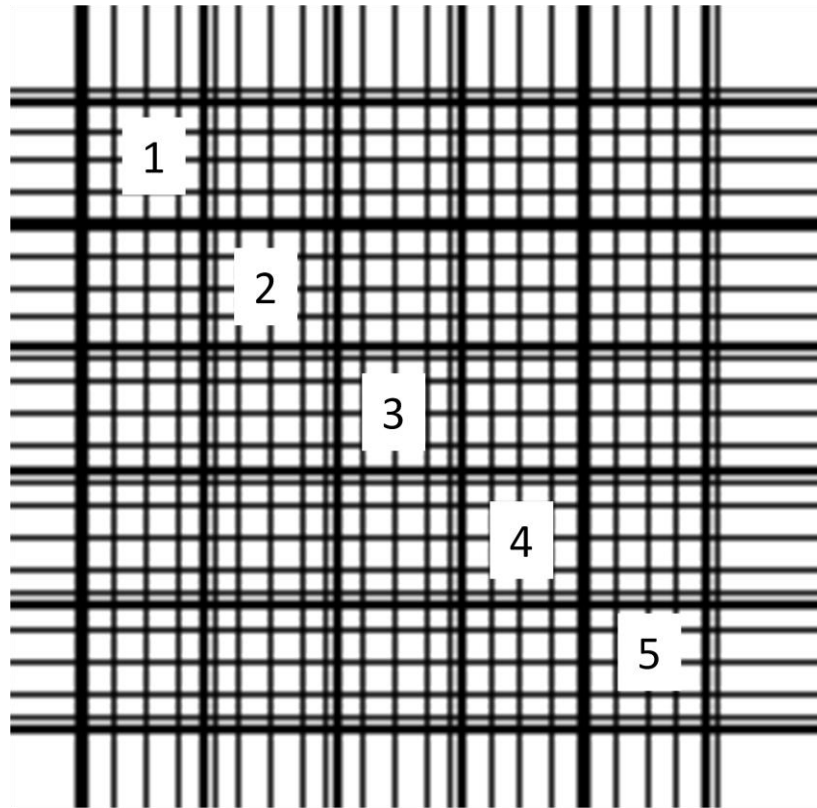
Figure 3: Counting grid of hemocytometer. The highlighted numbers represent the 5 individual squares counted for each replicate.

F. Transfection of parasites

Transfections were performed to introduce recombinant DNA into the parasites to make transgenic lines. The primary transgenic parasites generated during this work include TgAPE and TgAPE over-expressor parasite lines, a TgAPN conditional knockout, and the Shield-1 regulated TgAPN.

25 µg of plasmid DNA was used for each transfection. Plasmid DNA was propagated in *E. coli* bacteria and purified by maxiprep (Zymo Research # ZRC163686). Purified plasmid DNA was linearized by restriction digest and precipitated for transfection. The DNA was first extracted using phenol-chloroform to remove salts and restriction enzymes. An equal amount of phenol-chloroform and sample were mixed together by vigorous vortexing. The mixture was centrifuged at 13,000 rpm for 2 minutes to separate the aqueous and organic phases. The upper aqueous phase that contains the DNA was removed by pipetting. The DNA was precipitated by adding 2.5X cold 100% ethanol and 0.1X 3M NaOAc, pH 5.2 followed by mixing and incubation at 80°C for at 30 minutes. The sterile plasmid DNA was dissolved in 100 µM cytomix (2 mM EDTA, 120 mM KCl, 0.15 mM CaCl$_2$, 10 mM K$_2$HPO$_4$/KH$_2$PO$_4$, 25 mM HEPES, 5 mM MgCl$_2$·6H$_2$O; pH 7.6) freshly supplemented with 5 mM of glutathione and 2 mM of ATP [73].

Parasites were filter-purified and washed in cytomix three times before counting on a hemocytometer. 2X10^7 parasites in 300 µl cytomix were used for each transfection. The parasites and the plasmid DNA were mixed together by pipetting and transferred to a 2 mM gap electroporation cuvette (Fisher # 14-955-

128). Electroporation was done at the following settings: voltage=1500, resistance=25 ohms, capacity=25 μF. After electroporation, the cuvette was placed in the laminar flow hood for 15 minutes to allow the parasites to recover. The transfected parasites were passed onto a fresh monolayer with parasite media and allowed to replicate under standard culture conditions. For generation of clones, the transfected parasites were split into four flasks to reduce the chances of getting "sister" parasites. Selection in the appropriate drug was initiated following the first round of lysis post-transfection. Three rounds of drug selection were performed before the parasites were cloned. The drugs used for each transfection are shown in Table I.

Table I: Drugs used to select transgenic parasites.

Selection Agent	Concentration	Vehicle	Additional Information
Mycophenolic acid (MPA)	25 µg/ml	100% ethanol	For RHΔHX parasites when HXGPRT gene is utilized for selection. Must include XAN as a supplement
Xanthine (XAN)	50 µg/ml	fresh 0.5M KOH	Used in combination with MPA
6-thioxanthine (6TX)	320 µg/ml	fresh 0.5M KOH	For RHΔHX parasites prior to transfection to verify strain
Chloramphenicol (CAM)	20 µM	100% ethanol	CAT gene utilized for selection
Pyrimethamine (PYR)	1 µM	70% ethanol	Mutant pyrimethamine resistant DHFR gene utilized for selection

G. Cloning by limiting dilution

After three rounds of drug selection, parasites were cloned by limiting

dilution to obtain homogenous populations. After determining the count, the

parasites are diluted in media to yield approximately 1 parasite per well in a 96-

well plate containing confluent HFF cells. Drug selection continues throughout

the limiting dilution process. After approximately 10 days, plaques (areas of lysis)

will appear in some wells. Parasites arising from single plaques were picked and

inoculated into new HFF cells for expansion and subsequent analysis.

H. Parasite growth assays

Parasite growth assays were used to evaluate the viability of parasites

following treatment with DNA damaging agents or TgAPN inhibitors. Four

established *Toxoplasma* viability assays were used:

Plaque assay

Plaque assays were performed in 24-well plates of confluent HFF cells

based on standard protocols [76]. For each experiment a duplicate 24-well plate

was used. One of the plates was used as a reference plate to examine the

progression of the experiment during the experiment (typically lasting 7 days).

The other plate (experimental plate) was not to be moved for the duration of the

experiment to prevent parasites from being moved and forming secondary

plaques. 1,000 parasites are infected into each well and infected monolayers are

grown for 7 days (judging by progress of reference plate, which was viewed

throughout the experiment) at 37°C and 5% CO_2. Each experimental sample was

done in triplicate, taking up three wells in the plate. At the end of the 7 days or

when the reference plate wells indicate the desired plaque sizes, monolayers are fixed in ice cold (-20°C) methanol for 5 minutes after the culture media was aspirated. The methanol was aspirated and the monolayers are allowed to dry at room temperature. Crystal violet solution (1 µg/ml crystal violet, 25% ethanol, 1% ammonium oxalate) was used to stain the dried monolayers to facilitate visualization and counting of the plaques. Plaques are counted for each sample and the average of the triplicate scored as the plaque number.

B1 assay

The B1 assay is a PCR-based assay that amplifies the B1 region of the parasite genome [74,75]. This particular sequence is chosen for amplification because it is parasite-specific, i.e. the primers do not amplify host cell DNA. 1,000 parasites are infected onto a monolayer of HFF cells in a 24-well plate. The standard duration for the B1 assay is 5 days, unless stipulated otherwise. At the end of the fifth day, the samples are collected by aspirating the media and adding 400 µl of lysis solution (Qiagen, DNeasy kit (200 µl AL, 200 µl PBS, 20 µl proteinase K)) for 1 minute. Infected cells were dislodged by scraping and transferred into a collection tube. The lysate solution was heated for 10 minutes at 56°C. The heated lysate was mixed with 200 µl of 100% ethanol by vortexing. The entire mixture was transferred to a DNeasy spin column and centrifuged for 1 minute at 8,000g to bind the DNA to the column matrix. 500 µl of buffer AW1 was added to the column and centrifuged for 1 minute at 8,000g. 500 µl of buffer AW2 was added to the column and centrifuged for 3 minutes at 13,000g. 100 µl of elution buffer was added to the column and the samples were collected after 1

minute centrifugation at 8,000g. The eluted samples were heated to 95°C for 5

minutes to heat-inactivate proteinase K. The samples were diluted by 1:10 in

distilled water before proceeding to the real-time PCR phase of the experiment.

The real-time PCR master mix is as follows:

12.5 µl SYBR green mix (ABI # 4364344)

1.0 µl B1 forward primer (GGAGGACTGGCAACCTGGTGTCG)

1.0 µl B1 reverse primer (TTGTTTCACCCGGACCGTTTAGCAG)

9.5 µl Water distilled water

1.0 µl sample

24 µl of the master mix was pipetted into each sample well of a MicroAmp 96well

plate (Applied Biosystems # 4346906) followed by 1 µl of sample. All samples

were set up in triplicate. Standards of known parasite number (10,000, 5,000,

2,500 and 1,250 parasites) were also included in the setup. The plate was

covered by an adhesive optical membrane (Applied Biosystems # 401194). The

real-time PCR was performed on a StepOnePlus (Applied Biosystems) machine.

The amplification signal of the samples was quantified using the signal of the

known standards.

 Doubling assay

Parasite doubling assays were performed in T-25 cm^2 flasks containing

confluent HFF monolayers based on previously published methods [76]. $1X10^7$

parasites were inoculated into the flask and parasites that did not infect in the

first 4 hours are washed off by changing the media. The flasks were incubated at

37°C in a humidified incubator with 5% CO_2. Parasites within 50 random

vacuoles were counted at the indicated time points. A Leica DM IL inverted microscope was used to count the parasites at 100X [76].

 Monolayer disruption assay

HFF monolayer disruption assays were used to assess if compounds from the TgAPN inhibitor screen had activity against *Toxoplasma* tachyzoites *in vitro*. 3,000 parasites were added to each well of a 96-well plate containing confluent monolayers of HFF cells. A serial dilution (0.05 µM to 100 µM) of each inhibitor (or vehicle) was added to a row of infected wells. Assay plates were analyzed every 12 hours. The 96-well plates were incubated at 37°C in a humidified incubator with 5% CO_2 for the duration of the experiment, which typically lasts about 5 days.

2. Molecular biology and biochemical techniques

A. Cloning of TgAPE and TgAPN

A search of the *Toxoplasma* genome web site (ToxoDB.org) revealed that accession number TGGT1_098640/35.m00892 (chromosome XI) is a homologue of human APE1 (hAPE1, ExoIII class) and TGGT1_034710/80.m00015 (chromosome IX) a homologue of yeast APN1 (EndoIV class). The TgAPE and TgAPN open reading frames were amplified from an RH strain tachyzoite cDNA library. 5'- and 3'-rapid amplification of cDNA ends (RACE) was performed with the GeneRacer™ kit (Invitrogen # L1500-01) using tachyzoite mRNA as a template for reverse transcription (RT) with the provided random primers or oligo dT primer. RT-PCRs were performed using the SuperScript™ One-Step RT-PCR kit (Invitrogen # 45-0167) following manufacturer's instructions. Nested PCRs

were performed with the RACE to determine TgAPE and TgAPN untranslated regions (UTRs). TgAPN 5' RACE was performed using primer 5'-TTGACACTCGAGTGCTTCGCGTCC as the parental gene specific primer and 5'-TCCCACCCGCTTTTGTGTCTAATGT as the nested gene specific primer. TgAPE 5' RACE was done using primer 5'-TAGTTGCTCGATGAGGTCCCTCAG as the parental gene specific primer and 5'-CCTCTTCTCCCTGTGCTGAGTTCA as the nested gene specific primer. GeneRacer™ 5' primers were used as the forward primers in the initial and nested PCR reactions. TgAPN 3' RACE was done using primer 5'-CGATGCATATCAACGATTCTAAGGCT as the parental gene specific primer and 5'-GAAGCGGCCTGGACAGACACGA as the nested gene specific primer. TgAPE 3' RACE was done using primer 5'-CCATATCGTGTTGACGGAGACGCT as the parental gene specific primer and 5'-CTCGTGAGAATTTGCGGAGCTGGA as the nested gene specific primer. The GeneRacer™ 3' primers were used as the reverse primers in the initial and nested PCR reactions.

B. Purification of recombinant TgAPE and TgAPN

Recombinant TgAPE and TgAPN were produced in the laboratory of Dr. Millie Georgiadis (IUSM). An expression vector for TgAPE was constructed in pET28a including an N-terminal cleavable hexa-histidine tag using NdeI and NotI restriction sites. Following transformation into the Rosetta *E. coli* strain, a 1 liter culture was induced at 37°C for 4 hrs, after which cells were pelleted and stored at -80°C prior to purification. The cell pellet was resuspended in 25 ml of lysis buffer, Buffer A (50 mM Na phosphate pH 7.8, 0.3M NaCl) with 10 mM imidazole,

sonicated, and centrifuged at 35K for 35 min in an ultracentrifuge. The crude extract was incubated with 1 ml of a Ni-NTA agarose slurry for 45 minutes at 4°C, placed in a column, washed with 10 ml of wash Buffer A with 20 mM imidazole and eluted with 2 x 1.5 ml of elution buffer, Buffer A with 250 mM imidazole. Fractions were diluted to 0.05M NaCl with Buffer B (50 mM HEPES pH 7.5, 1 mM DTT) and applied to a Q-Sepharose column using an AKTA FPLC system. The protein was then eluted from the Q-Sepharose column using a NaCl gradient (0.05M NaCl to 1M NaCl). The fractions containing TgAPE were combined and digested overnight with thrombin in order to remove the N-terminal hexa-His affinity tag. The sample was then diluted to a final salt concentration of 0.05M NaCl with Buffer B and subjected to a NaCl gradient separation on the Q-Sepharose column as before. Fractions including TgAPE were combined, concentrated, and stored at -80°C. The purified sample is greater than 95% pure as judged by SDS-PAGE analysis.

An expression vector for TgAPN was constructed in pET15b using NdeI and BamHI. Expression and purification were similar to that described for TgAPE with the same buffers used for Ni-NTA affinity purification followed by S-Sepharose ion-exchange chromatography. The fractions containing TgAPN from the Ni-NTA purification were pooled, diluted in 50 mM HEPES pH 7.0, 1 mM DTT, and eluted using a NaCl gradient 0.05-1.0 M in the same buffer. Fractions including TgAPN were combined, concentrated, and stored at -80°C. The purified sample was approximately 90% pure.

C. DNA repair assays

Gel based DNA repair assays were carried out in the laboratory of Dr. Mark Kelley (IUSM). To test if the recombinant TgAPE and TgAPN exhibited AP endonuclease activity, we performed DNA repair assays using increasing concentration of purified recombinant protein. The DNA repair assay uses 5-hexachloro-fluorescein phosphoramidite (HEX)-labeled terahydrofurayl (THF) oligo. The oligonucleotide is 26 bp long and contains a THF, an abasic site analogue, in the middle. Upon DNA repair the AP endonuclease cleaves the oligonucleotide at the synthetic AP site (THF) yielding a 13mer fragment. The reaction products can then be visualized using a 20% polyacrylamide gel Increasing amounts of recombinant TgAPE were incubated in buffer A (50 mM HEPES, 50 mM KCl, 10 mM $MgCl_2$,1 µg/ml BSA, 0.05% Triton X-100, pH 7.5). Increasing amounts of recombinant TgAPN were incubated in buffer B (50 mM HEPES, 50 mM KCl, 20 mM EDTA,1 µg/ml BSA, 0.05% Triton X-100, pH 7.5). All reactions contained 1 mM DTT and 0.05 pmol HEX-TEF oligonucleotide in a total of 10 ml and were incubated at 37ºC for 15 minutes (Figure 4).

Fluorescent based DNA repair assays and enzyme kinetics were done in collaboration with Dr. Millie Georgiadis. AP endonuclease activity was measured in a fluorescent-based kinetic assay using a duplex DNA substrate containing a tetrahydrofuran abasic site mimic as the substrate. The oligonucleotides were diluted in water to a final concentration of 100 µM and annealed in a 4:5 ratio of fluorescein vs. dabcyl labeled oligonucleotides. Release of the fluorescein labeled oligonucleotide following cleavage at the abasic site by an AP

endonuclease is achieved by thermal denaturation at 37°C and followed in the

kinetic mode on a Tecan Ultra 384 instrument using an excitation wavelength of

485 nm and emission of 535 nm. TgAPN was assayed in 50 mM MOPS 7.5, 50

mM NaCl, and 0.8% DMSO. An optimal concentration of 4 nM TgAPN was

determined by varying the enzyme concentration with fixed substrate

concentration of 25 nM. The substrate was then varied from 0 to 75 nM in order

to determine V_{max} and K_m values for this enzyme. Similarly, 0.05 nM TgAPE was

used with substrate concentrations of 0 to 75 nM in 50 mM MOPS 7.5, 50 mM

NaCl, 1 mM $MgCl_2$, and 0.8% DMSO. DMSO was included in the assay in

preparation for screening of small molecule inhibitors dissolved in 0.8% DMSO

and found not to affect the activity of the enzyme. Kinetic parameters were

calculated from the average of quadruplicate measurements for each substrate

concentration using the Enzyme Kinetics module in SigmaPlot (SigmaPlot 11.2).
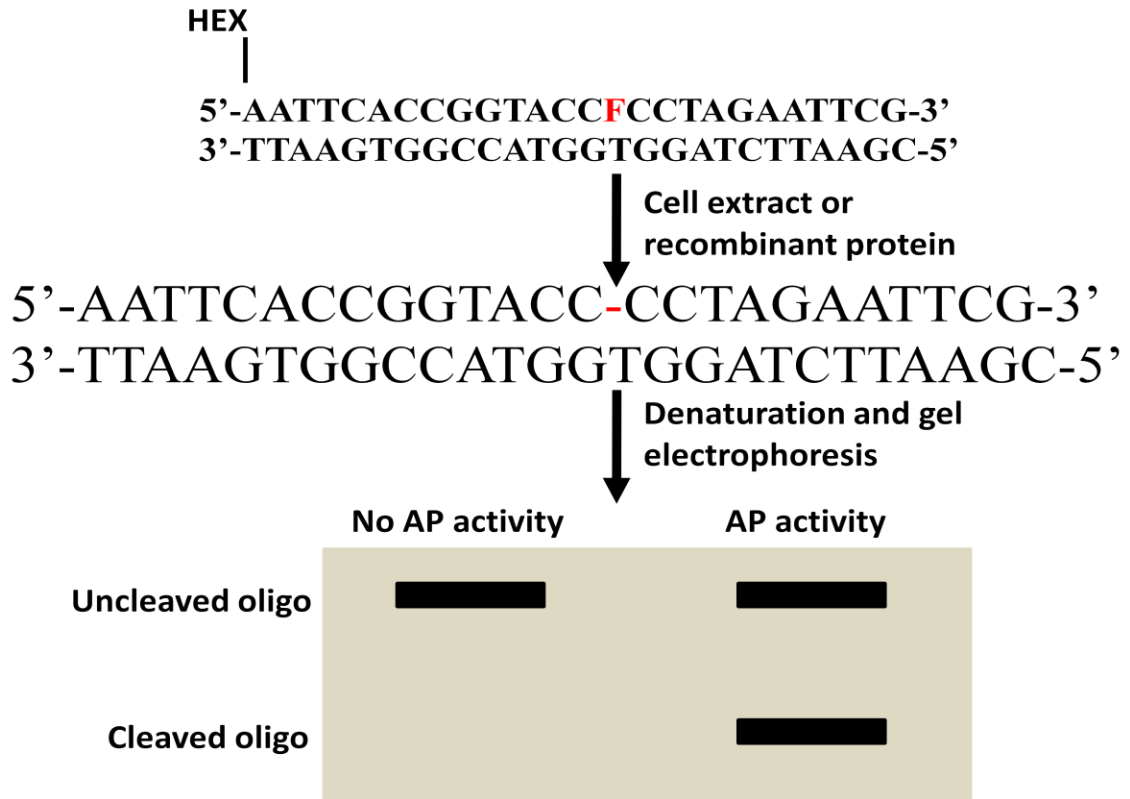
Figure 4: Schematic of a gel based DNA repair assay. A HEX labeled oligonucleotide with an abasic site mimic is cleaved in the presence of protein or cell extract with AP endonuclease activity. The cleavage product can be visualized after being denatured and resolved in a polyacrylamide gel. 5-hexachloro-fluorescein phosphoramidite (HEX). F=Terahydrofurayl (THF).

D. Generation of antisera recognizing TgAPE and TgAPN

Purified recombinant TgAPE and TgAPN (with N-terminal poly-his tag removed) were injected into rabbits for production of polyclonal antisera at Quality Controlled Biochemicals (Hopkinton, MA). Two rabbits were used for each of the proteins of interest. A pre-bleed was done prior to the injection of the rabbits with the antigen. Five other bleeds including a terminal bleed were done after injection with the antigen.

Western blot analysis of the raw serum collected from inoculated rabbits was performed to access reactivity against recombinant TgAPE or TgAPN protein and native *Toxoplasma* protein in parasite lysate. After establishing that the raw antisera recognized the respective recombinant and native protein using Western blot, the antisera were prepared for affinity purification.

The first stage of the affinity purification involves preparation of the purification column. The matrix used for the purification was the Affi-gel system (Biorad). Two matrices were used based on the isoelectric (PI) point of the respective recombinant proteins (TgAPE PI 6.53, TgAPN PI 9.04). Recombinant TgAPE was immobilized on Affi-gel 15 and recombinant TgAPN was immobilized on Affi-gel 10. 2 mg of each recombinant enzyme was dialyzed in 0.1 M phosphate buffer pH 7 or pH 8 for Affi-gel 15 and 10, respectively. The recombinant proteins (referred to as antigens) and beads were incubated together at 4°C overnight. The next day the matrix was washed with 10 ml 10 mM Tris pH 7.5 and 10 ml 100 mM glycine pH 3 to remove unbound antigen.

The next step of affinity purification involves purifying the raw serum over the column. The serum (10 ml) was dialyzed against (1,000 ml) 5 mM sodium phosphate pH 6.5 overnight. The dialyzed serum was pre-cleared over a DEAE matrix (Sigma # DF-100). After pre-clearing, the serum was rocked with the matrix overnight at 4°C. The matrix was then washed with 10 mM Tris by resuspending and centrifugation for 1 minute at 8,000 rpm. The antibodies are eluted with 10 ml 100 mM glycine pH 3. The eluted antibody solution was further concentrated using 30kD Amicon centrifugal concentrators (Amicon # UFC801024) to a volume of 2 ml. The affinity-pure antibodies were then quantified using the Pierce BCA protein assay kit (Thermo scientific # 23225). The affinity pure antibodies were evaluated by Western blot probing recombinant protein and *Toxoplasma* protein lysate.

E. Generation of parasites over-expressing TgAPE and TgAPN

FLAG-tagged TgAPE and TgAPN were ligated into the ptub$_{FLAG}$::HX expression vector using the NdeI and AvrII restriction sites (Bhatti and Sullivan, 2005). $_{FLAG}$APN was amplified using primers (sense) 5'-TCGATCGCATATGAAAATG*GACTACAAGGACGACGACGACAAG*ACGGCTGCAGCGTCCCTAAGAAAAACCAAGG and (antisense) 5'-TCGATCGCCTAGGTCACTCTTCGATGATGAATTTGTACATCATCTCC, where the underscored sequences represent the NdeI and AvrII restriction sites, respectively, and FLAG sequence is italicized. $_{FLAG}$APE was amplified using primers (sense) 5'-ATCATATGAAAATGGACTACAAGGACGACGACGACAAGTCTGTTCACAGAG

55

CAGTGAACTCA and (antisense) 5'-

ATCGAACCTAGGTCAACTGGAGGCCTGGACGTTTCGCG.

