Assessment of *Schistosoma mansoni* cAMP phosphodiesterases through complementation in a customised *Trypanosoma brucei* cell line.

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Abstract

The *Schistosoma mansoni* phosphodiesterase (SmPDE) enzymes have emerged as antiparasite targets because of their role in parasitic biology via cyclic nucleotides and differences with their mammalian counterparts. Previous studies conducted in the lab of De Koning described a characterization of the SmPDE family and showed that the SmPDE4A was able to replace the essential *Trypanosoma brucei* PDEB1/B2 system. However, it is still not clear whether there were other SmPDE proteins incompatible with the same system or negative results resulted from an inadequate construction design. Therefore, the goal of the current study was to continue investigating the ability of other SmPDE proteins to complement the essential function and use them for drug profiling. Unfused full-length constructions of SmPDE7var, SmPDE1, SmPDE8, and SmPDE9C were made and inserted into the conditional knockout *T. brucei* cells deficient in PDEB1/B2 under tetracycline inducible control. Simultaneously, catalytic domain constructs were prepared by introducing the catalytic domains of SmPDE4A, SmPDE7var, and SmPDE11 into the TbrPDEB1 construction to assess whether the negative results were linked to the specific localization or regulation of the SmPDE protein. It turned out that full-length constructions of all four proteins were able to complement the essential system. On the contrary, only SmPDE4A and SmPDE7var and not SmPDE11 worked as replacements of the catalytic domain. Late addition of tetracycline led to cell death and not arrest in proliferation. Finally, sensitivity of the created complementation lines was estimated using the dose–response assay in the presence of various drugs and calculating EC50 by fitting a nonlinear regression curve, reporting the value with its standard error and 95% CI. Pentamidine proved the highest activity, and experimental compounds showed construct-dependent potency differences in both systems. Thus, the research expanded the existing knowledge of complementation analyses of SmPDE and allowed to establish the relative importance of the two factors discussed.

Keywords: *Schistosoma mansoni*; phosphodiesterases; *Trypanosoma brucei*; functional complementation; catalytic-domain replacement; drug sensitivity profiling.

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Preface

In the intricate landscape of parasitology and drug development, the pursuit of effective therapies against infectious diseases demands innovative approaches and a deep understanding of the molecular mechanisms driving pathogenic organisms. This dissertation focuses into the realm of *Schistosoma mansoni*, a causative agent of schistosomiasis, one of the most prevalent and debilitating neglected tropical diseases globally.

The overarching goal of this research is to explore the potential of cAMP phosphodiesterases (PDEs) from *Schistosoma mansoni* as viable drug targets for chemotherapy. These enzymes play a crucial role in signal transduction pathways, making them enticing candidates for disrupting the life cycle of the parasite and, consequently, providing a novel avenue for therapeutic intervention.

Chapter 1 begins with an examination of the *S. mansoni* PDEs, specifically SmPDE7var, SmPDE1, SmPDE8, and SmPDE9C. The strategy involves cloning the full, untagged SmPDE open reading frames (ORFs) under the control of a tetracycline-inducible promoter in a specially engineered *Trypanosoma brucei* cell line. The development of this model, achieved through the deletion of essential loci and subsequent attempts at knockout, sets the stage for functional complementation experiments.

Chapter 2 opens the gateway to the experimental methodologies employed, where the minutiae of bacterial cultures, plasmid DNA preparation, and genomic DNA amplification set the stage for the subsequent chapters. The meticulous techniques described provide the scaffolding for the intricate molecular ballet performed in later stages.

Chapter 3 and 4 ushers in the generation of constructs, spotlighting the amplification and sub-cloning of SmPDEs into the final expression vector. The narrative details of the molecular biology, from re-amplifying and cloning to the digestion and ligation steps that bring these constructs to life.

The experimental landscape transforms once again in Chapter 5, where the catalytic domains of SmPDEs replace their counterparts in *T. brucei* PDEB1, a molecular metamorphosis that provides an improved complementation protocol

where the N-terminal domains and/or C-terminal sequence is essential for the function, localisation and regulation of the expressed PDE.

Chapter 5, the culmination of the research, explores inhibitor profiles of *Schistosoma* PDEs expressed in *T. brucei*. The evaluation of potential PDE inhibitors through *in vitro* assays and their subsequent testing in animal models adds a crucial translational dimension to the study, aiming to bridge the gap between bench and bedside.

In weaving this scientific tapestry, the dissertation aims not only to contribute valuable insights into the molecular underpinnings of schistosomiasis but also to lay the groundwork for the development of novel therapeutic strategies. It is our hope that the findings presented herein will spark further research endeavours and inspire future breakthroughs in the quest for combating neglected tropical diseases.

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I would like to acknowledge Infection, Immunity and Inflammation of the School of Life Sciences for providing the necessary resources, facilities, and research environment essential for the successful completion of this project.

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Finally, I would like to thank my family and friends for their unwavering encouragement, understanding, and patience throughout this demanding academic journey. Your support has been a constant source of motivation, and I am grateful for the sacrifices you have made to see me succeed.