The parasite strain used to generate the over-expressor parasite lines is RHΔHX. This is a parasite in which the HXGPRT (hypoxanthine-xanthine-guanine phosphoribosyltransferase) enzyme has been deleted [89]. Prior to any experiment, the RHΔHX parasites are selected using 6-thioxanthine (Acros organics # 241720010). 6-thioxanthine is a subversive substrate of HXGPRT as it is metabolized into a toxic metabolite. Parasites complemented with a plasmid containing HXGPRT can be selected by mycophenolic acid (Sigma # M3536) and xanthine (Sigma # X-4002) (Figure 5) because mycophenolic acid inhibits inosine monophosphate dehydrogenase (IMPDH) and parasites can only synthesize guanine nucleotides through the enzymatic action of HXGPRT in the presence of xanthine [77].

The N-terminal FLAG-tagged TgAPE and TgAPN constructs were linearized with NotI and electroporated into RHΔHX tachyzoites (Figure 6). Transgenic parasites were selected in mycophenolic acid (25 μg/ ml) and xanthine (50 μg/ ml) and cloned by limiting dilution. The individual clones were confirmed by IFA and Western blot. Anti-TgAPE and anti-TgAPN antibodies at 1:10,000 were used to determine if the transgenic clones were indeed over-expressing TgAPE or TgAPN.
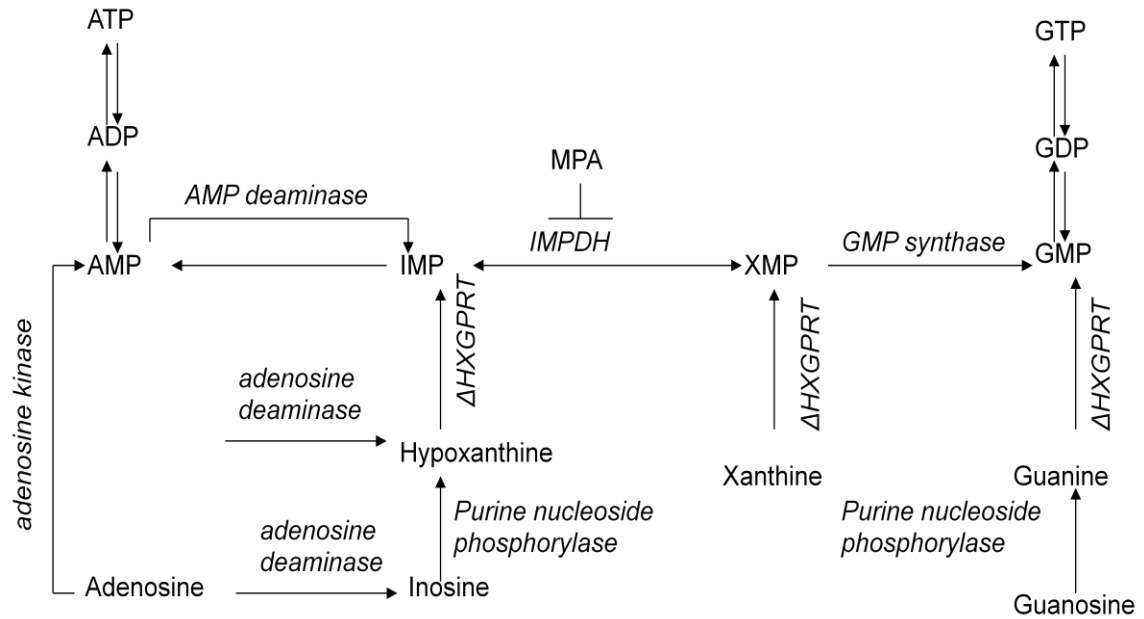
Figure 5: *Toxoplasma* purine synthesis pathway. *Toxoplasma* depends on the host cell for its purine requirements. Knocking out HXGPRT (hypoxanthine-xanthine-guanine phosphoribosyltransferase) prevents the parasite from utilizing guanine or xanthine to produce GTP. The parasite can overcome the loss of HXGPRT through the action of inosine-monophosphate dehydrogenase (IMPDH). Inhibition of IMPDH by addition of MPA (mycophenolic acid) results in the parasites being dependent on HXGPRT and xanthine for the production of guanine nucleotides. Therefore, the addition of MPA to RHΔHX parasites results in death, unless the parasites incorporated HXGPRT through transfection. RHΔHX parasites are resistant to 6TX (6-thioxanthine) due to the lack of HXGPRT. 6TX is metabolized by HXGPRT into a toxic metabolite that kills the parasite.

A.
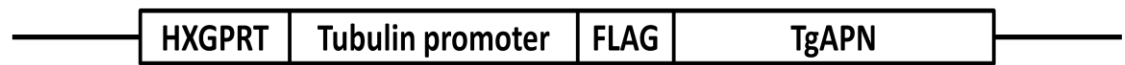
B.
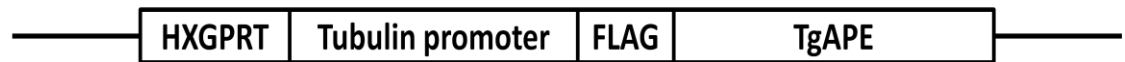
Figure 6: FLAG-tagged TgAPE and TgAPN constructs. Both TgAPE and TgAPN open reading frames were ligated into the ptub$_{FLAG}$::HX expression vector that utilizes a strong tubulin promoter. An N-terminal FLAG tag was used on each construct. A) FLAG tagged TgAPN. B) FLAG-tagged TgAPE. The ptub$_{FLAG}$::HX expression vector was made in a Bluescript vector backbone and contains a HXGPRT minigene.

F. Generation of the TgAPN conditional knockout

The TgAPN open reading frame with a C-terminal HA tag and an N-terminal FLAG tag was inserted into plasmids containing modified SAG1 and SAG4 promoters engineered to be regulated in a tetracycline (Tet)-off manner [78] (Figure 7). These plasmids also contain a minigene encoding chloramphenicol resistance for selection purposes [79]. The sites used in both the modified tet-based SAG1 and SAG4 plasmids were NsiI and PacI. The forward primer 5'-ATACCATCCTGCAGGAAAATGGCG*GAGCAGAAGCTCATCTCTGAGGAGGAC CTC*ACGGCTGCAGCGTCCCTAAGAAAAACC contained a SbfI restriction site that generates compatible ends with NsiI. The HA tag is in italics. The reverse primer was 5'-ATACCATTTAATTAATCA*CTTGTCGTCGTCGTCCTTGTAGTC*CTCTTCGATGA ATTTGTACATCATCTCC. The PacI site is underlined, while the FLAG sequence is in italics. 25 µg of vector was linearized using NotI and transfected into TATi parasites [78]. TATi parasites are engineered to express a transactivator protein that binds the modified SAG1 and SAG4 promoters to induce expression of the gene of interest, in this case TgAPN. In the presence of anhydrous tetracycline (ATc, Sigma # 37919-100MG-R) the transactivator is bound to the drug and cannot associate with the promoter elements. Following the first host cell lysis after electroporation, 20 µM chloramphenicol (Sigma # C0378) was added to select for the presence of the SAG1 or SAG4 plasmid. Three rounds of drug selection were performed before cloning by limiting dilution. Selected parasites

were evaluated using IFA and Western blot using FLAG antibody and protein specific antibodies.

A pDHFR plasmid containing a minigene encoding resistance to pyrimethamine was used to generate a knockout vector for TgAPN. The pDHFR vector encodes mutated dihydrofolate reductase, which confers resistance to pyrimethamine [80]. Two 3.5 kb genomic sequences flanking the TgAPN coding region were amplified from RH DNA using the Qiagen® LongRange PCR kit. HindIII and NotI sites were used to insert the 5' and 3' genomic flanking sequences into the vector, respectively. The forward primer for the 5' flanking sequence was 5'-GCAGTGAAGCTTCCGTCGCCTCTCGCGATGTTTTCCCGCCAGTCG and reverse primer was 5'-GCAGTGAAGCTTCGTAACCGTATCATGACATAGCACCACGAATCCGC. The HindIII sites are underlined. The forward primer of the 3' flanking sequence was 5'-GCAGTGGCGGCCGCGCTCGTTGAGGGGAGCCACGGCGCGGCAGACTTGG and the reverse primer was 5'-GCAGTGGCGGCCGCCGCAGGAGACTGGGGGTCCATTTCTACGCCATGCC. The NotI sites are underlined. Clones expressing ectopic, ATc-regulatable TgAPN driven by modified SAG1 or SAG4 promoters were transfected with 25 µg of the knockout vector after it was linearized using ScaI. 1 µM of pyrimethamine was included in cultures following the first lysis. Three rounds of drug selection were performed before cloning by limiting dilution. Clonal populations were

evaluated using PCR and Western blot. PCR using intron spanning primers
determined the disruption of the TgAPN genomic locus and Western blot
determined the loss of the native TgAPN protein.

A.

CAT	Tet-regulated promoter	HA	TgAPN	FLAG

B.

TgAPN 5' UTR	DHFR-TS*	TgAPN 3' UTR

X ... X

TgAPN 5' UTR	TgAPN	TgAPN 3' UTR

TgAPN 5' UTR	DHFR-TS*	TgAPN 3' UTR
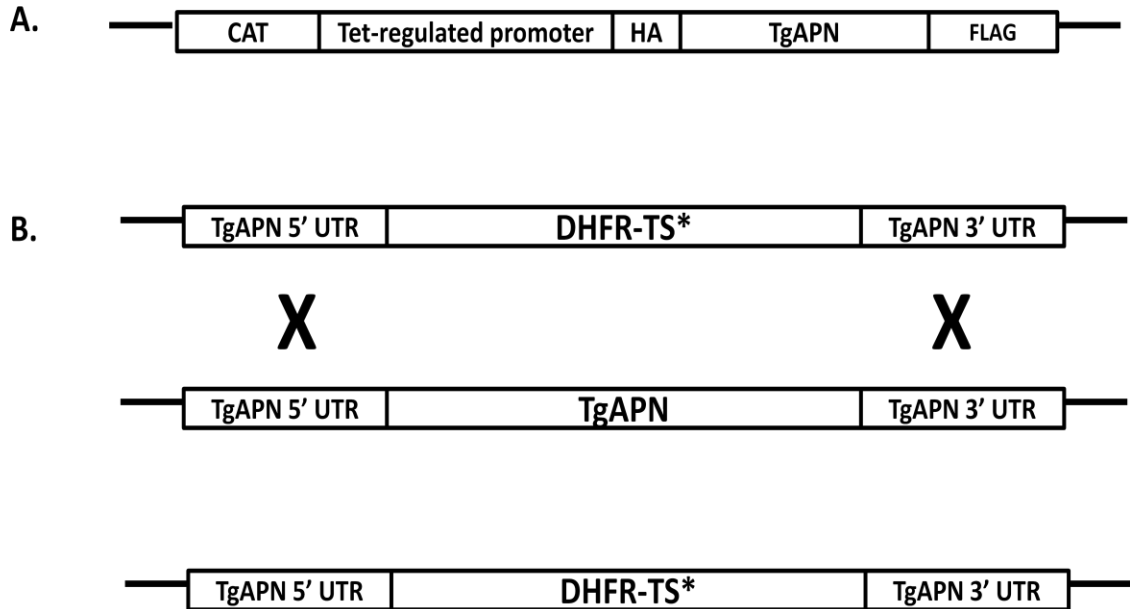
Figure 7: TgAPN conditional knockout constructs. A) The ectopic copy of TgAPN
was cloned into a SAG1 vector with a Tet-regulated promoter. The ectopic
TgAPN was HA and FLAG-tagged. Chloramphenical acetyltransferase (CAT)
conferred resistance to chloramphenical. B) TgAPN flanking sequence (UTR)
were cloned into a pDHFR vector with a mutant DHFR denoted by an asterisk (*)
that confers protection to pyrimethamine. The TgAPN genomic locus is replaced
by the construct by homologous recombination.

G. Generation of parasites expressing Shield-1 regulatable TgAPN protein

The 3' end of the endogenous TgAPN genomic locus was tagged with two hemagglutinin (HA) epitopes and a destabilization domain (DD) (Figure 8). 1,504 bp of the 3' end of the TgAPN genomic locus was amplified minus the stop codon using Phusion® High-Fidelity DNA Polymerase (New England Biolabs) and the following primers: (sense) 5'-TACTTCCAATCCAATTTAATGCCCTCCACAGTGTACAGACAGTTCCTCTAGGC and (antisense) 5'-TCCTCCACTTCCAATTTTAGCCTCTTCGATGAATTTGTACATCATCTCCG. The amplified product was cloned into the LIC-HA2X-DD-DHFR-TS vector as described [82]. The completed plasmid was linearized with BlpI and transfected into ΔKu80 RH strain parasites, which are more amenable to homologous recombination due to the reduced occurrence of non-homologous end joining [81,82]. 1 µM pyrimethamine was added to cultures after the first lysis to select for stable transgenics. Three rounds of drug selection were performed before cloning by limiting dilution. Pyrimethamine-resistant clones were examined for the loss of TgAPN-DD protein in the absence of Shield-1. Selected parasites were evaluated using PCR and Western blot using intron spanning primers and TgAPN antibody. 0.2 µM of Shield-1 was used to stabilize TgAPN-DD protein.
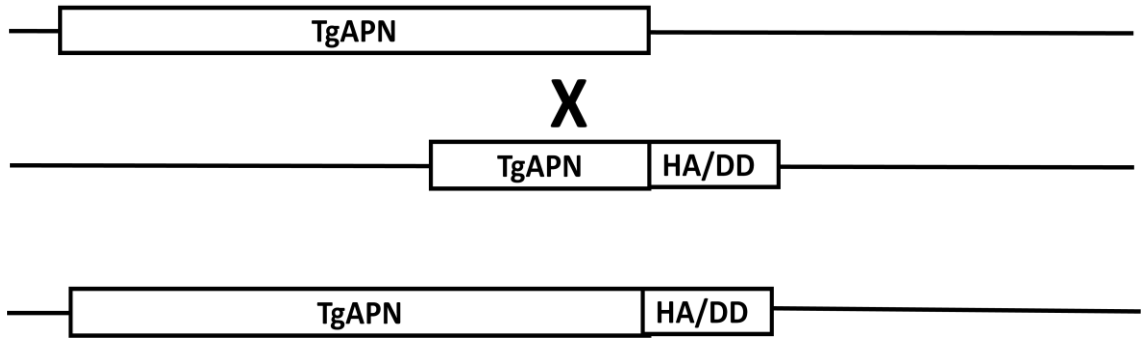
Figure 8: Endogenously tagging TgAPN with HA and the destabilizing domain.

1.5 Kb of the C-terminal end of TgAPN was amplified and cloned into the LIC-

HA2X-DD-DHFR*-TS. Through homologous recombination the DD was tagged

onto the C-terminal of the TgAPN gene

H. Immunofluorescence assays

HFFs grown in 12-well plates containing glass coverslips were used for immunofluorescence assays (IFAs). The host cell media was aspirated and replaced with parasite media. Parasites to be assayed were infected onto coverslips containing HFF monolayers. 24 hrs post-infection, the media is aspirated and the monolayer washed 3 times with sterile PBS before fixation with 1 ml of 3% paraformaldehyde for 15 min at room temperature. The infected cells were permeablized with 0.2% Triton X-100 in PBS for 10 min and blocked in 3% BSA (fraction V, OmniPur # 2910) in PBS for 30 min. Antibodies used in IFAs are represented in Table II. DNA intercalator 4', 6-diamidino-2-phenylindole (DAPI, Invitrogen # D3571) was applied at a concentration of 0.3 µM for 5 min as a co-stain to visualize nuclei. Secondary antibodies included Alexa Fluor 488 and 594 (Molecular probe) at 1:2,000 diluted in 3% BSA. Coverslips were prepared for viewing using 5 µl mounting media (50% glycerol containing 48 µg/ml Mowiol (Calbiochem # 81381) and 10 µg/ml DABCO (Sigma # 10981) to reduce photobleaching). Coverslips were mounted on glass slides to view using a Leica DMLB fluorescent microscope at 100X with a HCX Plan Apo oil immersion objective. Images were captured with a monochrome SPOT-RTSE camera and Spot Advance software (version 4.6). Images obtained are colored using Adobe Photoshop 7.0.

Table II: Primary and secondary antibodies used in IFAs.

Antibody Description	Source	Dilution
Primary Antibodies		
Anti-FLAG mouse, monoclonal	Sigma # F1804	1:5,000
Anti-HA (clone 3F10) rat, monoclonal	Roche # 11867423001	1:1,000
Anti-TgAPE rabbit, polyclonal	Custom	1:1,000
Anti-TgAPN rabbit, polyclonal	Custom	1:1,0000
Secondary Antibodies		
Alexa Fluor® 488 anti-rat, IgG (from goat)	Molecular Probes # A-11006	1:2,000
Alexa Fluor® 488 anti-mouse, IgG (from goat)	Molecular Probes # A-11017	1:2,000
Alexa Fluor® 488 anti-rabbit, IgG (from goat)	Molecular Probes # A-11070	1:2,000

Alexa Fluor® 594 anti-mouse, IgG (from goat)	Molecular Probes # A-11072	1:2,000

I. Immunoblotting

Affinity-purified antisera recognizing TgAPE and TgAPN were diluted in Tris-buffered saline tween (TBST) to a final concentration of 1:10,000. Additional antibodies used in this study included: anti-FLAG antibody (Sigma F1804) at 1:5000 and HA antibody (Sigma) at 1:2,000. Parasite protein lysates were made as described previously (section I of materials and methods). The protein lysates were reduced in 5% beta-mercaptoethanol and Nupage (Invitrogen # NP0007) sample buffer by heating at 95°C for 10 minutes. Reduced samples were loaded into 4-12% pre-cast SDS-PAGE gels (Invitrogen # NP0335) and run on a Biorad gel system at 200 volts for 50 minutes. After transfer, nitrocellulose membranes were blocked in 3% BSA for 2 hrs prior to being probed with the respective antibodies for two hours. Horseradish peroxidase conjugated secondary antibody (GE Health Care) was used as a secondary antibody at a dilution of 1:5,000. The signal was detected using an ECL chemiluminescent system (Thermo Scientific). Table III shows the antibodies used in Western blot.

Table III: Primary and secondary antibodies used in Western blot.

Antibody Description	Source	Dilution
Primary Antibodies		
Anti-FLAG mouse, monoclonal	Sigma # F1804	1:5,000
Anti-HA (clone 3F10) rat, monoclonal	Roche # 11867423001	1:3,000
Anti-tubulin rabbit, polyclonal	Custom	1:5,000
Anti-TgAPE rabbit, polyclonal	Custom	1:10,000
Anti-TgAPN rabbit, polyclonal	Custom	1:10,000
Secondary Antibodies		
ECL rat IgG, HRP-linked (from goat)	GE Healthcare # NA935	1:5,000
ECL mouse IgG, HRP-linked (from goat)	GE Healthcare # NA931	1:5,000
ECL rabbit IgG HRP-linked (from goat)	GE Healthcare # NA934	1:5,000

J. General PCR protocol and agarose gel electrophoresis

All primers used in these studies were obtained from Invitrogen in lyophilized state. Primers were resuspended to concentration of 100 µM in water distilled water. Only plugged tips were used in handling PCR reagents at a designated PCR station to minimize contamination. Reactions were set up on ice to maintain the integrity of the reagents. Phusion® High-Fidelity DNA polymerase (New England Biolabs # F530S) was used for amplification unless stated otherwise. PCRs were set up in 50 µl volumes in thin walled 0.5 µl tubes (Fisher # AB-0350). The PCR was performer using an Eppendorf thermocycler (Eppendorf, Mastercycler). The general PCR set up was as follows:

1. 98°C for 30 seconds

2. 98°C for 10 seconds

3. 60°C for 30 seconds

4. 72°C for 60 seconds

Repeat steps 2 to 4

5. 72°C for 10 minutes

6. 4°C holding temperature

PCR samples were analyzed using agarose gel electrophoresis. 1% agarose gels containing 0.5 µg/ml ethidium bromide (ICN Biochemicals # 806808) ran at 130 volts to separate DNA. Agarose gels were placed on UV transilluminator to visualize the DNA intercalated by ethidium bromide. DNA bands were excised and purified using the Invitrogen DNA extraction kit (K210012). PCR samples were prepared for sequencing by cloning them into TOPOII-Blunt (PCR) vector

(Invitrogen # K2800-20). Sequencing was performed using M13 forward and M13

reverse or T7 and T3 primers. All nucleotide sequencing was performed on both

strands at the Indiana University Biochemistry Biotechnology Facility

(Indianapolis, IN).

K. Real-time PCR

Real-time PCR was performed to quantify the mRNA levels of TgAPE and

TgAPN in tachyzoites. Total RNA was obtained from RH strain parasites using

the Qiagen RNeasy kit (Qiagen # 74106) and cDNA was reverse synthesized

using Omniscript reverse transcriptase (Qiagen # 205111). The cDNA was used

for the real time PCR reaction. The inverse value CT numbers obtained were

used to generate data.

Relative real-time PCR was performed for the conditional knockout

parasite with and without anhydrous tetracycline. cDNA was once again

synthesized using Omniscript reverse transcriptase (Qiagen # 205111). Tubulin

(type, GenBank number, primers) was used as a normalization control in the

PCR. The cDNA synthesis was set up as follows:

2 µl 10x Buffer RT

2 µl dNTP Mix

2 µl Oligo-dT primer

1 µl RNase inhibitor

1 µl Omniscript Reverse Transcriptase

12 µl RNase-free water

1 µl template RNA

The reaction mixture was incubated at 37°C for 1 hour and then the reverse transcriptase was inactivated at 95°C for 5 minutes. All reactions were performed in triplicate, using the 7500 real-time PCR system and analyzed using SDS software version 1.2.1 (Applied Biosystems).

L. Protein quantification

Protein quantification was performed using the Pierce BCA protein assay kit (Thermo scientific # 23225). Protein lysates were made as previously described (in section I of materials and methods). 25 µl of each sample was loaded into 96-well plates in triplicate. 200 µl working reagent, made from mixing reagent A and B 50:1 was added to each sample and incubated at 37°C for 30 minutes. After allowing the samples to cool to room temperature, the plate was read on a Magellan plate reader. The amount of protein in the sample was determined based on a standard curve derived from known BSA standards.

M. Transformation into *E. coli*

Vials containing ligation mixtures are placed on ice. One vial of Oneshot Top Ten cells (Invitrogen # C505003) for each reaction is thawed on ice. 5 µl of each ligation mixture was added directly to the *E. coli* and mixed gently by taping. Excessive agitation will rapture the fragile competent cells. The bacteria and DNA mixture was incubated on ice for 20 minutes before heat-shocking for 30 seconds in a 42°C water bath. After heat-shock, the bacteria were put on ice. 250 µl of pre-warmed S.O.C. medium (0.5% yeast extract, 8.6 mM NaCl, 2.5 mM KCl, 20 mM MgSO$_4$ and 20 mM Glucose) was added to each vial. Transfected samples were placed in a shaking incubator at 300rpm and 37°C for 45 minutes.

100 µl of transfected *E. coli* was plated onto an agar plate with the appropriate antibiotic. Plates were incubated inverted at 37°C overnight.

3. High-throughput screen for TgAPN inhibitors

A. Screening library

The ChemDiv library of diverse drug-like compound was used to screen for the TgAPN inhibitors. The compounds in this library obey the Lipinski rule of five with good ADME (absorption, distribution, metabolism and distribution) profiles. The Lipinski rule of five states that drugable compounds should have not more than 5 hydrogen bond donors (nitrogen or oxygen atoms with one or more hydrogen atoms), not more than 10 hydrogen bond acceptors (nitrogen or oxygen atoms), a molecular weight under 500 Daltons and a octanol-water partition coefficient log *P* less than 5. The library is formatted and available for screening in 384 well plates. The screen was carried out at the Chemical Genomics Core Facility in collaboration with Dr. Millie Georgiadis.

B. Drug screening assay for TgAPN inhibitors

The assay used a fluor-quenched pair of oligonucleotides; 5-F-GCCCCCXGGGGACGTACGATATCCCGCTCC-3' and its annealed complement oligonucleotide 3-Q-CGGGGGCCCCCTGCATGCTGCTATAGGGCGAGG-5', where F is fluorecein, Q is dabcyl, and X is terohydrofuran, an abasic site analog. In preliminary studies, varying amounts of recombinant TgAPN and 100 nM of substrate (oligonucleotide) are incubated together at room temperature to measure fluorescence as a function of time. Cleavage at the abasic site will result in the F-GCCCCC product and a time dependant increase in fluorescence

following the dissociation of the oligonucleotide duplex. The increased fluorescence is due to increased distance between the fluorescein and dabcyl. While associated in the oligonucleotide duplex, the fluorecein signal is quenched in by the dabcyl due to their close proximity. The high-throughput assay was done in a buffer containing: 50 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 50 mM NaCl and 2 mM DTT. The Genesis Workstation 150 that has three liquid handling and robotic arms for handling the 384 well plates was used to screen the inhibitor reactions. The fluorescence readings were monitored continuously through the 30 minute duration of the experiment using a SpectraMax plus 384 plate reader Molecular devices) and Softmax pro software in the kinetic mode at 495 excitation and 530 emission. 10 µM of compounds were initially screened in the reaction and compared to a control sample with no inhibitor to determine the level of inhibition. Compounds that had inhibitory activity against recombinant TgAPN had reduced fluorescent emission compared to the control sample.

C. Evaluation of high-throughput screen data

For each of the assay plates, 16 values of the control were obtained by measuring the fluorescence emitted. The assay reagents were dispensed exactly the same way in the sample wells as well as control wells to minimize variability. In addition to calculating the standard deviation among the controls and sample, a statistical Z factor, commonly used in high-throughput screens was also determined. The Z factor is a measure of data scatter and reproducibility in the high-throughput screen. Typically the Z factor varies between 0 to 1 with 1 being a perfect reaction and a score greater than 0.5 is considered acceptable.

Potential hits were also compared to the positive control, lucanthone, which
inhibits both human and *Toxoplasma* TgAPE.

Once the compounds with inhibitory activity against TgAPN were
identified in the high-throughput screen, their inhibitory activity is evaluated using
a standard AP endonuclease assay. The DNA repair assay uses 5-hexachloro-
fluorescein phosphoramidite (HEX)-labeled terahydrofurayl (THF) oligo. The
oligonucleotide is 26bp long and contains a THF, an abasic site analogue, in the
middle. Upon DNA repair the AP endonuclease cleaves the oligonucleotide at the
synthetic AP site (THF) yielding a 13mer fragment. The reaction products can
then be visualized using a 20% polyacrylamide gel. Different concentrations of
the inhibitors were added to determine the inhibition of recombinant TgAPN AP
endonuclease activity.

**D. Evaluating the inhibitory activity of compounds on *Toxoplasma*
tachyzoites**

Compounds were that determined to have inhibitory activity against
TgAPN were further tested against *Toxoplasma* tachyzoites. Initial studies were
done using monolayer disruption assay (discussed under growth assays).
Concentrations of the inhibitors ranging from 0.05 to 100 μM were added to
3,000 parasites in 96 well plates containing confluent human fore skin fibroblasts.
The assay was evaluated for 5 days to determine inhibition of parasite growth.
Host cell and parasite toxicity were determined as well as any of the compounds
that crystallized out of solution when diluted in parasite media.

Inhibitors that had no observable toxicity to host cells and inhibited parasite growth were further tested using more quantitative growth assays such as plaque assays and B1 assays describe previously under growth assays. 24 well plates of confluent human fore skin fibroblasts were infected with 1,000 parasites as well as different concentration of inhibitors determined by the data from the monolayer disruption assay. Data is collected on day 5 and day 7 for the B1 and plaque assay, respectively. In all assays, DMSO, in which the compounds were dissolved in, was used as a negative control. Pyrimethamine, a potent anti-*Toxoplasma* drug, was used as a positive control.

E. Determining host cell toxicity by TgAPN inhibitors

Preliminary toxicity of the TgAPN inhibitors on human foreskin fibroblast was done by observing the host cell monolayer under a microscope. The toxicity was determined by comparing the host cell monolayer infected with *Toxoplasma* tachyzoites alone with a monolayer infected with *Toxoplasma* and an inhibitor as well as a control of an uninfected confluent monolayer. The monolayers were fixed with 100% ice cold methanol for five minutes at the end of the experiment (5th day).

4. Miscellaneous

A. Chemicals

Methyl methanesulfonate (MMS) was obtained from Sigma (M4016) in DMSO at a concentration of 11.8 M (1.3 g/ml) and stored at 4°C. Shield-1 (CheminPharma, New Haven, # CIP-S1) was dissolved in 100% ethanol at a

concentration of 0.5 mM and stored at -20°C. 200 nM Shield-1was added to designated parasite cultures to stabilize the indicated fusion protein.

B. Statistics

All viability assays and DNA damage assays were performed at least three times. The results were reported as the mean standard deviation of the three independent experiments. Statistical significance was determined by the Student t-test or ANOVA (analysis of variance).

C. Bioinformatics

Bioinformatics was performed using http://www.ToxoDB.org for DNA and protein sequence evaluation (ToxoDB accession numbers 35.m00892 and 80.m00015 for TgAPE and TgAPN, respectively). DNA and protein sequences were also analyzed for homologues using BLAST (Basic local alignment search tool) programs at http://www.ncbi.nlm.nih.gov. Multiple sequence alignments were compiled using Vector NTI 9.0 (Informax) and ClustalW (http://www.ch.embnet.org/software/ClustalW).

CHAPTER 3: RESULTS

1. Characterization of *Toxoplasma* AP endonucleases

A. Apicomplexan parasites have ExoIII and EndoIV AP endonucleases

A search of the *Toxoplasma* genome web site (ToxoDB.org) revealed that accession number TGGT1_098640/35.m00892 (chromosome XI) is a homologue of human APE1 (hAPE1, ExoIII class) and TGGT1_034710/80.m00015 (chromosome IX) a homologue of yeast APN1 (EndoIV class). Dr. Sullivan subsequently cloned the full-length cDNA and confirmed the sequences for each (GenBank accession numbers are HM593513 for TgAPE and HM593514 for TgAPN). 5'- and 3'-RACE (rapid amplification of cDNA ends) were performed, revealing that TgAPE has a 5'UTR (untranslated region) of 273 nt and 3'UTR of 320 nt (Figure 9); TgAPN has a 5'UTR of 254 nt and 3'UTR of 424 nt (Figure 10).

TTTTCTCTTGTCCGCGTCTCGCGTCGTCTAAGTGCGCCTCTCTGTCTCTGTCTCTTTTCGCCGACTTTGTT
GTCTTCGGACTCGCAGACTCTGTCCCCTCCCCAGGCCTCGCTCTACTTTATCTCCGTTTTTCTGTGCGTTT
TCTTTCGGTTCCATCACCTCTGATCGTGCGTCGTCTCGACCGCGGAACTCTGCTGCTCGCTGTCTTCTGGA
CTTCTCTCCCGACTCTTCTCCGCTCGACCTCGTGTCTCGCCTGCGTACAAGTGCTCATCA

ATGTCTGTTCACAGAGCAGTGAACTCAGCACAGGGAGAAGAGGAAACTGAGGGACCTCATCGAGCAACTAC
AACAGAACTCAACTCATCCGCTGCTTCTCTCTCCTCCTCTCCTTCGTCTGCTCCTTCTTGCCCGGTTTCTA
CTTCAGCTATGCTGCAACGCGCCTCTGATTCGTCCTCTTCCGGCGCTCCGCCTGTCGATTTGAATCCTTCT
CGTGAGACTCGCGAGACGGAGGAGGACGGGGAGGGAGAACTCGAACGGAGAACAGAAGGACCGAAGCGAAA
GGCGCCTTTGAGCATCGTCACCTGGAATGTGAACAGCATCGCAGCGCGAATTCGAGACAGCCGCCAGTGGT
TCTACTTCTCGCGTTTTCTTCAAAAGATCGATCCAGACATTCTTTGTCTCCAAGAAGTCAAACTCGCGGCT
CATGGCCCCCCGGGCGCAAAGAGAGGCGACGGCATGCCGCGAGACCATGGAAGAATAAAGGACTCGGACAA
AGTCAGTAGCGTGGAAGCGCGAGAACTTCGCGAGGCACTCCACACTCTGCTTCCCAACCACAGTCTCCTCA
TCTCGCTGGCAGACTGGAGATATAGCGGACAAATGATGTTCATCAGAAAGGATGTCCAGGTTTGCTCCCTG
CGCTACAACCTGTCCCTCGACGGCTGCCCCGCGCATGAACATGATTTCGAAGGGCGCGTCATTCTGGCGGA
ATTCGAGGCCTTTTGTGTTCTTACCACGTACAGTCCCAACAACGGCGCAACACCGAAGTCGTTCGAGAGAC
GGCGGCTCTGGGATGAACGCATGCTGCAGTTCGTTACTCAATTGAAGAAGCCGCTGGTGTGGGTCGGAGAT
CTGAACTGCGCCCCAGAGGATATCGACCTCTCGGATCCGGATCAGTTTCGCTCGGTCATCCACGAAACCGC
AGACGGCACCATTGATCCGGACAACATTGGACAAGCCGGGTGCACAGATGCCGAGCGGCGGCGTTTTCGGG
CCATTCTCGAGAGGGGGAACCTCGTCGATGCCTTTCGAGAACTGCACCCGCGAACGGAGCCCCCTCCCCTG
GAGAGCGCCGAGTACTCTTGGCGTGGCTTCGGAGGGTCCGGGTCCCGGGGTCTGCTGAGAGGTTTGGGAAT
GCGACTCGACCATATCGTGTTGACGGAGACGCTGATGCCCGCAGTGGAACTCGTGAGAATTTGCGGAGCTG
GAAAGTCAAAGGCGAATTTTTTTGGATCTGATCACTGCCCGGTTCTCGTTCGTTTCAAAGAAAAAGAAATA
TCGGCGTTACCTGCGGTCGTTTGCGAGGCACTGCGCTCCTCTGCTCCTGCGCCAGCTGCGAAGAAGCGGCA
GGACGATTGCCGGTTAGATTCTTTCTTCGCGGTGAAGCGTCGAGCGCAATTGCCAGGTGCAGCGGACCAGA
AAAGGAAGAAGCCGGAGGAAGTTGTCATCTCTGATTCGTCAGACGAGGAGCGGAAAACAAGCCAGAAGATC
GCGCTGCATGCAAGTCTCGAGAAACCGCGAAACGTCCAGGCCTCCAGTTGA

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AGAGGAACTGAGCGTCTCGAGGAAACGGAATACTCGGAGTTAGACGACTGGTGTAACTGTCGAAGGTCTGC

AAAACAGGACAGAGACACACAGAGCTGGCGGGGGTGAGAAGAGAGATGATGGTACGGGGGAAGAAGAAAAC

GAATCGAAGAAAGGGAAGCGGCAGAAGCTCTCGGGGTGAGGGAGGCACGTGAGGCCTTCAAACCGGACAGA

AAAAGTGAGAGAGGGTGACGAGGGAATCTGAAGTATTTAAAAGACCGGGCAACGAGAATGTCGAGGTCAAA

CAAAGAAGTTGTCCAAGTCCAATCTGTCTTTGCGTC
```

Figure 9: Nucleotide sequence of TgAPE. TgAPE gene was cloned and the sequence was verified. The 5' and 3'UTRs (grey) were determined by rapid amplification of cDNA ends (RACE). Full length *Toxoplasma* mRNA was reverse transcribed and cDNA was used in RACE reactions to amplify the TgAPE untranslated regions. The 5' UTR is 273 nt and 3'UTR is 320 nt.

ATGACGGCTGCAGCGTCCCTAAGAAAAACCAAGGGTGCTGACGCTGCTGCGTCGCAGGGTCGGGCCGGGCa
GCAGCCCCGCGGCGTAAAGCGAGATCAGGCAGATTCCCCAGGTGAACTGCCAGCGACTGAGGAGACTACAA
GTCCGATTTCTAGCGTTTCTCGGGGCGTCTCCGGGTCTGGAAAACCAGTCGTGTCGAAACGGATCAAAACA
GAGCCTGACACCCAGACGACCAGTGCCCATTAGACACAAAAGCGGATGGGACAGCGGACGCGAAGCACTCG
AGTGTCAAGAAAcCGGCGATGAAATCGGAGGCACATGAGGGCAACCATAATGGCACAGAGGGCGAAATGAA
GCAGAAGGTGAATGCGAAGGGGGCGAAAGTTAAGGGTGAGACCGGTGATGAGGCAGACGACACGCAAGAGG
GAGAAGCCAAGAATGGAGGCAAGGCACCGAAGCGATCAGCTGGCAAGAAACAATCAACTGCAGGAAACTTG
CCGACGGAGGTCGACGAAGTTTTTCTCAGGCATCGAGCTGTGGCTGAGAAGAGCCGGAAGTTCCTCGGcGC
TCATATATCAGCTGCTGGAGGTGTTCAGAACGCTCCAGTGAACTGCCTGGCCATCGGAGGACAAGCTTTCG
CTTTCTTTTTGAAGAATCAGCGGCGCTGGGACTCACCGCCGATAAGCGACGAATCAGCAGATGGCTTCAAA
GCCGAGGTGGCGAAGCTGAAGCTCGATGGCCCCGAGCATATCTTGCCTCACGGCTCCTACCTGATCAACTT
AGCGAACCCAGACGCGGCGAAACGCAAGGTGTCCTACAACGCGTTTCTTGACGACCTGCAGAGGTGCGAGC
AGATCGGCGTTCACCGATACGTCTTTCACCCAGGTTCGACGGTTGGCCAGTGCACaAAGGAAGAAAGCATC
AGACATATTGCAGAATGTCTCAATAAGGCAATAGCGGCAACCAAGAGCGTCACGATTCTACTCGAAAACAT
GGCGGGACAGAAGAACGTGCTCTGCAGCGAGTTCGAGGATCTCCGAGATATAATTGCTCTCGTGGACAGGA
AGGACCGCATCGGTGTCTGCCTGGATACGTGCCATCTGTATTCTGCCGGTCATGACATCAAAACGGAAGAA
AAATTTGAAGCGGTGATGAAAAAGTTCGACAGCATCGTTGGCATGAAGTATCTGAAGGCGATGCATATCAA
CGATTCTAAGGCTCCTCTAGGAAGCGGCCTGGACAGACACGAGCACCTCGGAAAGGGCACTCTCGGAATGG
CCCCGTTCAAGTTCATTATGCAGCACCCGACGTGGTTCAAGGACATGCCCCTCGTCCTAGAGACGCCCGAT
GTCGACAACAGTGGTCCAGCCATGTGGCGCAAAGAAACGGAGATGATGTACAAATTCATCGAAGAGTGA

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GTGAGAGTGCGGTGCGAATTTCCGGTCTCCGTCGGGTGAAGAAAATACACTTCTTGACTGGCAGATGGTGC

GAAACACATGGGCACACCACTAGTGATGTTTGCGTCAATTTCCGTTTGCACTCGAATGCCGTATTCAAAAC

GCTTCTGATGTTGGCGTAGTGTGAATATTTCAGTCAGAATCAGTGTTGTACGAACTACCTCCGCAACGGCT

TTCATCAAGAAACTGC
```

Figure 10: Nucleotide sequence of TgAPN. TgAPN gene was cloned and the sequence was verified. The 5' and 3'UTRs (grey) were determined by rapid amplification of cDNA ends (RACE). Full length *Toxoplasma* mRNA was reverse transcribed and cDNA was used in RACE reactions to amplify the TgAPN untranslated regions. The 5' UTR is 254 nt and 3'UTR is 424 nt.

BLASTp searches with the deduced amino acid sequence for TgAPE reveal most significant matches to other apicomplexan APE1 homologues, followed by plant species (rice, sorghum, and *Arabidopsis*), which are ~35% identical and ~50% similar. In contrast, TgAPE is less similar to hAPE1. TgAPE is comprised of 513 amino acids while hApe1 only has 318 residues; TgAPE contains 10 insertions and one deletion as compared to the human sequence, which may have important structural consequences. A PSI-BLAST alignment of TgAPE to hAPE1 sequences is shown in (Figure 11). Most but not all of the residues thought to play an important role in the repair active site are conserved in TgAPE, including E96, Y171, N174, D210, N212, D282, and H309 (numbering refers to the human sequence). Notably, three residues that are not conserved are W280 and F266, which form the walls of the abasic deoxyribose binding site, and R177, which fills the position of the missing base in the duplex. These residues in TgAPE are M379, S361, and A253, respectively. The fact that some of the residues in the active site are not conserved suggests there may be important structural differences in the repair active site. Furthermore, amino acid sequence comparison of human APE1 against homologues in mouse, *E. coli* and *Toxoplasma* were done showing percent homology. Mouse (*M. musculus*) APE1 showed the most homology with 93% identity followed by *E. coli* (27%) and *Toxoplasma* (39%), respectively (Figure 11B).

A.

Score = 116 bits (290), Expect = 3e-24, Method: Composition-based stats.
Identities = 110/354 (31%), Positives = 160/354 (45%), Gaps = 87/354 (24%)

```
TgAPE   76    ETEEDGEGEL------ERRTEGPKRKAPLSIVTWNVNSIAARIRDS-RQWFYFSRFLQKI    128
              + E  GEG        + +T     + A L I +WNV+ + A I+     W     +++
hAPE1   34    DKEAAGEGPALYEDPPDHKTSPSGKPATLKICSWNVDGLRAWIKKKGLDW------VKEE     87

TgAPE   129   DPDILCLQEVKLAAHGPPGAKRGDGMPRDHGRIKDSDKVSSVEARELREALHTLLP--NH    186
               PDILCLQE K + +  P                              EL+E    LP +H
hAPE1   88    APDILCLQETKCSENKLPA------------------------ELQE-----LPGLSH    116

TgAPE   187   SLLISLADWR-YSGQMMFIRKDVQVCSLRYNLSLDGCPAHEHDFEGRVILAEFEAFCVLT    245
                  + +D   YSG  +  R+   C L+ +    G    EHD EGRVI+AEF++F ++T
hAPE1   117   QYWSAPSDKEGYSGVGLLSRQ----CPLKVSY---GIGDEEHDQEGRVIVAEFDSFVLVT    169

TgAPE   246   TYSPNNGATPKSFERRRLWDERMLQFVTQL--KKPLVWVGDLNCAPEDIDLSDPDQFRSV    303
               Y PN G       E R+ WDE   +F+  L  +KPLV  GDLN A E+IDL +P
hAPE1   170   AYVPNAGRGLVRLEYRQRWDEAFRKFLKGLASRKPLVLCGDLNVAHEEIDLRNPK-----    224

TgAPE   304   IHETADGTIDPDNIGQAGCTDAERRRFRAILERGNLVDAFRELHPRTEPPPLESAEYSWR    363
                             N   AG T  ER+ F  +L+    L D+FR L+P T      A   W
hAPE1   225   -----------GNKKNAGFTPQERQGFGELLQAVPLADSFRHLYPNT-----PYAYTFWT    268

TgAPE   364   GFGGSGSRGLLRGLGMRLDHIVLTETLMPAVELVRICGAGKSKANFFGSDHCPV    417
                + S    + +G RLD+ +L+ +L+PA     +C + K ++   GSDHCP+
hAPE1   269   YMMNARS----KNVGWRLDYFLLSHSLLPA-----LCDS-KIRSKALGSDHCPI    312
```

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B.

Exonuclease III family of AP endonucleases
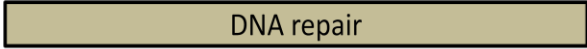
Species	Domains	% Identity
H. sapiens	REF / DNA repair	100%
M. musculus	REF / DNA repair	93%
E. coli	DNA repair	27%
T. gondii	DNA repair	39%
P. falciparum	DNA repair	36%

Figure 11: TgAPE homology. A) PSI-BLAST sequence alignment of TgAPE and hAPE1. B) Diagram showing Exonuclease III family AP endonuclease proteins and domains. Diagram showing the homology of Exonuclease III family AP endonuclease proteins and domains. All proteins were compared for homology to the human *(H. sapien)* APE1.

BLASTp searches with the deduced 472 amino acid sequence of TgAPN reveal that the most significant matches are to other apicomplexan APN1 homologues, followed by fungal species (e.g. *Chaetomium globosum*, *Fusarium graminearum*, *Neurospora crassa*, *Cryptococcus neoformans*) which are ~40-50% identical and ~60-70% similar. Residues 187 to 463 of TgAPN are 43% identical to *E. coli* EndoIV, which includes 285 residues. Residues involved in coordinating the three metal ions in the active site of *E. coli* EndoIV are conserved in TgAPN. These residues include H255, H295, and E331 (numbering for TgAPN), which coordinate Zn_1, E448, H392, and D365, Zn_2, and D415, H417, and H368, Zn_3. In addition, recognition loop residues, R222 and Y258 are also conserved suggesting that mechanistically TgAPN is very similar to *E. coli* EndoIV. In contrast to the APE family enzymes in which the larger TgAPE has a number of sequence inserts throughout the enzyme the contribute to its significantly larger mass, the difference in size between TgAPN and *E. coli* endonuclease IV results primarily from an N-terminal insertion in TgAPN. The amino acid sequence homology of *S. cerevisiae* APN1 was compared to other organisms including *Toxoplasma. C. elegans* and *E. coli* showed 41% identity, while *Toxoplasma* had 47% identity to yeast APN1 (Figure 12).

A.

Score = 236 bits (601), Expect = 2e-60, Method: Composition-based stats.
Identities = 121/277 (43%), Positives = 176/277 (63%), Gaps = 7/277 (2%)

```
TgAPN    187  KFLGAHISAAGGVQNAPVNCLAIGGQAFAFFLKNQRRWDSPPISDESADGFKAEVAKLKL  246
              K++GAH+S AGG+ NA +    I   AFA F KNQR+W + P++ ++ D FKA   K
EndoIV   2    KYIGAHVSRAGGLANAAIRAAEIDATAFALFTKNQRQWRAAPLTTQTIDEFKAACEKYHY  61

TgAPN    247  DGPEHILPHGSYLINLANPDAAKRKVSYNAFLDDLQRCEQIGVHRYVFHPGSTVGQCTKE  306
                 + ILPH SYLINL +P     + S +AF+D++QRCEQ+G+    FHPGS + Q ++E
EndoIV   62   TSAQ-ILPHDSYLINLGHPVTEALEKSRDAFIDEMQRCEQLGLSLLNFHPGSHLMQISEE  120

TgAPN    307  ESIRHIAECLNKAIAATKSVTILLENMAGQKNVLCSEFEDLRDIIALVDRKDRIGVCLDT  366
              + +   IAE +N A+  T+ VT ++EN AGQ + L  +FE L  II  V+ K R+GVC+DT
EndoIV   121  DCLARIAESINIALDKTQGVTAVIENTAGQGSNLGFKFEHLAAIIDGVEDKSRVGVCIDT  180

TgAPN    367  CHLYSAGHDIKTEEKFEAVMKKFDSIVGMKYLKAMHINDSKAPLGSGLDRHEHLGKGTLG  426
              CH ++AG+D++T + E     F  VG KYL+ MH+ND+K+  GS +DRH  LG+G +G
EndoIV   181  CHAFAAGYDLRTPAECEKTFADFARTVGFKYLRGMHLNDAKSTFGSRVDRHHSLGEGNIG  240

TgAPN    427  MAPFKFIMQHPTWFKDMPLVLETPDVDNSGPAMWRKE    463
                F++IMQ    F  +PL+LET +     P +W +E
EndoIV   241  HDAFRWIMQDDR-FDGIPLILETIN-----PDIWAEE    271
```

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Endonuclease IV family of AP endonucleases

Species	Domains	% Identity
S. cerevisiea	DNA repair	100%
C. elegans	DNA repair	48%
E. coli	DNA repair	41%
T. gondii	DNA repair	41%
P. falciparum	DNA repair	40%

Figure 12: TgAPN homology. A) PSI-BLAST sequence alignment of TgAPN and yeast *Saccharomyces cerevisiae* APN1 (Endo IV). B) Diagram showing the homology of Exonuclease III family AP endonuclease proteins and domains. All proteins were compared for homology to the *S. cerevisiae* APN1.

Previous studies and comparative genomics reveal additional insight into the AP endonucleases in Apicomplexa. Biochemical evidence suggested that *Plasmodium falciparum* (malaria) contains both Mg^{2+}-dependent (APE1) and Mg^{2+}-independent (APN1) AP endonuclease activities (Haltiwanger et al., 2000a); we have verified that the *Plasmodium* genome sequence harbors genes for both APE1 (chr3.glimmerm_170) and APN1 (chr13.genefinder_182r) homologues. Survey of the *Cryptosporidium* genome also reveals both APE1 (CpIOWA_EAK87392) and APN1 (CpIOWA_EAK88592) homologues. Phylogenetic analysis shows that apicomplexan APN1 homologues form a clade with yeast APN1, while apicomplexan APE1 homologues are more closely related to those found in plants rather than mammals (Figure 13).
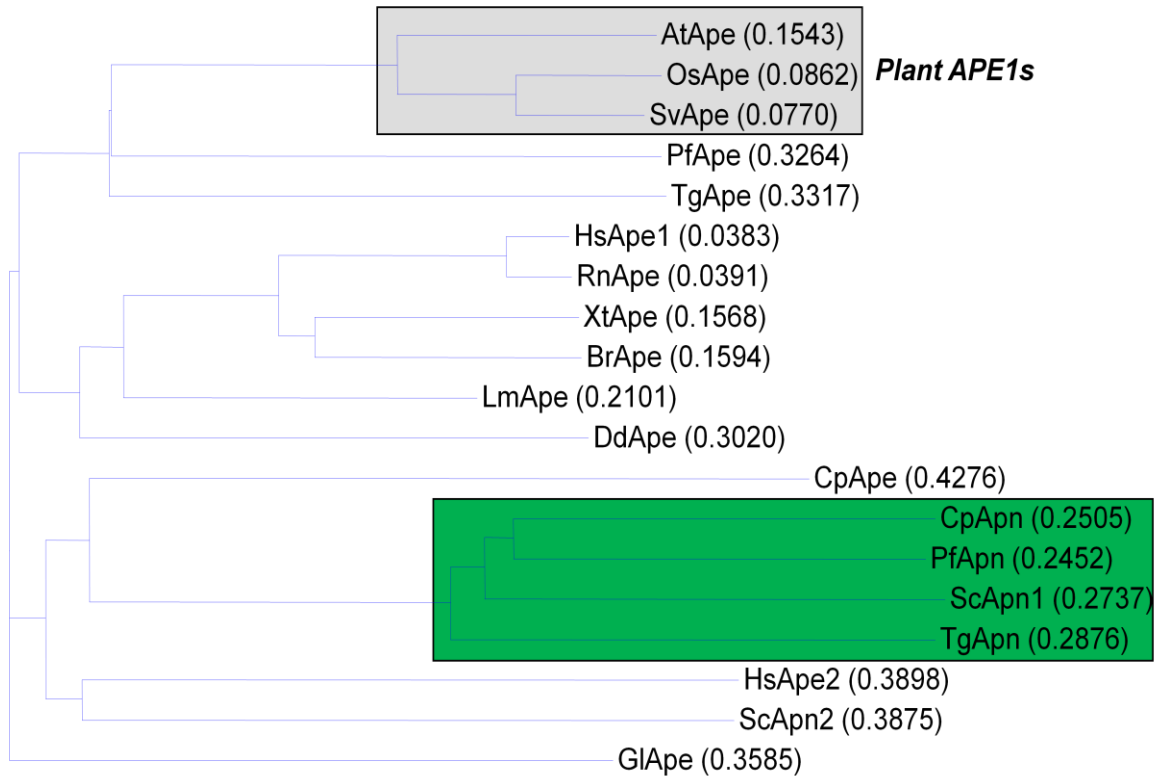
Figure 13: Phylogenetic analysis of APE1 and APN1 homologues. Dendogram was composed using AlignX module of Vector NTI Advance 9.0 software (Informax). Gray box indicates plant APE1 homologues; light green box indicates apicomplexan APN1 homologues. *At=Arabidopsis thaliana; B=Brachydanio rerio (zebrafish); Cp=Cryptosporidium parvum; Dd=Dictyostelium discoideum (slime mold); Gl=Giardia lamblia; Hs=Homo sapiens; Lm=Listeria monocytogenes; Pf=Plasmodium falciparum (malaria); Os=Oryza sativa (rice); Rn=Rattus norvegicus; Sc=Saccharomyces cerevisiae; Sv=Sorghum vulgare; Tg=Toxoplasma gondii; Xt=Xenopus laevis.*

B. Recombinant TgAPE and TgAPN

TgAPE and TgAPN were cloned and expressed in *E. coli*. The recombinant proteins were purified and analyzed on an SDS-PAGE gel and determined to be the sizes predicted on http://www.toxodb.com. TgAPE recombinant protein was observed as a doublet between 54- 57 kDa, while TgAPN was observed at 51 kDa (Figure 14).
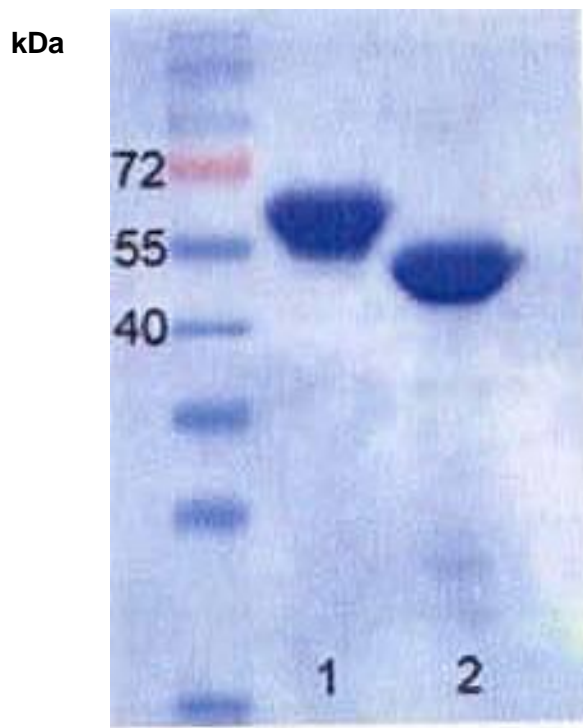
kDa

Figure 14: Purified recombinant TgAPE and TgAPN SDS-PAGE analysis. TgAPE (lane 1) and TgAPN (lane 2) expressed in Rosetta *E. coli* and purified using nickel affinity chromatography. (Millie M. Georgiadis, unpublished data).

C. TgAPE and TgAPN AP DNA repair activities

We expressed recombinant TgAPE and TgAPN in *E. coli* and purified the proteins for enzymatic analysis. The DNA repair assay utilizes a 5'-hexachloro-fluorescein phosphoramidite (HEX)-labeled terahydrofuranyl (THF) oligo (the 26 bp oligonucleotide substrate contains a single THF residue in the middle, yielding a HEX-labeled 13mer fragment upon repair). THF is a stable synthetic abasic site. The reaction products are visualized after resolving on 20% polyacrylamide gels. As predicted from their structural similarities to hAPE1 and yeast APN1, the TgAPE enzyme should require Mg^{+2} while TgAPN should be Mg^{+2}-independent. As shown in Figure 15, TgAPE is capable of cleaving the THF oligo as evidenced by the accumulation of the lower 13 bp product with increasing concentrations of enzyme and is only active in the Mg^{+2}-containing buffer. TgAPN also displays AP endonuclease activity and is active in the presence or absence of Mg^{+2} (Figure 15).

We measured total AP endonuclease activity levels in wild-type *Toxoplasma* lysate using the AP endonuclease gel assay (Figure 15). The percent activity was expressed as a percent of the value of the cleaved oligo to the total value of the cleaved and uncleaved oligo. TgAPN (Mg^{2+}-independent) activity levels were measured by using Mg^{2+}-free buffer containing EDTA. In both cases, a dose-dependent increase of activity was seen with increasing amounts of parasite lysate present in the reaction. The results demonstrate that TgAPN contributes substantially to the total amount of AP endonuclease activity detected in parasite lysate.
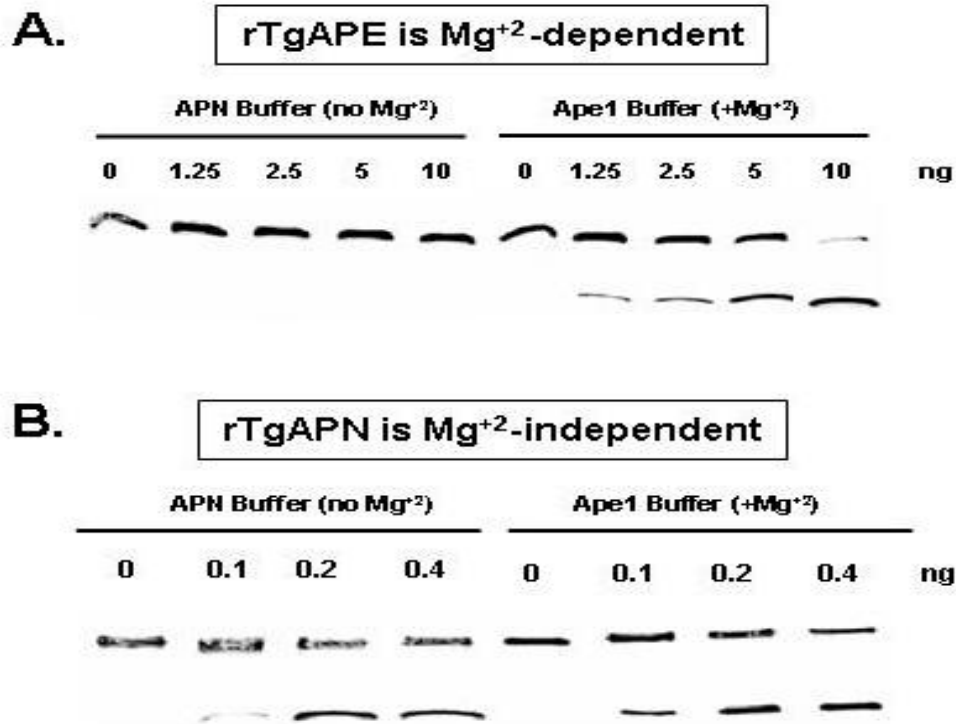
Figure 15: AP endonuclease assay. A) Increasing amounts of rTgAPE were incubated in buffer A (50mM HEPES, 50mM KCl, 10mM $MgCl_2$,1 μg/ml BSA, 0.05%TritonX-100, pH 7.5). B) Increasing amounts of rTgAPN were incubated in buffer B (50mM HEPES, 50mM KCl, 20mM EDTA,1 μg/ml BSA, 0.05%TritonX-100, pH 7.5). All reactions contained 1mM DTT and 0.05 pmol HEX-TEF oligonucleotide in a total of 10 μl and were incubated at 37ºC for 15 minutes. AP endonuclease assays were performed in the laboratory of Dr. Mark Kelley.
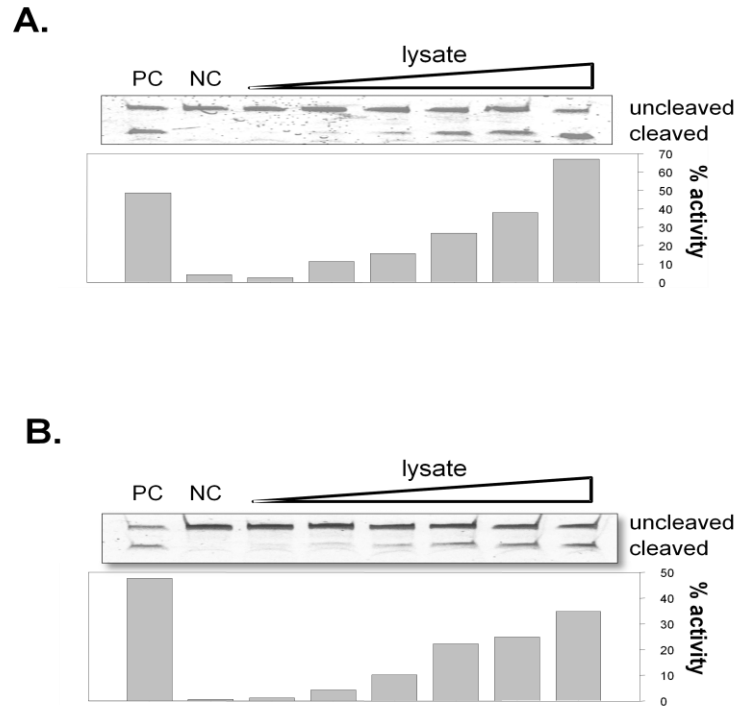
Figure 16: AP endonuclease activities in *Toxoplasma* lysate. A) Total AP endonuclease activity in wild-type *Toxoplasma* lysate, performed in presence of 1.0 mM MgCl$_2$. Lane 1, 10 nM recombinant human APE1 (PC, positive control); lane 2, DNA alone (NC, negative control); lanes 3-8, increasing amounts of *Toxoplasma* lysate (100, 250, 500, 1000, 1500 and 3000 ng, respectively). The columns on the graphs below each gel correspond to the percent activity of the sample directly above and are representative of the average percent activity of two independent assays. B) APN activity levels in parasite lysate, as determined by degree of cleaved oligonucleotide. AP endonuclease assays were performed in the presence of 10 mM EDTA to chelate Mg^{2+}. Lane 1, 10 nM recombinant TgAPN (PC, positive control); lane 2, DNA alone (NC, negative control); lanes 3-8, increasing amounts of *Toxoplasma* lysate (100, 250, 500, 1000, 1500 and 3000 ng, respectively).

Further steady state enzymatic characterizations of TgAPN and TgAPE were performed in the lab of Dr. Millie Georgiadis using a fluorescent-based solution assay as described in the Materials and Methods (Section III). The THF-containing oligonucleotide substrate concentration was varied from 0 to 75 nM, and Michaelis-Menton K_m values of 26.2 and 21.1 nM and V_{max} values of 1458 and 110,070 RFU/μg/s, respectively for TgAPN vs. TgAPE, were calculated (Figure 17), where RFU is a relative fluorescent unit. Thus, while the relative binding affinities of each enzyme for the substrate are similar, the turnover rate is approximately 75 times faster for TgAPE.
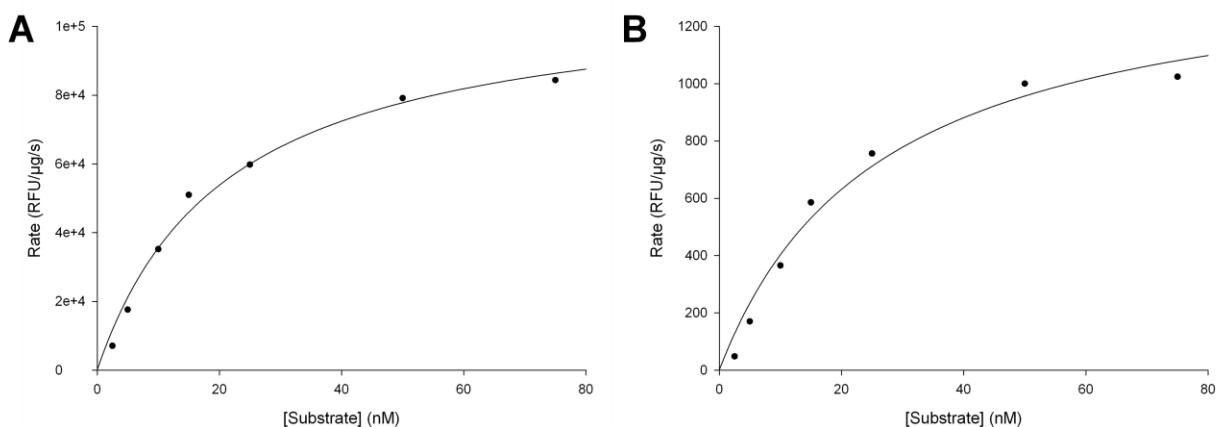
Figure 17: TgAPE and TgAPN AP endonuclease assays. A fluorescent-based solution assay was used to characterize the steady state kinetic properties of TgAPE (A) and TgAPN (B). Using optimized concentrations of TgAPE (0.05 nM) and TgAPN (4 nM) and substrate concentrations from 0 to 75 nM, K_m values of 21.1 and 26.2 nM, respectively, were determined. V_{max} values for the two enzymes differ by a factor of 75, with TgAPE exhibiting the more rapid rate of endonuclease activity.

D. Size of *Toxoplasma* AP endonucleases

Polyclonal antisera were generated in rabbits using the recombinant

TgAPE and TgAPN purified from bacteria as antigen. Polyhistidine tags were

removed by proteolytic cleavage prior to immunization. Antibodies were affinity-

purified and used to probe Western blots containing lysates from *Toxoplasma*

tachyzoites or the recombinant protein as control (Figure 18). Recombinant

TgAPE migrates at the expected MW of 57 kD, but also appears at 52 kD,

possibly a breakdown or proteolytic product. Native TgAPE expressed in

Toxoplasma also migrates as two proteins as seen when produced in *E. coli*,

making it likely that this is protein processing rather than an alternatively spliced

product. Native TgAPN migrates slightly higher than the expected 51 kD in

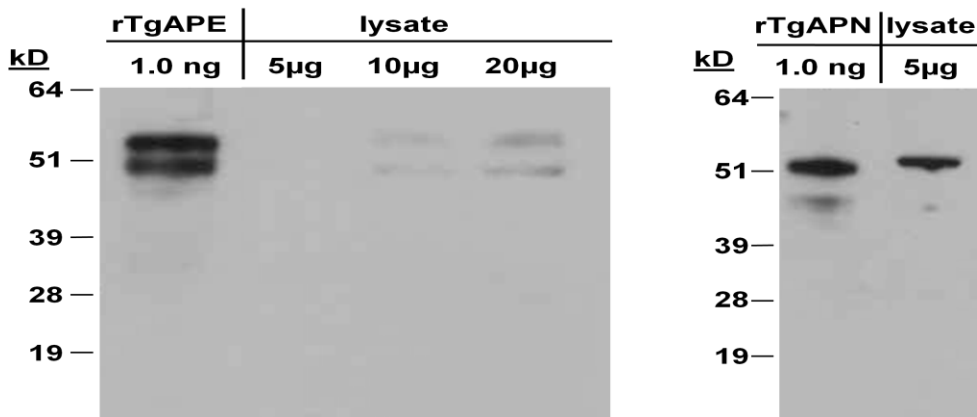*Toxoplasma* lysate, possibly due to a post-translational modification(s).

Figure 18: Size of TgAPE and TgAPN. Western analysis with affinity-purified anti-

TgAPE or anti-TgAPN, each used at a 1:10,000 dilution. The designated amount

of *Toxoplasma* protein lysate is shown. Recombinant (r) protein expressed and

purified from *E. coli* was run as control.

E. Localization of *Toxoplasma* AP endonucleases

TgAPE and TgAPN antibodies were used in immunofluorescence assays (IFA) to localize the native protein in *Toxoplasma* tachyzoites growing inside their host cells. Native TgAPE is present in the parasite nucleus and cytoplasm while native TgAPN is nuclear (Figure 19). Arrowheads point to potential TgAPE present in the apicoplast, a specialized organelle housing a 35 kb extrachromosomal element. We did not detect TgAPN in organelles housing extrachromosomal DNA, such as the apicoplast or mitochondria.
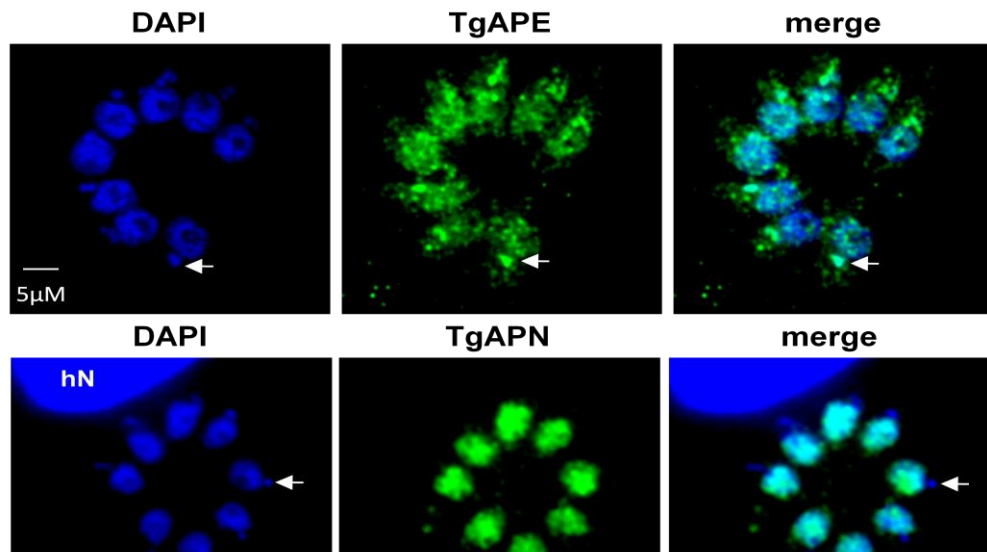

Figure 19: Localization of *Toxoplasma* AP endonucleases. IFA of intracellular parasites probed with 1:1,000 dilution of anti-TgAPE (top panels, green) or 1:10,000 dilution anti-TgAPN (lower panels, green). 4',6-diamidino-2-phenylindole (DAPI) was used as a co-stain to highlight the nuclear compartment (blue). hN=host cell nucleus. Arrowheads point to parasite apicoplast organelle.

F. TgAPN is more abundant than TgAPE in tachyzoites

In other species possessing more than one AP endonuclease, one tends to be the predominant enzyme that is critical for BER [59]. We therefore sought to establish which AP endonuclease was predominant in *Toxoplasma* tachyzoites. An analysis of the expressed sequence tags (ESTs) data at the ToxoDB Release 6.0 (http://www.toxodb.org) shows that only 6 ESTs represent TgAPE whereas >65 ESTs were identified for TgAPN, indicating that TgAPE message levels are very low compared to those encoding TgAPN (Figure 20). Real time PCR analysis also shows more TgAPN message than TgAPE.
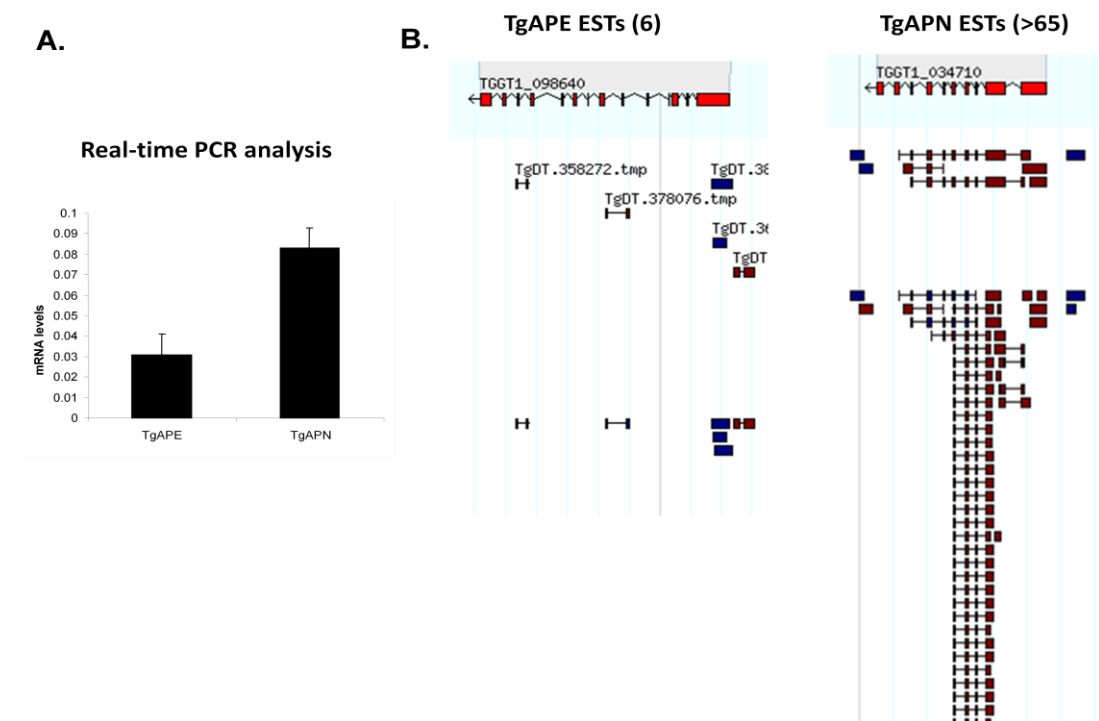

Figure 20: Levels of TgAPE and TgAPN mRNA. A) Real time PCR data showing levels of TgAPE and TgAPN mRNA. The values represent the inverse Ct numbers of the respective message. B) Partial screen shot of the EST data for TgAPE and TgAPN from http://www.toxodb.com.

Analysis at the protein level also supports that TgAPN is the more prevalent AP endonuclease. Proteomics data at the ToxoDB mirrors what we observed for ESTs in that a large number of peptides in various independent datasets (e.g. generated by Wastling, Hu, or Einstein labs) suggest TgAPN is present in abundance at the protein level; in contrast, only 5 peptides have been reported matching to TgAPE (Figure 21A). Immunoblots of tachyzoite lysate with antibodies to TgAPE and TgAPN further support that TgAPN is the more abundant protein (Figure 21B). Considered together, these multiple lines of evidence indicate that TgAPN is more abundant than TgAPE at both the RNA and protein levels in tachyzoites.
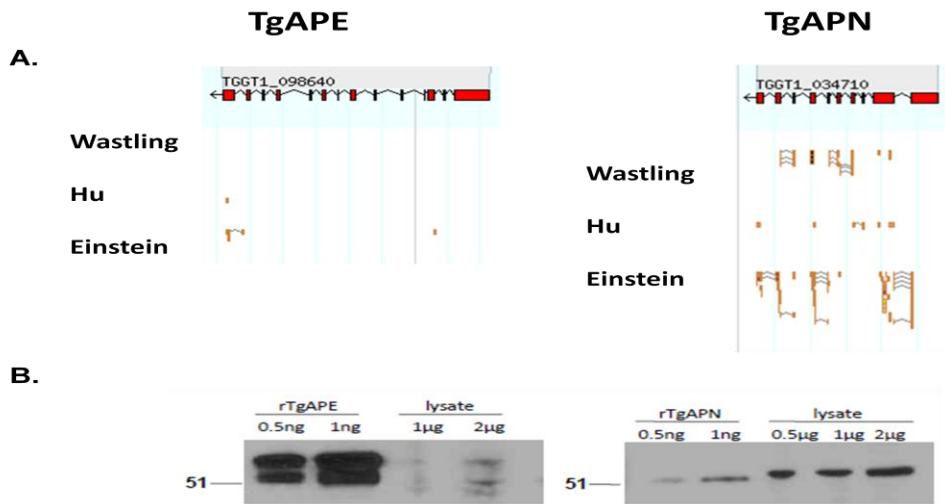

Figure 21: Levels of TgAPE and TgAPN protein. A) A partial screenshot of proteomics data available from http://www.toxodb.com for TgAPE and TgAPN. B) Western analysis with affinity-purified anti-TgAPE or anti-TgAPN, each used at a 1:10,000 dilution. The designated amount of *Toxoplasma* protein lysate is shown. Recombinant (r) protein expressed and purified from *E. coli* was run as control.

G. Over-expression of TgAPE and TgAPN

To determine if over-expression of TgAPE or TgAPN confers protection against DNA damage, we expressed recombinant FLAG-tagged versions of TgAPE or TgAPN (tagged at N-terminus) in RH strain tachyzoites (FLAGAPE or FLAGAPN). Expression of each ectopic copy was driven by the same strong tubulin promoter as previously described [90]. Western blot analysis showed that both selected transgenic clones were expressing more TgAPE or TgAPN protein than wild-type parasites. Immunoblots also show that there is more FLAGAPE compared to FLAGAPN in the over-expressing lines (Figure 22). Also note that only the full-length TgAPE appeared on the immunoblot probed with anti-FLAG, suggesting that the shorter form of TgAPE is missing a portion of its N-terminal region.
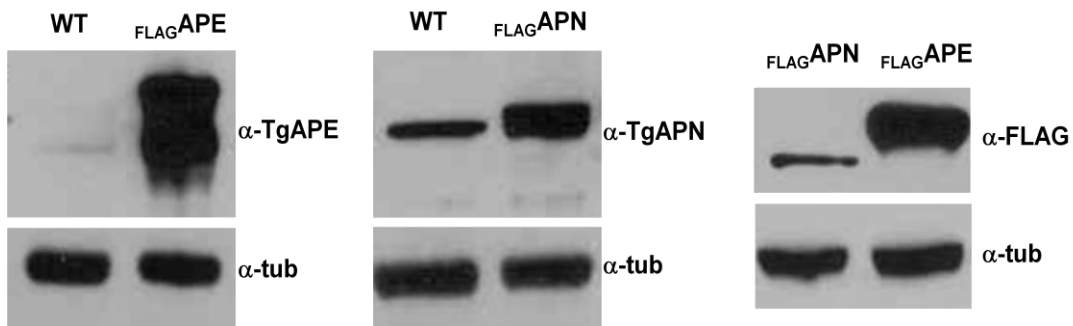
Figure 22: Expression levels of ectopic FLAG-tagged AP endonucleases. Ten µg of parasite lysate was loaded for immunoblotting with anti-TgAPE (1:10,000), anti-TgAPN (1:10,000), or anti-FLAG (1:5,000). Anti-tubulin (1:5,000) was used to check protein loading.

100

We also performed IFAs to determine the subcellular localization of each tagged *Toxoplasma* AP endonuclease (Figure 23). When anti-APE or anti-APN antibodies are used in IFAs of the parasites over-expressing FLAG-tagged TgAPE or TgAPN, the staining pattern is identical to that seen for wild-type parasites. Moreover, anti-FLAG IFAs of parasites expressing FLAGAPN confirm nuclear localization of this protein. In contrast to IFAs with anti-APE, anti-FLAG IFAs of parasites expressing FLAGAPE show almost exclusive localization to the nucleus. This suggests that the cytosolic staining for native TgAPE is due to the fact that anti-APE recognizes both full-length and truncated forms. In other words, full-length TgAPE is nuclear, but the shorter TgAPE (which anti-FLAG would not recognize) is cytoplasmic (Figure 23 B).

A.
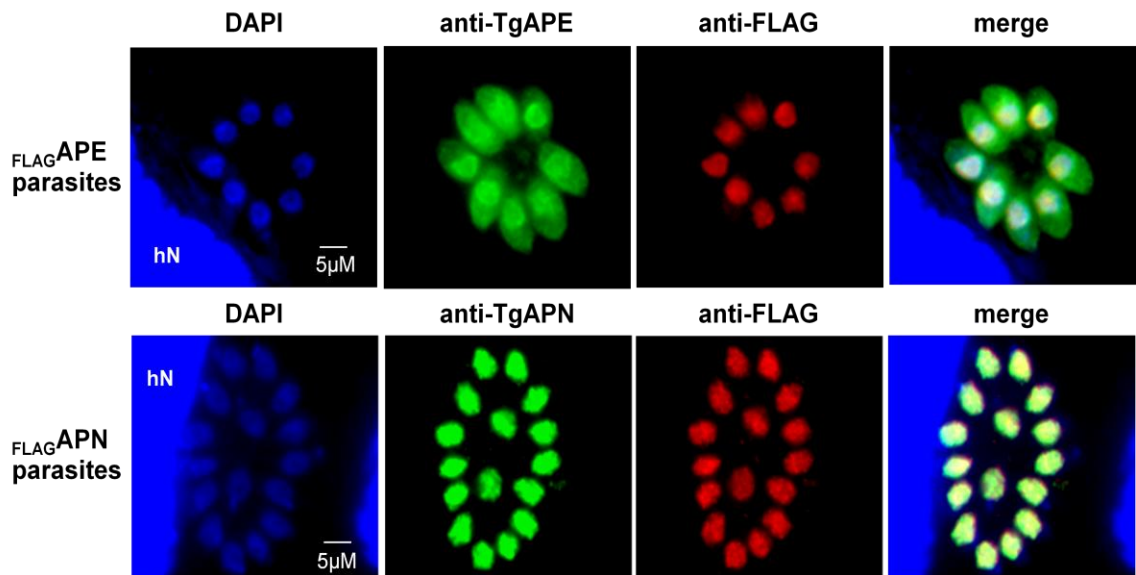
B.
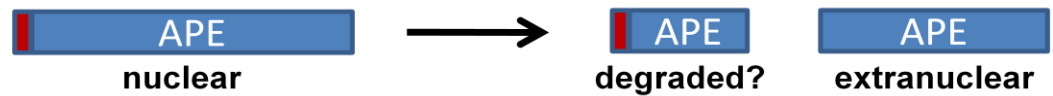
Figure 23: Localization of FLAG-tagged AP endonucleases. A) IFA was performed on intracellular parasites expressing FLAGAPE (top panels) or FLAGAPN (bottom panels). Parasites were probed with anti-FLAG (1:5,000, red) and either anti-TgAPE (1:1,000, green) or anti-TgAPN (1:10,000, green). DAPI was used as a co-stain to highlight the parasite nucleus (blue). B) Diagram depicting TgAPE cleavage products and possible localization in *Toxoplasma* tachyzoites.

H. Parasites over-expressing TgAPN, but not TgAPE, are protected from DNA damage mediated by MMS

We examined if our transgenic parasites over-expressing recombinant forms of each AP endonuclease under control of the same heterologous promoter (tubulin) exhibited cytoprotection against the alkylating agent methyl methanesulfonate (MMS), a well-established DNA-damaging agent used to study BER. Using three independent growth assays, we found that *Toxoplasma* tachyzoites exposed to MMS have increased viability only when TgAPN is stably over-expressed; no protection is conferred when TgAPE is over-expressed (Figure 24). Figure 24A is a PCR-based assay for the parasite-specific B1 sequence that monitors growth over 5 days following exposure to an insult of 400 µM MMS for 2 hrs. A plaque assay performed at day 7 following 400 or 800 µM MMS produced similar results (Figure 24B). Finally, a *Toxoplasma* doubling assay performed on parasites over a time course following exposure to 800 µM MMS verified that those over-expressing TgAPN were remarkably refractory to this insult (Figure 24C). It should be noted that the growth rates of each over-expressing line are virtually identical to each other as well as wild-type under vehicle-treated conditions.
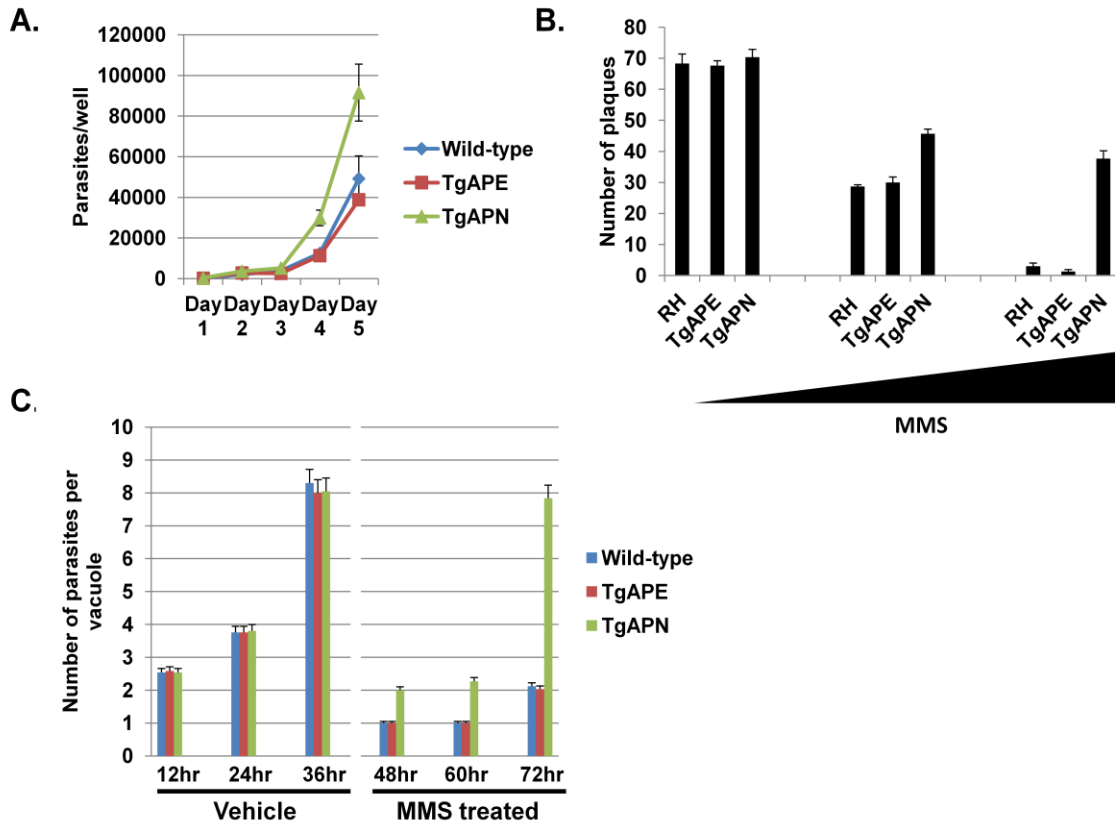
Figure 24: DNA damage recovery assays. Extracellular parasites (wild-type, WT), $_{FLAG}$APE and $_{FLAG}$APN) were subjected to DNA damage induced by MMS or vehicle control (DMSO) for 2 hr and then allowed to infect fresh HFF monolayers under normal culture conditions. A) Parasite growth was monitored by PCR-based B1 assay following exposure to 400 µM MMS. B) Parasite growth was measured using a plaque assay in either 400 or 800 µM MMS. C) Parasite doubling assays were performed following exposure to 800 µM MMS. Error bars represent standard deviation. Significance determined by t-test (P<0.05).

I. TgAPN conditional knockout

To definitively demonstrate that TgAPN is vital for *Toxoplasma* DNA repair, we designed experiments to ablate or diminish TgAPN levels in the parasite. We attempted to knockout the TgAPN locus in type I RH strain parasites using conventional homologous recombination. No viable clones were obtained, possibly because the TgAPN gene is essential. To test this idea, we created a conditional knockout for TgAPN in the TATi parasite background, which expresses a transactivator protein that can be regulated by anhydrotetracycline (ATc) [78]. Ectopic $_{HA}$-APN$_{FLAG}$ driven by the ATc-responsive SAG1 promoter was stably expressed in TATi parasites (Figure 25 and 26).
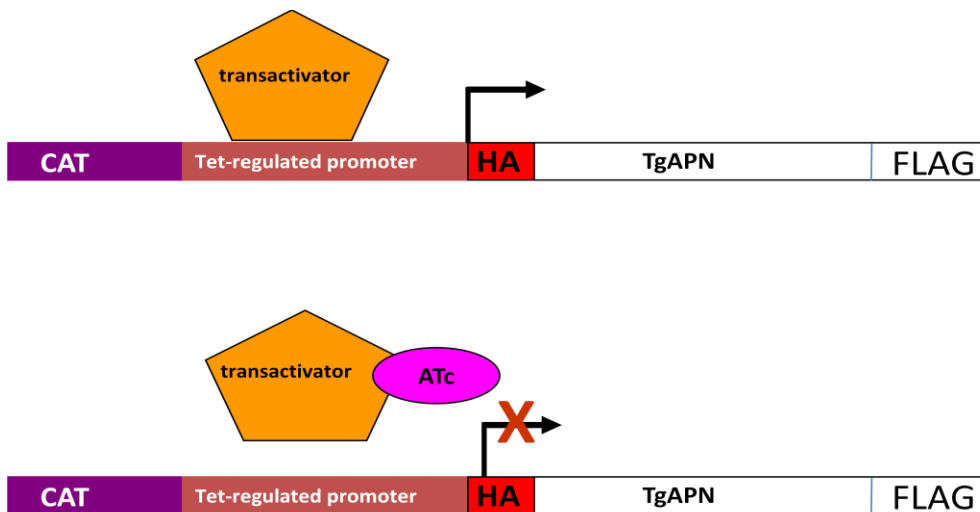
Figure 25: Diagram representing the tet-regulatable TgAPN ectopic gene. The tet-transactivator binds to the tet-regulatable promoter and the TgAPN is expressed. When anhydrous tetracycline (ATc) is added, it binds the tet-transactivator, preventing association with the tet-regulated promoter and repressing expression of TgAPN. CAT=chloramphenical acetyltransferase. HA and FLAG are tags used to detect ectopic TgAPN.
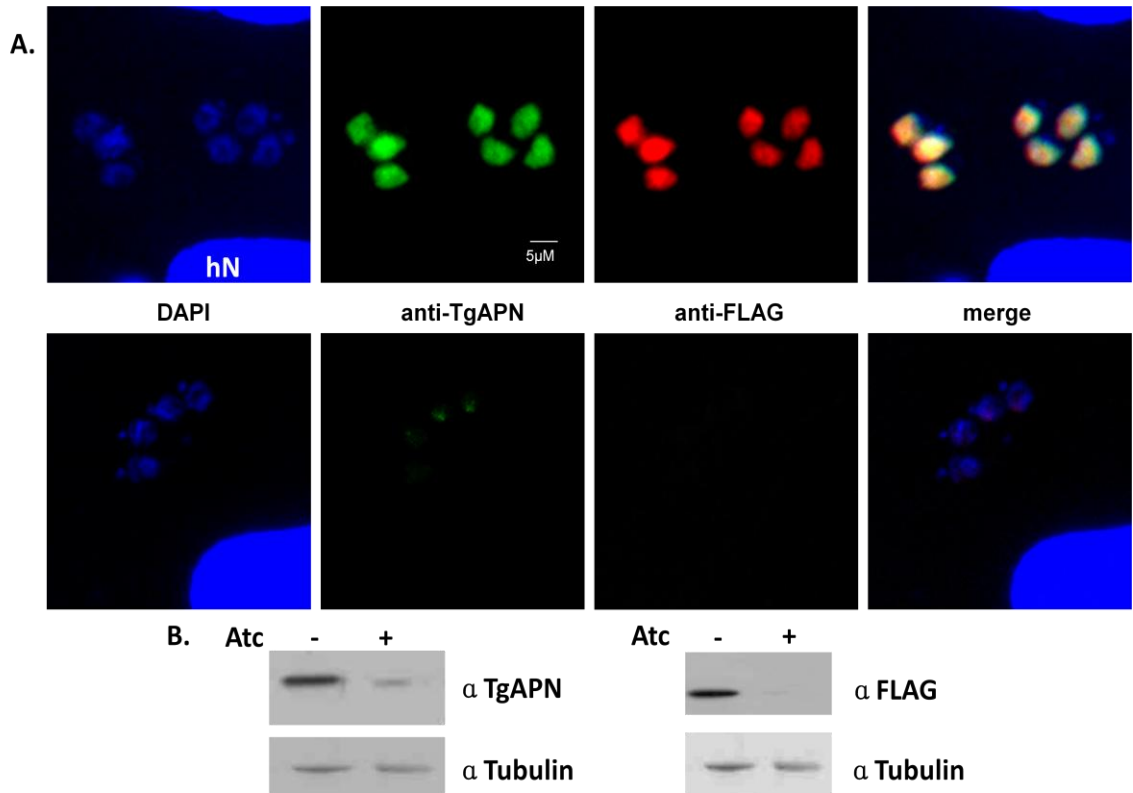
Figure 26: Expression of ectopic TgAPN is regulated by ATc. A) IFAs showing

regulatable TgAPN in the presence or absence of 1 μg/ml ATc. B and C)

Western blots of parasite lysates in the presence or absence of ATc. TgAPN

antibody=1:10,000 (green), FLAG antibody=1:5,000 (red), tubulin antibody=

1:5,000 DAPI was used as a nuclear co-stain (blue).

Next, the entire endogenous locus encoding TgAPN was eliminated through homologous recombination in the tet-regulatable clone shown in Figure 27. 3.5 kb of DNA was amplified from the flanking regions of the TgAPN genomic locus. These pieces were ligated to a vector conferring pyrimethamine resistance and transfected into the parasites. Over 130 pyrimethamine-resistant clones were screened and a single knockout was obtained and verified by PCR as well as Western blotting (Figure 27). Two sets of intron spanning primers (ISP1 and ISP2, which amplify 400 bp and 600 bp fragments, respectively) were used to screen the clones for the conditional knockout by PCR (Figure 27A and B). The knockout could be detected by the absence of an amplicon when using the intron spanning primers. TgAPN 5' UTR primers, which amplify a 400 bp fragment, were used as a positive control to ensure the presence of quality DNA template. Western blot analysis using anti-TgAPN confirmed the knockout, showing loss of the native protein band in the knockout parasite (Figure 27C). The wild-type TgAPN protein was detected as a single band at the expected size. The TgAPN+ ectopic TgAPN clone exhibits two bands with the higher MW band being the ectopic copy and the lower MW band being the native protein. In the conditional knockout, only the ectopic regulatable TgAPN is detected.
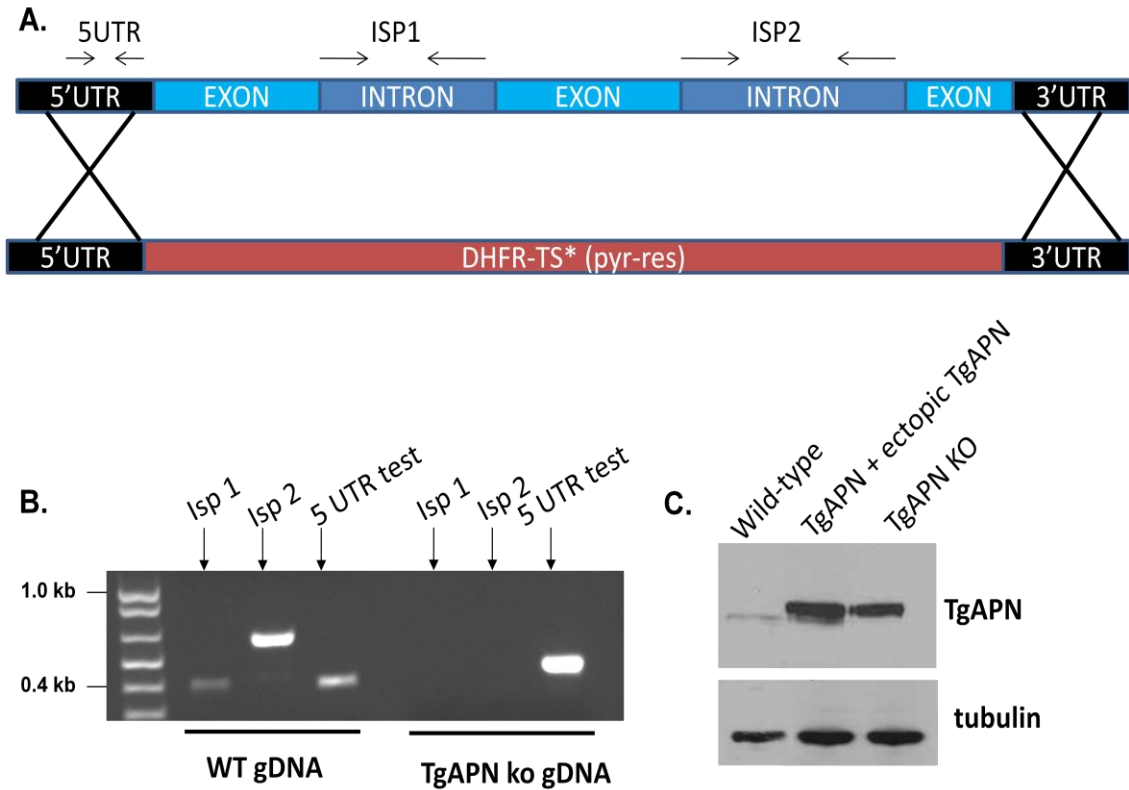
Figure 27: Conditional knockout of TgAPN. A) Diagram of knockout vector (red) and homologous recombination with the TgAPN genomic locus (blue). Intron spanning primers (ISP). B) PCR screening of TgAPN conditional KO using intron spanning primers. The 5'UTR primer set was used as a positive control to confirm the quality of the DNA. C) Western blot confirmation of TgAPE conditional KO. 10 µg of protein was used for each sample. Antibody concentration used was: TgAPN antibody 1:10,000. Tubulin antibody 1:5,000.

Unfortunately, addition of 1.0 µg/ml ATc for 48 hrs did not significantly reduce the amount of ectopic TgAPN in the conditional knockout clone below the level of wild-type parasites, as determined by relative real-time RT-PCR as well as Western blot. Both real-time PCR and Western blotting show a significant reduction of ectopic TgAPN, but not less than wild-type parasites (Figure 28). The problem appears to be that the ectopic TgAPN is expressed at dramatically higher levels than endogenous TgAPN, making it difficult to down-regulate protein to levels below normal. Similar problems using the tet-based SAG promoters have been reported [91].
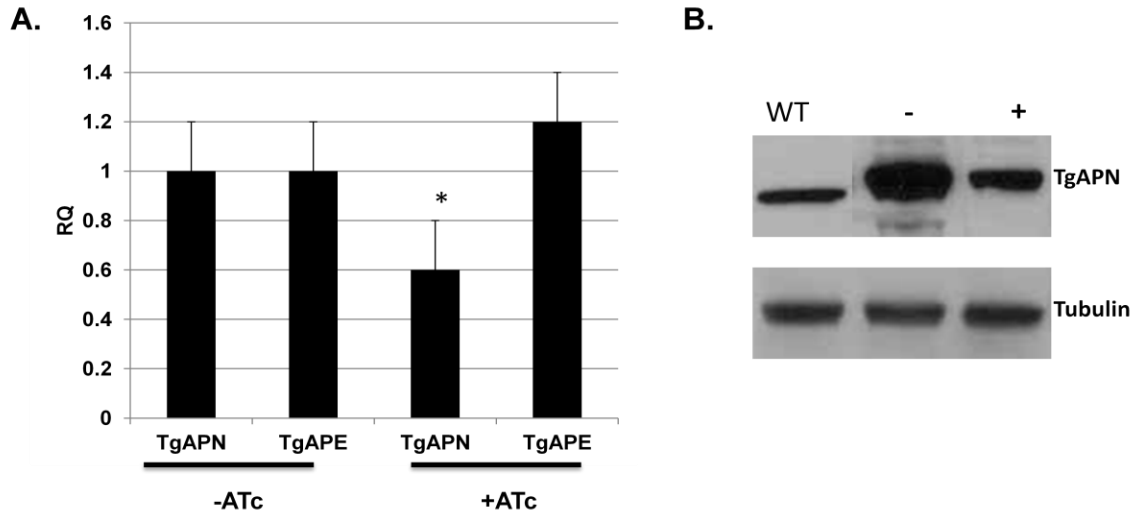
Figure 28: Down-regulation of ectopic TgAPN in the conditional knockout. A)

Relative real-time RT-PCR of conditional KO in the presence or absence of 1

µg/ml ATc. Tubulin was used to normalize the samples. RQ values represent

relative quantification to tubulin. Error bars represent standard deviation. The

asterisk denotes statistical significance determined by t-test. P< 0.05.B) Western

blot of WT and conditional KO in the presence or absence of 1 µg/ml ATc. 10 µg

of protein lysate was used for each sample. TgAPN antibody was used at

1:10,000. Tubulin antibody was used at 1:5,000.

Higher levels of anhydrous tetracycline did not yield greater down-regulation of TgAPN compared to 1.0 µg/ml. Toxicity studies showed that exceeding 1.4 µg/ml of ATc began to exert toxicity on the TATi parental parasite strain (Figure 29).
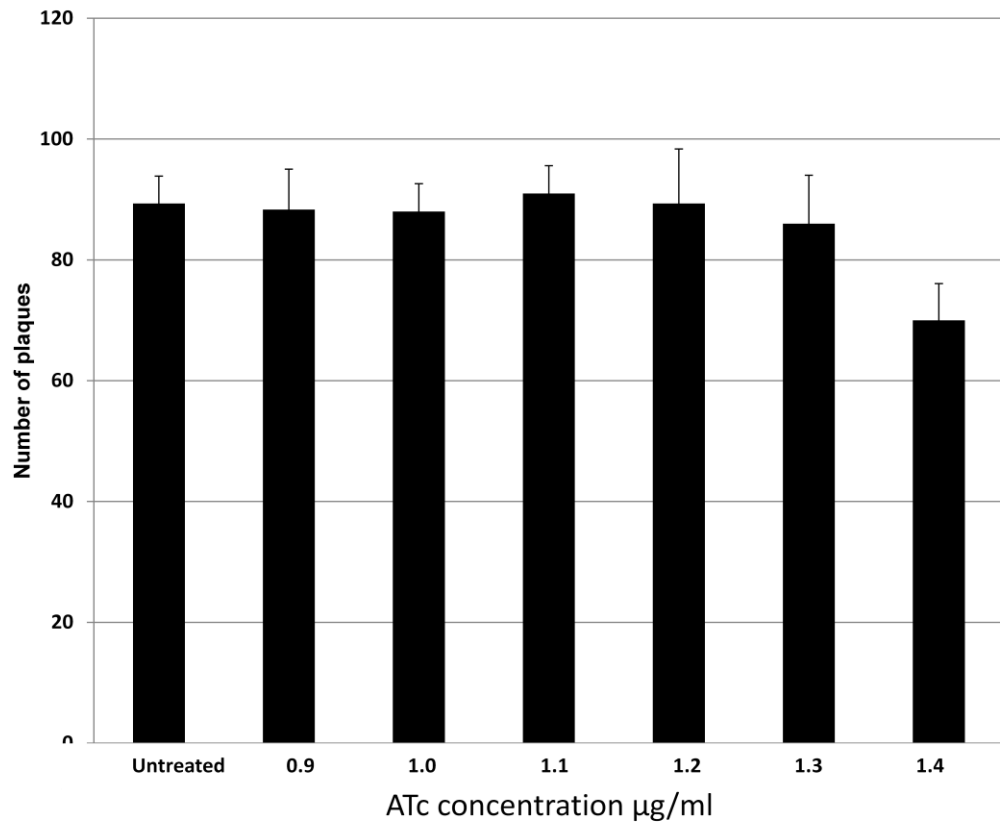
Figure 29: Anhydrous tetracycline toxicity. 1,000 parasites were allowed to infect host cell monolayers and exposed to different concentrations of ATc continuously for 7 days. ATc was added to cultures at the beginning of the experiment during parasite infection. After one week, infected monolayers were fixed in methanol and parasite plaques counted. All samples were performed in triplicate and the average was used as the final measurement. Error bars represent standard deviation.

To test if reduced TgAPN increases susceptibility to MMS, intracellular parasites were treated with 1 µg/ml ATc for 24 hours and treated for two hours extracellularly with MMS before being placed into culture for a plaque assay. A modest slow growth phenotype accompanied the conditional knockout cultured in1.0 µg/ml ATc following pre-treatment with 400 µM MMS, consistent with the idea that TgAPN is important for *Toxoplasma* DNA repair (Figure 30).
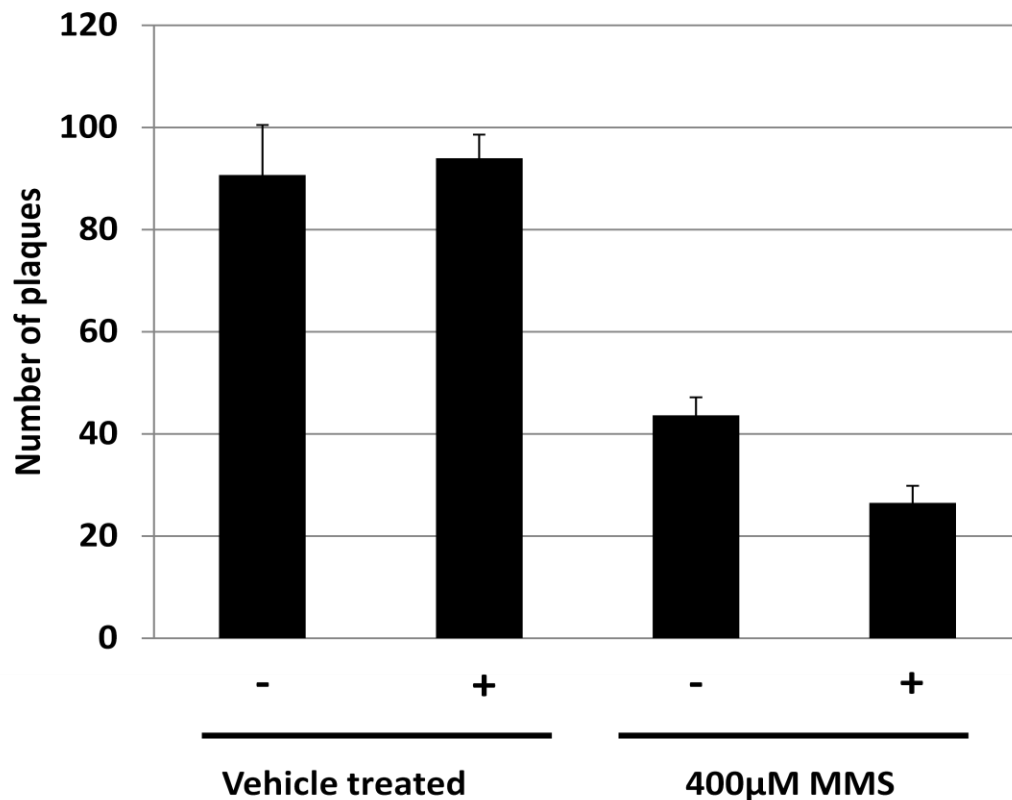
Figure 30: MMS DNA damage recovery assay. 1,000 parasites were treated with 400 µM MMS for 2 hours prior to infection of host cell monolayers. 1.0 µg/ml of ATc was added to the parasites at the time of infection to deplete TgAPN. Infected cells were fixed and parasite plaques counted on the seventh day post-infection. The error bars represent standard deviation.

J. Impaired response to DNA damage in TgAPN knockdown parasites

As an alternative approach to evaluate if reduced levels of TgAPN would result in greater sensitivity to MMS, we used a ligand controlled destabilization domain. We engineered a "knock in" vector designed to place a fusion tag onto the C-terminal end of TgAPN consisting of two HA epitopes and a destabilization domain (2xHA-DD), which allows for regulated expression of the fusion protein through addition of the stabilizing ligand Shield-1 [92]. RH strain parasites lacking Ku80 (Ku80-KO), which display a significantly higher degree of homologous recombination [93], were used to create *Toxoplasma* parasites expressing endogenous TgAPN fused to the 2xHA-DD tag (called TgAPN-2xHA-DD, Figure 31). We were able to obtain several clones tagged in this fashion that behaved similarly. The ability to regulate levels of TgAPN-2xHA-DD protein with Shield-1 was verified using IFA and immunoblotting (Figure 32A and B).
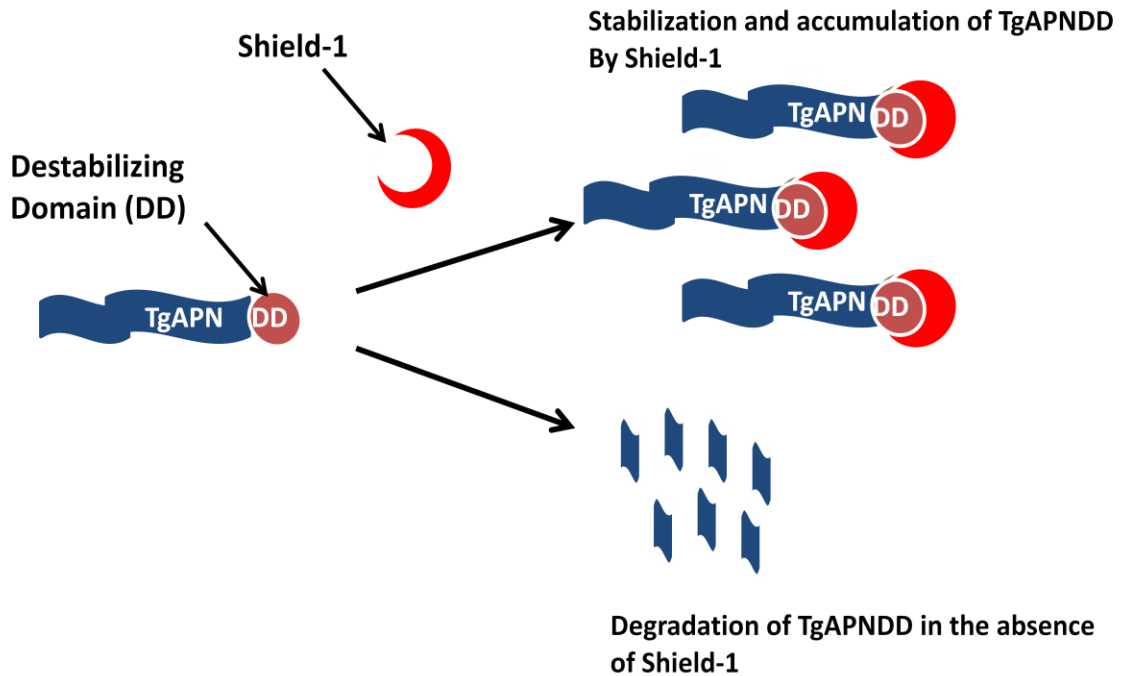
Figure 31: Diagram of Shield-1 regulation of TgAPN. The TgAPN gene is tagged at the 3'end to encode a protein with 2xHA and the destabilization domain (DD) fused to the C-terminus. The TgAPN-2xHA-DD fusion protein is expressed and sent to the proteosome for degradation in the absence of Shield-1. In the presence of Shield-1, the destabilizing effect is inhibited, allowing the protein to accumulate.
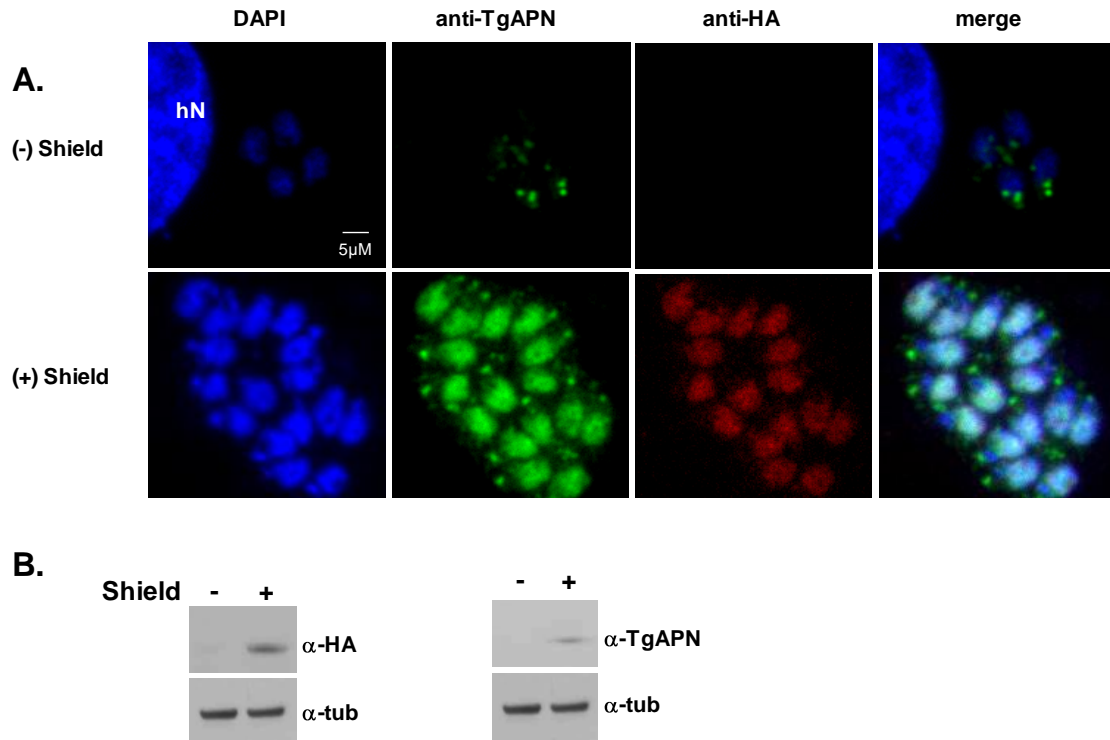
Figure 32: Knockdown of TgAPN protein using ligand-controlled destabilization domain. A parasite clone was generated to express endogenous TgAPN fused to a tag containing two HA epitopes and a destabilization domain (2xHA-DD). A) TgAPN-2xHA-DD is rapidly degraded unless stabilized through the inclusion of 100 nM Shield-1 in the media. IFA of TgAPN-2xHA-DD parasites grown with (+) or without (-) Shield, probed with anti-TgAPN (1:10,000, green) or anti-HA (1:2,000, red). DAPI was used as a nuclear co-stain (blue). hN=host cell nucleus. B) Western blot analysis of TgAPN-2xHA-DD parasites grown with (+) or without (-) Shield, probed with anti-HA (1:3,000, left) or anti-TgAPN (1:10,000, right). 10 µg of parasite lysate was used for the immunoblots. Tubulin was monitored as a loading control (1:5,000).

TgAPN-2xHA-DD parasites cultured under normal conditions without Shield (TgAPN knockdown) are viable, as reported for other species [94]. We then analyzed the ability of purified, extracellular TgAPN knockdown parasites to recover from a direct 2 hr exposure to 25 µM MMS. Relative to the Ku80-KO parental line, the TgAPN knockdown parasites were modestly impaired in recovering from the 25 µM MMS pre-treatment for 2 hours (Figure 33A). As shown, the parental Ku80-KO parasites show ~40% reduction in growth in the presence of 100 µM MMS continuously for 7 days. In contrast, TgAPN knockdown parasites show ~80% reduction in growth in the presence of 100 µM MMS (Figure 33B). Together these data further support that TgAPN plays a role in the DNA damage response in *Toxoplasma*.
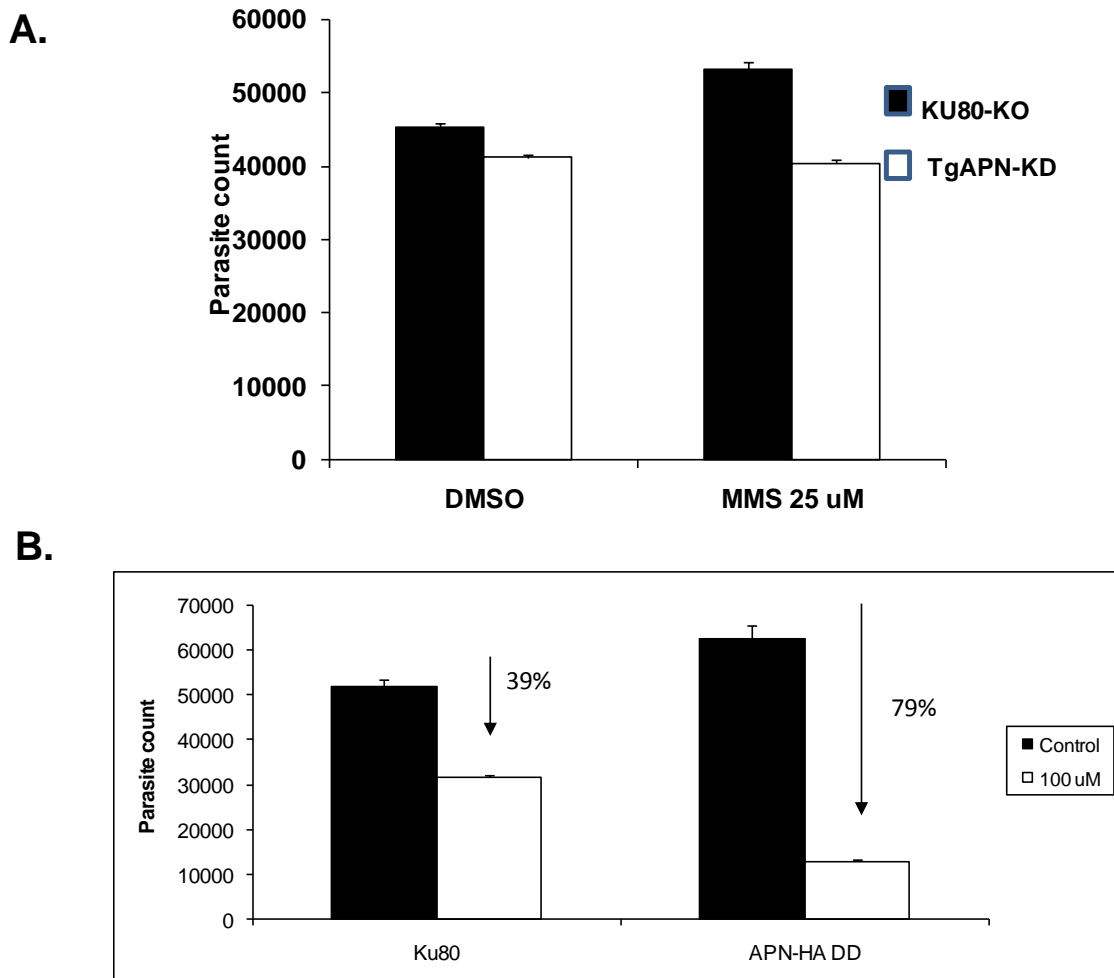
Figure 33: TgAPN knockdown parasites impaired response to DNA damage. A)

Parental (Ku80-KO) and TgAPN-2xHA-DD parasites cultured without Shield

(TgAPN knockdown, KD) were purified and subjected to 25 μM MMS for 2 hrs

prior to being placed back in culture to infect fresh host cells. Growth was

monitored on day 5 using the PCR-based B1 assay. B) Parental (Ku80-KO) and

TgAPN-2xHA-DD parasites cultured without Shield were allowed to infect HFF

monolayers incubated in the presence of 100 μM MMS (white bars) or vehicle

control (black bars). Growth was monitored on day 7 using the B1 assay. Error

bars represent standard deviation.

K. Levels of TgAPE in TgAPN knockdown parasites

      The levels of TgAPE in the TgAPN conditional knockout (see Figure 22) and the TgAPN knockdown parasites were analyzed to check if an upregulation of TgAPE occurred that might compensate for the loss of TgAPN. Protein levels of TgAPE were determined in the conditional knockout cultured with or without anhydrous tetracycline. Immunoblots show that levels of TgAPE protein remain unchanged in parasites with diminished levels of TgAPN (Figure 34A). Immunoblots were also performed for the TgAPN-2x-DD knockdown parasites under the control of the destabilization domain. The data suggest that TgAPE is not upregulated when TgAPN is depleted. (Figure 34 B). However, these studies do not rule out the possibility that there may be overlapping roles for TgAPE and TgAPN.
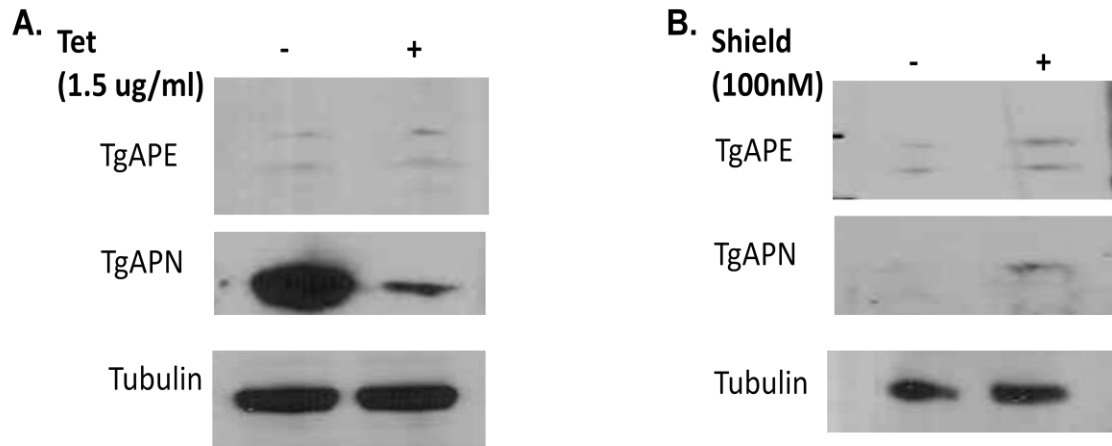
Figure 34: Level of TgAPE protein in parasites with down-regulated TgAPN. A) TgAPE levels in tet-based TgAPN conditional KO. 1 μg/ml ATc was used to reduce the levels of TgAPN. Intracellular parasites were treated with ATc for 48 hours before the Western blots were performed. B) TgAPE levels in TgAPN-2xHA-DD parasites with diminished levels of TgAPN. 100 nM Shield-1 was added to cultures for 48 hours prior to harvesting the parasites for Western blotting. TgAPN antibody used at 1:10,000, TgAPN antibody used at 1:10,000 and tubulin antibody used at 1:5,000.

2. Pharmacological inhibition of TgAPN

This section describes our pharmacological approach to inhibit the function of TgAPN using small molecule compounds identified in a high-throughput screen. The screen against recombinant TgAPN activity identified nine compounds with potent inhibitory activity. Compound 4491-0277 was found to be the most potent inhibitor of *Toxoplasma* growth *in vitro*. Moreover, 4491-0277 is not overtly toxic to human host cells.

A. Characterization of TgAPN inhibitors

Prior to the start of this project, there were no inhibitors of APN from any species. In collaboration with Dr. Millie Georgiadis, a high-throughput screen for a specific inhibitor of TgAPN was carried out using the ChemDiv library with over 60,000 compounds. The library is comprised of "drugable" compounds that follow the Lipinski's rule of five: not more than 5 hydrogen bond donors (nitrogen or oxygen atoms with one or more hydrogen atoms), not more than 10 hydrogen bond acceptors (nitrogen or oxygen atoms), a molecular weight under 500 Daltons and a octanol-water partition coefficient log *P* less than 5. The compounds also possess good ADME (absorption, distribution, metabolism and excretion).

The drug screen used a fluor-quenched pair of oligonucleotide with a synthetic abasic site. One strand of the oligonucleotide had a fluorecein molecule and the other dabcyl, a quencher. The fluorescence of the fluorecein is quenched by the close proximity to the dabcyl while associated in the oligonucleotide duplex. When cleavage at the abasic site mimic (terohydrofuran) occurs, it

results in disassociation of the oligonucleotides, thereby increasing fluorescence as the fluorecein and the dabcyl move further away from each other.

The initial screen yielded 9 promising compounds that had strong inhibitory activity against recombinant TgAPN. The nine compounds were tested against *Toxoplasma* tachyzoites using a standard monolayer disruption assay. Pyrimethamine, a known inhibitor of *Toxoplasma* proliferation, was used as a positive control for the assays while DMSO was the vehicle for the compounds. A know inhibitor of human APE1 was also tested to analyze its specificity and cross reactivity with *Toxoplasma* AP endonucleases Table IV shows the results of the initial characterization of the compounds.

Table IV: Effect of TgAPN inhibitors on *Toxoplasma* viability.

Conc. (µM)	100	46.5	21.6	10.0	4.7	2.2	1.0	0.47	0.22	0.10	0.05	No drug
4872-0414	Toxic	2	1.5	0	0	0	0	0	0	0	0	0
4593-1532	0	0	0	0	0	0	0	0	0	0	0	0
4491-1565	Toxic	Toxic	Toxic	3	2	1.5	0	0	0	0	0	0
K780-0462	0	0	0	0	0	0	0	0	0	0	0	0
2825-0093	0	0	0	0	0	0	0	0	0	0	0	0
C699-0451	Toxic	Toxic	3	2	1	0	0	0	0	0	0	0
3057-1537	Toxic	Toxic	Toxic	Toxic	0	0	0	0	0	0	0	0
3230-2363	0.5	0	0	0	0	0	0	0	0	0	0	0
4491-0277	Toxic	Toxic	Toxic	Toxic	3	2	1	0.5	0	0	0	0
5015-0007	Toxic	Toxic	Toxic	Toxic	Toxic	0	0	0	0	0	0	0
Pyrimethamine	Toxic	Toxic	3	3	3	3	3	2	0	0	0	0
DMSO	0	0	0	0	0	0	0	0	0	0	0	0

All compounds were initially dissolved in DMSO and serially diluted in DMEM media to the test concentrations. 3,000 parasites were added to each well in 96 well plates with confluent host cells in presence of compound or vehicle control. The parasites were allowed to grow for 5 days before monolayer disruption was scored as follows: 3=maximum parasite death, 2=moderate parasite death, 1=minor parasite death, 0=no effect on parasites, Toxic=toxic to host cells. The effect of the inhibitors on the parasite viability was determined subjectively under a microscope.

B. Further characterization of compounds

Based on the results of the monolayer disruption assays, three compounds most effective in inhibiting parasite proliferation included: 4491-0277, C699-0451 and 4491-1565. These three compounds were further characterized using the more quantitative PCR-based B1 assay (Figure 35). Concentrations tested were based on the results of the initial monolayer disruption assay (Table IV). All compounds resulted in a reduction of parasite viability in a dose-dependent manner. Compound 4491-0277 had the highest inhibitory activity on parasite proliferation with an IC50 of 0.4 µM based on the B1 assays done.

The structure of the TgAPN inhibitors: 4491-0277, C699-0451 and 4491-1565 are structurally similar to each other. 4491-0277 and 4491-1565 retain the most structural similarity, while C699-0451 retains the related ring structure (Figure 36).

A.
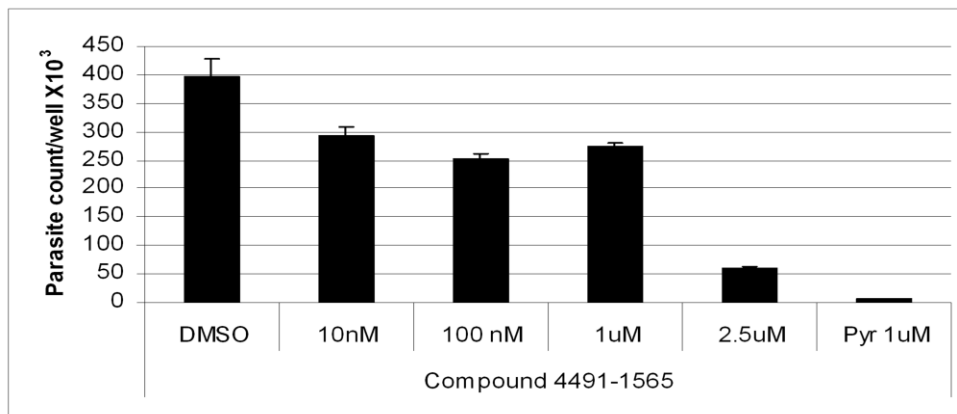
B.
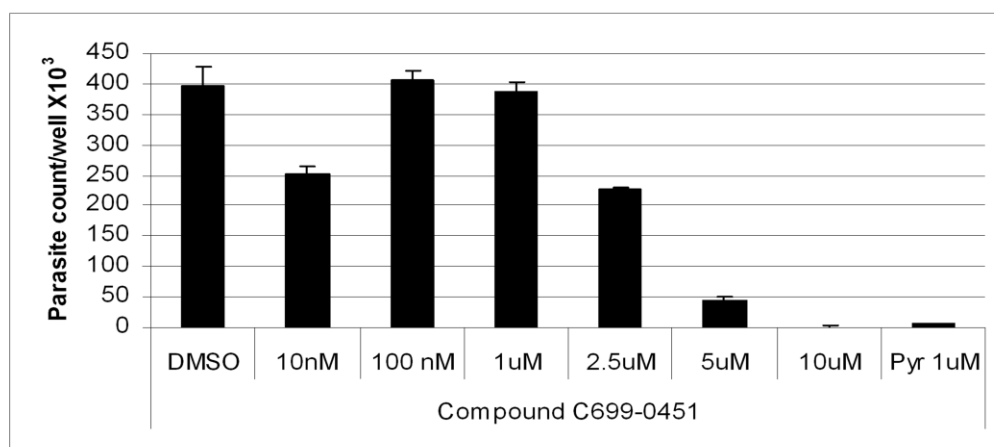
C.
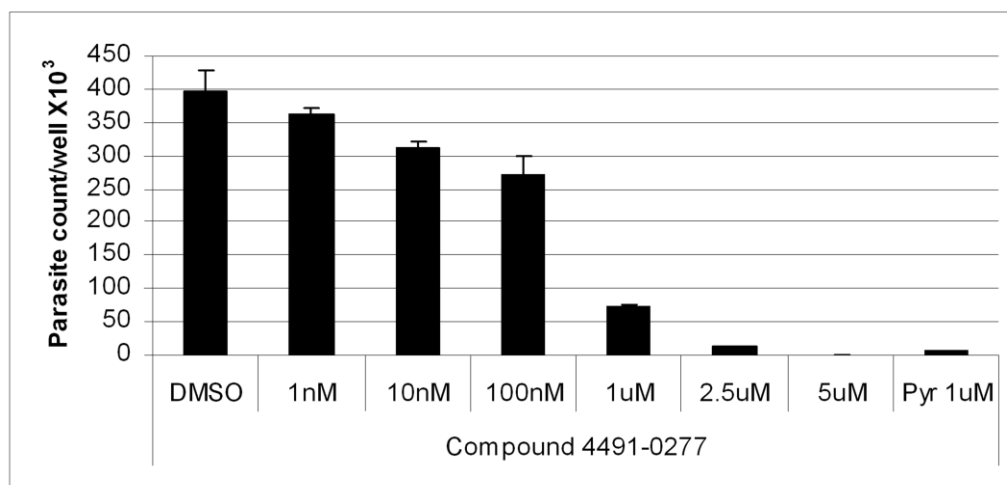
Figure 35: B1 assay of TgAPN inhibitors. 1,000 parasites were added to each well of a 24 well plate with confluent host cells. Compound dilutions were made in DMSO. Serial dilutions of different concentrations of inhibitors were made in DMEM media and added to the host cells and parasites. The cultures were incubated for 5 days and then DNA was isolated from the parasites using DNeasy kit and used for the B1 assay. 1.0 μM of pyrimethamine was used as a positive control and DMSO as a negative control. Error bars represent standard deviation.

A.

4491-1565

B.

C699-0451

C.
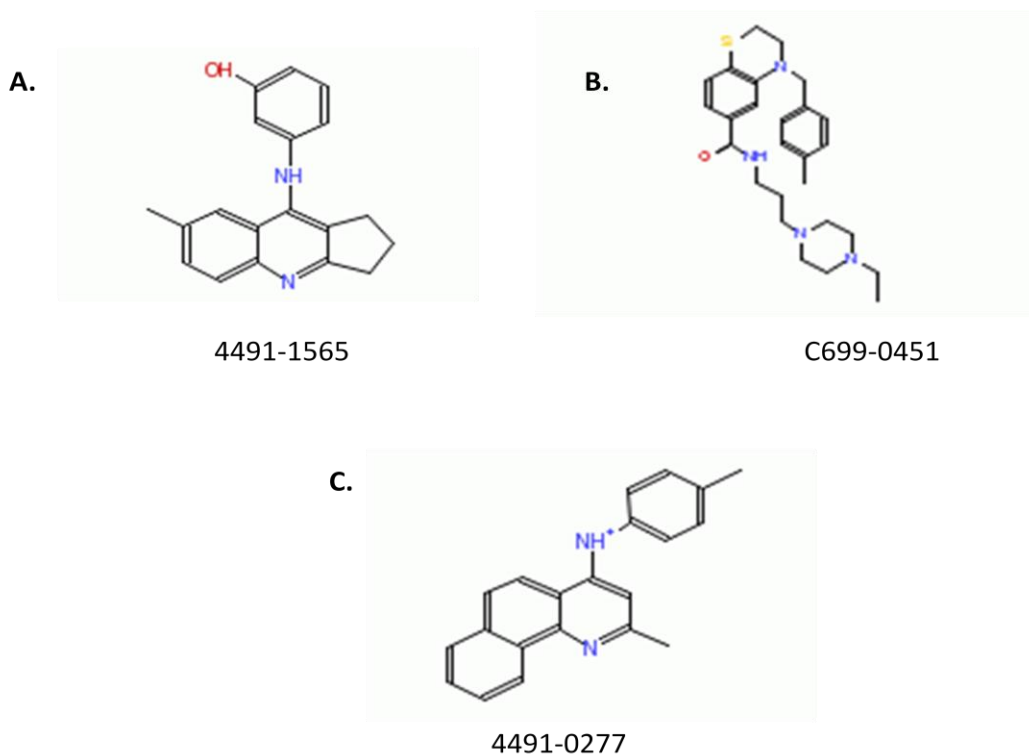
4491-0277

Figure 36: TgAPN inhibitor structures. TgAPN inhibitor structures obtained from the ChemDiv library.

We have also examined the impact of compound 4491-0277 on

Toxoplasma using a plaque assay, which allows us to observe if there is overt

toxicity to the HFF host cells. The plaque assay was done by allowing 1,000

parasites to infect the HFF monolayer in the presence of 1.0 µM of compound

4491-0277. The compound 4491-0277 was added to the culture at the time of

infection and left on through the duration of the experiment (5 days). Infected

host cell monolayers were analyzed under a microscope to determine parasite

and host cell viability. An uninfected confluent monolayer was included as a

control. Consistent with data from the B1 assay, *Toxoplasma* treated with 1.0 µM

of the compound 4491-0277 ceased to replicate (Figure 37, right panel). It is also

important to note that the infected host cell monolayer treated with 4491-0277

showed no overt damage (Figure 37, right panel). These findings suggest that

TgAPN inhibitor 4491-0277 stops tachyzoite proliferation *in vitro* at

concentrations that do not appear to cause overt toxicity to the human host cells.

Normal uninfected monolayer **Untreated parasites destroy host cell monolayer** **Inclusion of APNi 4491-0277 prevents Toxo growth without damage to host cell**
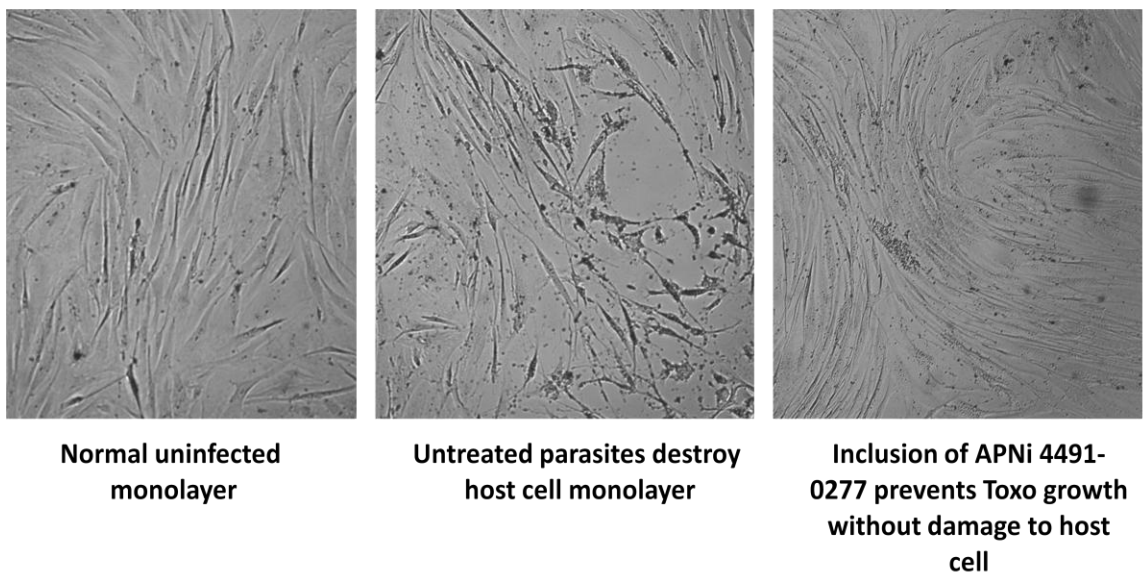
Figure 37: Compound 4491-0277 toxicity to host cells. Photomicrographs of host cell monolayers response to parasite infection and compound 4491-0277. 1,000 parasites were infected onto host cell monolayers and cultured for 5 days. 1.0 µM of 4491-0277 was added to the culture at the start of infection. At the end of the experiment, infected monolayers were fixed using methanol and analyzed under a microscope (X40 magnification).

CHAPTER 4: DISCUSSION

1. Aim 1: Determine the role of TgAPN in *Toxoplasma* in DNA damage

A. Summary of Aim 1 results

We have identified two AP endonucleases in *Toxoplasma*: TgAPE and TgAPN. TgAPN is a homologue of yeast and bacterial endonuclease IV enzymes. TgAPE, a homologue of Exo III enzymes, is expressed as two forms, one of which is a truncated version. TgAPE localizes to the parasite nucleus as well as the cytoplasm. TgAPN localizes only to the nucleus of the parasites. Native TgAPN is larger than rTgAPN, suggesting a post-translational modification may occur within parasites. There is a great disparity in the abundance of the two *Toxoplasma* AP endonucleases. TgAPN is more abundant than TgAPE at both mRNA and protein levels. Both enzymes show the expected enzymatic activity, although recombinant TgAPE displays higher substrate turnover than TgAPN *in vitro*. This higher turnover of TgAPE may indicate that the enzyme can process more abasic sites than TgAPN, but further study is required to determine if this occurs *in vivo*. TgAPN over-expression confers protection against MMS. A complete knockout of TgAPN did not yield any viable parasites necessitating a conditional knockout approach. Depletion of TgAPN in *Toxoplasma* tachyzoites results in increased susceptibility to MMS.

B. Impact of *Toxoplasma* infection

A number of protozoal species in phylum Apicomplexa have a tremendous impact on human health and livestock. In addition to *Toxoplasma*, other important pathogens in this phylum include *Plasmodium*, *Cryptosporidium*, and

Eimeria. In humans, the relationship between immunosuppression and occurrence of severe toxoplasmosis is well recognized. In human immunodeficiency virus (HIV)-infected patients, the incidence of toxoplasmic encephalitis is a leading cause of death [15]. *Toxoplasma* infection is also an important health risk in organ transplants [20]. Congenital toxoplasmosis also occurs and might lead to abortion or birth defects in newborns [8]. *Plasmodium* is the causative agent of malaria and kills up to 2.7 million persons per year, over 75% of them children [95]. *Cryptosporidium* causes acute diarrheal illness that can be extremely serious in the young and immunocompromised. *Eimeria* is a coccidian parasite of poultry, causing losses in excess of $3 billion annually to US agribusiness [96]. A combination of too little research and rapid spread of resistance to frontline drugs is grossly hindering our control of these infectious diseases.

C. *Toxoplasma* susceptibility to DNA damage

Like other apicomplexans, *Toxoplasma* is an intracellular parasite likely to be exposed to an oxidative burst generated by the host immune response in addition to reactive oxygen species (ROS) generated through its own metabolism. ROS including hydrogen peroxide, the superoxide radical and the hydroxyl radical, are produced as byproducts of oxygen metabolism in all cells. In addition, immune effector cells including neutrophils, eosinophils, and macrophages release superoxide anion radicals as part of the oxidative burst during a microbial infection [47]. A common feature among the different ROS types is their capacity to cause oxidative damage to proteins, DNA, and lipids.

The resulting microenvironmental oxidative stress is toxic to various parasites and, in order to counteract this, pathogens are equipped with specific ROS-detoxifying mechanisms as well as DNA repair mechanisms [47]. Most intracellular parasites studied possess antioxidant and DNA repair enzymes. *In vitro* assays have implicated the role of ROS in the toxoplasmacidal activity of human monocytes and interferon gamma-activated human macrophages. ROS induces DNA damage, which includes a multitude of oxidized base lesions, abasic (AP) sites, single- and double-strand breaks containing 3′ sugar fragments or phosphates and all of these are invariably cytotoxic and/or mutagenic if not repaired. Nearly all oxidatively induced DNA lesions (except double-strand breaks), as well as single strand breaks, are repaired via the DNA base excision repair (BER) pathway in organisms ranging from *E. coli* to mammals [51].

D. *Toxoplasma* AP endonucleases

Repair of abasic sites is achieved through BER, and AP endonucleases are the rate-limiting enzymes in this pathway. Limited work has been performed to date to characterize AP endonucleases in protozoa. AP endonucleases have been studied in kinetoplastid parasites *Trypanosoma cruzi* and *Leishmania major* [71]. Over-expression of the *L. major* AP endonuclease (LMAP), which is an APE1 (ExoIII) homologue, protects the protist from DNA damage. The crystal structure for LMAP has recently been solved, and it largely resembles other APE1 orthologues including TgAPE. It has been reported that *Plasmodium falciparum* predominantly uses long-patch BER as opposed to short-patch BER, which is the predominant pathway in most other species including human [51].

Our study confirms the presence of two AP endonucleases in apicomplexan *Toxoplasma gondii*, the Mg^{2+}-dependent TgAPE and Mg^{2+}-independent TgAPN.

TgAPE is a homologue of the human APE1 and TgAPN is a homologue of *S. cerevisiae* APN1.TgAPN is absent in mammals, which makes it a promising drug target. Although TgAPE has a human homologue, it is considerably divergent and can also be considered a possible drug target. TgAPE does not have a redox domain as its human counterpart. Interestingly, TgAPE is more closely related to plant APEs than metazoans. ExoIII and EndoIV enzymes have no structural similarities to each other [59,60], which makes it unlikely that TgAPE and TgAPN have structural similarities. The only similarity between TgAPE and TgAPN is they both remove abasic sites. The presence of two structurally different enzymes with the same function is an example of convergent evolution. The evolution of two distinct proteins to catalyze the same enzymatic reaction supports the importance DNA repair in *Toxoplasma.*

E. Size and localization of TgAPE

While TgAPE does not appear to be abundant or important in protecting *Toxoplasma* from MMS-mediated DNA damage in tachyzoites. I speculate that TgAPE may be critical during another life cycle stage. Another interesting finding from our studies is that TgAPE exists as two forms, the shorter one being in the cytoplasm and possibly the apicoplast. The presence of truncated APE1 in the cytoplasm is not without precedent; the distribution of human APE1 (hAPE1) in the nucleus, mitochondria, and cytosol is in a dynamic equilibrium [83]. Several recent studies indicated the presence of nuclear hAPE1 in the mitochondria

(mtAPE), suggesting that hAPE1 is not altered after its mitochondrial import [83]. The full length hAPE1 has an N-terminal nuclear localization sequence. Deletion of the first 33 amino acids of hAPE1 results in translocation to the mitochondria, and increases the specific activity of mtAPE (truncated hAPE1) by 3-fold [83]. Interestingly, cytoplasmic localization of hAPE1 has been observed in several tumors and correlates with a poor prognosis [97]. Localization of TgAPE to the apicoplast could be a significant finding as this plastid-like organelle houses its own 35 kb genome that is essential for *Toxoplasma* viability. Genes encoding proteins involved in DNA replication or repair are absent from the apicoplast genome [66]. Certain genes encoding plastid DNA replication enzymes are, however, present in the nuclear genome, with the protein being imported into the plastid post-translationally. This lends evidence to the possibility of TgAPE being translocated into the apicoplast to serve as a repair enzyme for apicoplast DNA. In addition to its endonuclease activities, hAPE1 can regulate transcription by virtue of its redox activity; the TgAPE homologue, however, has a valine residue in the position equivalent to cysteine residue 65, which is essential for redox activity in the human enzyme [84].

F. Size and localization of TgAPN

The identification of TgAPN as a major AP endonuclease important for DNA repair in *Toxoplasma* is the first demonstration that such enzymes may be attractive drug targets against intracellular protozoan pathogens. TgAPN is more abundant than TgAPE at both protein and mRNA levels. In other species, it has been shown that there is usually a dominant AP endonuclease that handles the

bulk of DNA repair [71]. Native TgAPN appears larger than the rTgAPN, which may suggest post-translational modifications (PTMs). Although no PTMs have been identified for APN1 homologues to date, several have been identified for hAPE1. Human APE1 has previously been shown to be ubiquitinated, phosphorylated, nitrosylated, and acetylated [98]. Such PTMs are involved in the function of the protein as well as its localization. TgAPN, like its yeast homologue, localizes to the nucleus (Figure 19). The APN1 C-terminus is rich in basic amino acids and includes two lysine/arginine clusters related to the nuclear transport signals of some other proteins. Deletion of the C-terminal sequences associated with nuclear localization resulted in the accumulation of cytoplasmic APN1 [99]. These delocalized derivatives also failed to restore wild-type resistance to oxidative or alkylating agents in mutant strains [85]. *APN1* deficient cells (*apn1Δ*) show increased mutation frequencies in mitochondrial DNA (mtDNA), suggesting that *APN1* is also important for mtDNA stability [85]. In our studies, no appreciable levels of TgAPN were detected outside the nucleus of the parasite.

G. Enzymatic analysis of TgAPE and TgAPN

Our findings show that both recombinant TgAPE and TgAPN are enzymatically active. Like its human homologue, TgAPE is Mg^{2+} dependent and requires the presence of magnesium ions for DNA repair activity. The divalent metal ion dictates the directionality of bond cleavage by stabilizing the O3′ leaving group, either by direct ligation or via a water molecule in the first hydration shell of a magnesium ion [51]. APN1 homologues require zinc ions to

have enzymatic activity [51]. Although TgAPN displays DNA repair activity without zinc ions, it is thought that the Zn^{2+} ions are incorporated into the enzyme during folding in the bacteria. APE requires two magnesium ions while APN requires three zinc ions [51]. Using the recombinant proteins, we were able to establish that both enzymes had similar Michaelis-Menton K_m values of 26.2 and 21.1 nM for TgAPN and TgAPE, respectively. The V_{max} values were 1458 and 110,070 RFU/µg/s for TgAPN and TgAPE, respectively, indicating that the turnover rate was approximately 75 times faster for TgAPE (Figure 17). These data suggest that despite the low abundance of TgAPE in tachyzoites, TgAPE may play an important role in DNA damage repair in *Toxoplasma.* Furthermore, AP endonuclease assays performed using *Toxoplasma* lysate in the presence or absence of EDTA (to chelate Mg^{2+}) demonstrated that TgAPN does not account for 100% of the total AP endonuclease activity.

H. TgAPN over-expression protects parasites from MMS

Cells would be expected to be protected from DNA damaging agents when AP endonucleases are over-expressed. Furthermore, expression of *Trypanosoma cruzi* and *Leishmania major* AP endonucleases in *E. coli* deficient in DNA repair confers protection against DNA damaging agents. Over-expression of TgAPN conferred protection against MMS. The increased levels of TgAPN protein in the over-expressor facilitated an increased capacity to repair DNA damage compared to the wild-type parasites. Over-expression of TgAPE surprisingly did not yield any protection to MMS. There are a few possibilities that may explain this result. Unlike APN1 homologues, APE1 enzymes do not

possess nucleotide incision repair. Nucleotide incision repair activity (NIR), which can directly incise 5′ to specific oxidized bases, e.g., α-deoxyadenosine (α-dA/T) or dihydrouridine (dHU/G), to create a 3′-hydroxyl group for DNA repair synthesis [94]. Another explanation for TgAPE over-expression failing to confer protection to MMS is that necessary co-factors or other proteins needed to facilitate TgAPE function are not expressed to sufficient levels. Finally, we only tested the recovery to MMS-mediated damage. It is possible that TgAPE is reserved for responding to DNA damage mediated by other agents.

I. TgAPN conditional knockout

In humans, the knockout of the main AP endonuclease is not viable and only cells with low levels of APE can escape death [51]. In mammals, complete deletion of APE1 is embryonically lethal [51]. Attempts to make a knockout of TgAPN in tachyzoites have been unsuccessful. A conditional knockout approach to disrupt TgAPN was successful, demonstrating that the TgAPN genomic locus can be disrupted and highlighting the probability that TgAPN is essential for parasite survival. APN1 and APN2 have been knocked out in *S. cerevisiae* producing viable yeast cells. APN1 has also been knocked down in *Caenorhabditis elegans*. In both cases, the depletion of APN did not prove lethal but the cells were particularly susceptible to DNA-damaging agents. The conditional knockout strategy that we used to diminish TgAPN levels was a tet-off system [51]. The conditional knockout was created in a special RH-based parasite line (TATi), which has been transfected with tet-transactivator, tTA, composed of a TetR-VP16 fusion. The regulatable copy of TgAPN was made in a

vector containing TetOSAG1 promoter elements. Although the presence of TetO

promoter elements attenuates the SAG1 (*Toxoplasma* surface antigen 1)

promoter, it still proved to be stronger than the endogenous TgAPN promoter.

Consequently, expression of ectopic TgAPN was higher than wild-type levels.

Down regulation of ectopic TgAPN could be achieved through the inclusion of

ATc in the culture, but TgAPN levels did not fall significantly below wild-type

levels. Inability to completely down-regulate protein levels using the Tet-off

system has been previously documented in *Toxoplasma* [78]. The inability to

deplete TgAPN levels in the conditional knockout limited the use of the clone to

study susceptibility of the parasites to DNA-damaging agents, but our data did

show a mild impaired DNA damage response upon depletion of TgAPN.

J. TgAPN knockdown using a destabilization domain

An alternative to the conditional knockout is the destabilization domain,

which does not rely on a strong exogenous promoter. The native TgAPN is

tagged at the C-terminus with a destabilizing domain (DD), which ensures that

wild-type levels of TgAPN are maintained in the parasite; the TgAPN-DD fusion

can then be depleted to an amount lower than wild-type using a ligand that binds

the DD [78]. The DD utilizes an engineered version of human FKBP12 (FK506

binding protein) fused to the N or C terminus of a target protein, promoting

degradation of the fusion protein [92]. FKBP, or FK506 binding protein, is a family

of proteins that have prolylisomerase activity, thus their destabilizing effect on

proteins. Prolylisomerase enzymes interconvert the *cis* and *trans* isomers of

peptide bonds with the amino acid proline [100]. Proline is unique among amino

acids in having a relatively small difference in free energy between the *cis* configuration of its peptide bond and the more common *trans* form. Proline has an unusually conformationally restrained peptide bond, this because proline has a cyclic structure with its side chain bonded to its secondary amine nitrogen. FKBP12 is notable in humans for binding the immunosuppressant molecule tacrolimus (originally designated FK506), which is used in treating patients after organ transplant and patients suffering from autoimmune disorders [92]. In the presence of a ddFKBP ligand, degradation is mitigated, allowing regulation of protein levels in cells. Shield-1, a cell-permeable ligand, binds tightly to the destabilizing domain and prevents degradation, providing small molecule control over intracellular protein levels [100]. Our findings show that the levels of TgAPN were significantly decreased when tagged with a destabilization domain in the absence of Shield-1. Trace amounts of TgAPN were detected by Western blot. Immunofluorescence showed that some TgAPN protein was found in what appears to be cytoplasmic vesicles. The vesicular localization of TgAPN could represent the protein in the proteosome being degraded. Despite the low levels of TgAPN still left in the parasite, little or no TgAPN was detected in the nucleus, its target organelle. The TgAPN-DD parasites are viable under normal culture conditions, consistent with the yeast APN1 knockout [101]. Viability of the knockdown parasites decreases significantly in the presence of MMS, illustrating the reliance *Toxoplasma* places on TgAPN for DNA repair.

K. TgAPE does not compensate for loss of TgAPN

To rule out the upregulation of TgAPE to compensate for the loss of TgAPN, mRNA and protein levels were analyzed. In other species, such as *S. cerevisiae*, loss of one AP endonuclease can be offset by another AP endonuclease to preserve DNA integrity. The viability of TgAPN knockdown parasites could be explained by the fact that both TgAPE and TgAPN carry out the same function of repairing abasic sites. The levels of TgAPE did not increase in either the conditional knockout or the TgAPN-DD clone. On the other hand, we have demonstrated that although TgAPE has very low abundance in the parasite, it has a very high activity in repair of abasic sites compared to TgAPN *in vitro*. In other words, TgAPN is a very active enzyme that can repair DNA damage at very low levels. To completely rule out TgAPE compensation following loss of TgAPN, a double knockout of both enzymes might be necessary. This approach is complicated by our inability to isolate viable parasites following attempts to knockout TgAPE or TgAPN. Therefore, a knockdown approach would have to be pursued, which suffers the caveat of not fully depleting protein levels. Despite these caveats, a double knockdown of TgAPE and TgAPN is expected to dramatically impact parasite growth, especially following DNA damage.

L. Conclusion

In conclusion, the genetic approach supports the hypothesis that the base excision repair pathway is important for *Toxoplasma* survival in an intracellular environment. AP endonucleases are the rate limiting enzymes in BER and targeting the abundant abasic endonuclease (TgAPN) would render the parasites

unable to survive in the intracellular environment. Humans do not possess APN1 homologues, and the TgAPN enzyme is structurally unrelated to human APE1, making it an attractive drug target.

Although TgAPN has been shown to be important for DNA repair, the importance of TgAPE in *Toxoplasma* DNA repair cannot be overlooked. The TgAPN knockdown under the control of the destabilizing domain did not definitively prove that TgAPN is an essential gene. However, disrupting the TgAPN genomic locus in the conditional knockout and inability to knock out the TgAPN gene with conventional homologous recombination shows that TgAPN is likely to be essential. There is the possibility that TgAPE might be compensating for the loss of TgAPN despite no increase in TgAPE protein. In other species, such as *E. coli,* loss of one AP endonuclease is compensated by another AP endonuclease. This redundancy might explain why *Toxoplasma* is viable even with very low levels of TgAPN.

Both TgAPN and TgAPE catalyze the same enzymatic reaction, cleaving the phosphate backbone of DNA 5' of the abasic site. Enzymatic activity assays have shown that both recombinant as well as native TgAPE and TgAPN have activity. Whether there is a predominant AP endonuclease operating in *Toxoplasma* remains to be resolved. Native TgAPN appears to be more active when enzyme activity is tested from *Toxoplasma* lysate, i.e. TgAPN shows greater than 50% of total activity when TgAPE is inhibited by magnesium chelation. Determining the activity of TgAPN by inhibiting TgAPE activity in *Toxoplasma* lysate assumes no other enzyme other than TgAPN and TgAPE can

process abasic sites in the parasite. There may be other enzymes that in the parasite including glycosylases that can process abasic sites. Recombinant TgAPE protein turns over substrate 75 times faster than recombinant TgAPN. The apparent discrepancy between recombinant and native proteins enzymatic activity could be an *in vitro* artifact, or could be explained by the possibility that TgAPN might be post-translationally modified, which in turn might augment its activity.

TgAPE may be an important DNA enzyme involved in apicoplast DNA repair. Curiously, over-expression of TgAPE provided no protection from MMS-induced DNA damage in the parasite. This result could be explained if the primary role of TgAPE is to repair apicoplast DNA. Moreover, it would be expected that the higher substrate turnover observed *in vitro* would not necessarily translate into an *in vivo* setting if TgAPE only repaired apicoplast DNA.

TgAPE is considerably diverse compared to the human APE1 and, if found to be critical for *Toxoplasma* proliferation could also be further explored as a drug target against *Toxoplasma* infection.

M. Future studies

The first study will be to test if the TgAPN knockdown parasites are viable in an *in vivo* environment. Mice will be infected with the destabilization domain knockdown parasites and a control mouse group infected with an equal number of wild-type parasites. The result expected is that the TgAPN knockdown parasites would be more susceptible to the mouse immune system. The mice

infected with TgAPN knockdown parasites would thus be expected to survive longer after infection compared to mice infected with wild-type parasites.

The size disparity between the recombinant TgAPN and the larger native TgAPN suggests post-translational modification(s) (PTMs). An investigation to determine the post-translational modification would be important in understanding the regulation and function of the protein. Post-translation modifications can be detected using specific antibodies to a variety of PTMs. To determine PTMs on TgAPN using specific antibodies, the native TgAPN protein will first be immunoprecipitated from wild-type parasite lysate using the TgAPN antibody and specific antibodies used to identify the PTMs on Western blots. An alternative approach to determine PTMs on TgAPN would be to use mass spectroscopy. Mass spectroscopy is a powerful tool used for protein identification, structural characterization, and identification of post-translational modifications.

Finally, investigation into the cleavage and localization of TgAPE would be interesting. Human APE1 is cleaved at the N-terminus and localized to the mitochondria [86]. TgAPE is also cleaved at the N-terminus and this may affect its localization to other organelles such as the apicoplast. Different truncations at the N-terminus of TgAPE coupled with tags on both the N-terminal and C-terminal ends would help determine localization as well as cleavage points. The localization can be determined by immunofluorescence and the cleavage products monitored by Western blotting. Localization of a DNA repair enzyme in

the apicoplast would be an important finding as there is no knowledge on DNA repair mechanisms of apicoplast DNA.

2. Aim 2: Pharmacological inhibition of TgAPN

A. Summary of Aim 2 results

Work in Aim 2 identified a small molecule, 4491-0277, that can inhibit TgAPN enzymatic activity as well as the growth of *Toxoplasma in vitro*. Compound 4491-0277 was identified through a high-throughput screen against rTgAPN enzymatic activity. 4491-0277 was able to inhibit parasites at concentrations that did not produce overt toxicity to the human host cells.

B. Limitation of current drug treatments against toxoplasmosis

The frontline treatment for *Toxoplasma*, pyrimethamine and sulfadiazine, is limiting for a number of reasons. Both drugs target the folic acid synthesis pathway needed for both DNA and RNA synthesis. Pyrimethamine inhibits dihydrofolate reductase (DHFR), while sulfadiazine inhibits dihydropteroate synthase (DHPS). The drugs cause severe side effects, particularly after prolonged use, which is required in cases of chronic toxoplasmosis. The inhibition of folic acid synthesis results in hematological problems such as bone marrow suppression, characterized by megaloblastic anemia and leucopenia. Sulfadiazine, a sulfonamide, can cause life threatening skin conditions such as Steven-Johnson syndrome and toxic epidermal necrolysis. The adverse effects are more pronounced in the immunocompromised, the patient population that most commonly suffers from recurring episodes of acute toxoplasmosis. Additionally, some strains of *Toxoplasma* have developed resistance to the

current drugs. There is also a paucity of treatments that can be safely administered during pregnancy to treat toxoplasmosis acquired *in utero*. Thus, there is an urgent need to identify and develop new drug targets against *Toxoplasma* infection. Additionally, it is hoped that such novel therapies would also have utility against other apicomplexan parasites, particularly if the therapies are targeted against crucial molecular targets that are similar in protozoa.

Our genetic studies have shown that the base excision repair pathway is important in the viability of parasites in their intracellular environment. Disrupting BER by depleting TgAPN resulted in increased susceptibility of *Toxoplasma* to DNA damage. Thus, TgAPN may be a promising drug target against *Toxoplasma* infection. Targeting TgAPN in *Toxoplasma* is likely to be specific because humans and other mammals do not have an APN1 homologue. Inhibiting TgAPN with small molecule inhibitors would therefore not be expected to have such severe side effects as those seen in targeting the folate synthesis pathway.

C. TgAPN as a probable drug target

DNA repair machinery has been targeted in the treatment of various types of cancers [69]. Some treatments induce direct DNA damage to overwhelm repair mechanisms resulting in death of tumor cells [69]. Inhibition of the BER has also been explored widely as a drug target against cancer [69]. Several compounds have been developed to inhibit glycosylases to sensitize cancer cells to alkylating agents [102]. The most convincing studies on targeting BER in cancer have been done by inhibiting human APE1 in brain tumors to sensitize the cancer cells to radiation [70]. Inhibiting AP endonuclease has thus been

proven in a clinical setting to be a good drug target. No work has been done in parasites to disrupt their DNA repair pathways pharmacologically. Cancer cells are very similar to pathogenic microbes in some respects. Like cancer cells, *Toxoplasma* is proliferative, which in theory would cause increased aberrations in its DNA. *Toxoplasma* and cancer cells are also under constant pressure by the immune cells likely to cause DNA damage. Treatment of *Toxoplasma* infection is similar to cancer in that the treatment should be able to prevent or minimize the toxicity to normal human cells by only targeting the rogue cells (*Toxoplasma* and cancer cells).

D. Identification of small molecules that inhibit TgAPN

To study the viability of TgAPN to function as a drug target against *Toxoplasma*, a high-throughput screen was performed. The high-throughput screen was conducted in collaboration with Drs. Mark Kelley and Millie Georgiadis against rTgAPN enzymatic activity. The ChemDiv library of drugable compounds was used in identifying TgAPN inhibitors. The fluorescent-based screening assay described in detail in the materials and methods identified 9 compounds that had inhibitory activity against rTgAPN. The compounds identified were tested against wild-type RH parasites *in vitro*. Two of the nine compounds tested showed significant activity against *Toxoplasma* tachyzoites but no observable toxicity to the host cells. Further characterization showed that compound 4491-0277 was more potent than compound C699-0451 in its ability to inhibit parasite growth. Compound 4491-0277 had an IC_{50} of 0.4 µM. The human APE1 inhibitor was also tested against *Toxoplasma* tachyzoites *in vitro*.

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The compound proved effective in killing the host cells (human foreskin fibroblasts) and only had an effect on the parasites at high concentrations. This result highlights differences between the human and parasite AP endonucleases and the possibility of targeting them specifically with minimal off target effects. Unlike the TgAPN knockdown parasites, compound 4491-0277 inhibits parasite growth without having to induce DNA damage. The most likely explanation for this is that 4491-0277 inhibits both TgAPE and TgAPN or might have off-target effects.

E. Conclusion

The pharmacological approach supports the finding that TgAPN is an integral enzyme in *Toxoplasma's* ability to repair DNA damage. Additionally, the use of compound 4491-0277 to inhibit TgAPN has highlighted the possibility of targeting *Toxoplasma* DNA repair machinery to treat infection, possibly providing a new approach that overcomes the limitations of the current drug treatments targeting the folate synthesis pathway.

F. Future studies

More complete toxicological assays are required to determine if compound 4491-0277 is non-toxic to human cells. Preliminary studies could employ the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to determine cytotoxicity of the compound, and comet assays could be performed to determine genotoxicity. The effect of the compound on replicating human cells would also have to be evaluated to determine if there is any inhibition of cellular growth. If 4491-0277 proves to have negligible toxicity on mammalian cells,

mouse studies can be performed. Mice can be infected with wild-type parasites and treated with compound 4491-0277 to determine if the compound can reduce parasite infection *in vivo*.

Structural studies to determine the mechanism by which compound 4491-0277 inhibits TgAPN should also be performed. Such studies will help identify or design other compounds that may be more potent at inhibiting TgAPN. Crystal structures of TgAPN interacting with 4491-0277 may help establish how the two bind. Furthermore, compounds 4491-1565 and C699-0451 have structural similarities with compound 4491-0277 and can be modified by altering their moieties to increase their inhibitory activity against *Toxoplasma*.

CHAPTER 5: SUMMARY

This dissertation has characterized TgAPN, one of the two AP endonucleases in *Toxoplasma gondii*. The study identifies two AP endonucleases in *Toxoplasma*, a homologue of the human APE1 (TgAPE) and a homologue of yeast APN1 (TgAPN). TgAPN is the more abundant AP endonuclease and is absent in humans, making it an attractive candidate as a drug target. TgAPN over-expression protects parasites against MMS, while TgAPE confers no protection. The data also suggests that TgAPN is an essential gene due to the inability to generate a genetic knockout. Supporting this idea, it is possible to disrupt the genomic locus to create a conditional knockout in the presence of ectopic TgAPN. Knocking down the TgAPN protein levels using a destabilization domain results in viable parasites that are hypersensitive to the DNA-damaging agent MMS. We have also identified the first APN1 inhibitor, 4491-0277. Compound 4491-0277 inhibits *Toxoplasma* growth and does not have any overt toxicity to the host cells *in vitro*. In conclusion, TgAPN has been identified as an important enzyme for *Toxoplasma* DNA repair and can be pharmacologically inhibited to serve as a drug target against *Toxoplasma* infection.

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Curriculum Vitae

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Dalmasso MC, **Onyango DO**, Naguleswaran A, Sullivan WJ Jr., Angel SO. (2009) *Toxoplasma* H2A Variants Reveal Novel Insights into Nucleosome Composition and Functions for this Histone Family. *Journal of Molecular Biology.* 392: 33-47.