Author's Declaration

I, declare that this thesis is my original work, and all the content presented herein is the result of my independent research conducted under the supervision of Harry de Koning. I have not submitted this work for any other academic qualification, and it has not been previously published in any form.

I acknowledge the sources of information and references cited throughout the thesis, and I have complied with all relevant academic and ethical standards in the conduct and reporting of this research.

Maha Abdullah Aloraini

Definitions / Abbreviations

Abbreviations

Abbreviation	Full term / meaning
AC	Adenylate cyclase
AMP	Adenosine monophosphate
bp	Base pairs
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CI	Confidence interval
dKO	Double knockout
DNA	Deoxyribonucleic acid
EC_{50}	Half maximal effective concentration
GAF domain	Regulatory domain found in certain cyclic nucleotide phosphodiesterases
gDNA	Genomic DNA
GFP	Green fluorescent protein
KO	Knockout
ORF	Open reading frame
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PZQ	Praziquantel
RNAi	RNA interference
sKO	Single knockout
SE	Standard error

SmPDE	*Schistosoma mansoni* phosphodiesterase
TbrPDEB1	*Trypanosoma brucei* phosphodiesterase B1
TbrPDEB2	*Trypanosoma brucei* phosphodiesterase B2
Tet	Tetracycline
WT	Wild type

Definitions of key technical terms

Term	**Definition**
Functional complementation	Restoration of an essential biological function in a host cell by expression of a heterologous gene or protein.
Heterologous expression	Expression of a gene from one organism in a different organism or cell system.
Catalytic-domain replacement	A strategy in which the catalytic domain of one enzyme is substituted with the catalytic domain of another enzyme while preserving the host scaffold.
Catalytic compatibility	The ability of an introduced catalytic domain or enzyme to provide sufficient enzymatic activity in the host system.
Regulatory context	The structural and cellular framework that influences enzyme localisation, control, and function within the host cell.
Nonlinear regression	A curve-fitting method used here to derive EC_{50} values from cell-based dose-response data.
Drug sensitivity profiling	Comparative assessment of cellular response to compounds using fitted EC_{50} values and related dose-response measures.

Chapter 1: Cloning and functional complementation of *Schistosoma mansoni* cyclic nucleotide phosphodiesterases in *Trypanosoma brucei*

1.1 Definition

Schistosomiasis is a parasitic neglected tropical disease caused by several species of trematodes belonging to the genus *Schistosoma* (Moreira-Filho et al., 2021). Parasitic flatworms of the genus *Schistosoma* cause the disease after penetrating the human body through the skin. Flatworms spread through contaminated water and food. They use water snails as intermediate hosts before finding their way to humans and other mammals. Schistosomiasis is recognized as a major global health issue, particularly affecting tropical and subtropical regions. (Moreira-Filho et al., 2021; Munday et al., 2020; Saidu et al., 2023).

According to the World Health Organization (World Health Organization: WHO, 2021), approximately 229 million people are currently infected with this parasite worldwide. Among them, about 20 million people are facing serious repercussions due to advanced schistosomiasis. According to WHO, almost 99 million individuals were treated around the world, with the African region shouldering 88% of the worldwide burden (World Health Organization: WHO, 2018). In this line, more efforts should have been made to decrease these numbers. As far as we know, schistosomes don't possess the genetic adaptability that microbes have. This lack of genetic plasticity could contribute to the increased severity of schistosome infections, although this relationship has not been fully understood or defined yet (Wilson, 2012).

In today's context, the schistosome species that are recognized as harmful to humans include *S. japonicum* (identified in 1904), *S. mansoni* (discovered in 1907), *S. intercalatum* (recognized in 1934), *S. mekongi* (identified in 1978), and *S. haematobium*, which was first described in 1852 (Inobaya et al., 2014). The prevalence of schistosomiasis is notably high in impoverished regions of tropical South America, Africa, the Arabian Peninsula, and Asia, as reported by the Centers for Disease Control and Prevention (CDC, 2019). The two main species, *S. mansoni* and *S. haematobium*, are frequently reported in the African region, Arabian Peninsula as well as South America (especially *S. mansoni*). On the other hand, *S. japonicum* is mostly found in China, the Philippines, and Indonesia (Magalhães et al., 2014). In addition, *S. intercalatum*, *S. mekongi*, and *S. guineensis* are known to be prevalent in

restricted areas (World Health Organization: WHO, 2018). Due to the high egg and adult worm burden in the water bodies, children are more likely to get infected due to regular communal and fun ventures into infected water bodies. However, in adults and teenagers, the likely routes of transmission of this disease include fishing and farming (McManus et al., 2018). In this line, the most prevalent targets of infection are the intestine (small and large), liver, kidney, and immune system. I will describe the life cycle of the parasite in detail in the next sections, particularly "Life Cycle of *Schistosoma*".

1.2 History

The oldest written literature about schistosomiasis has been found in Egypt where many medical papyri dating from around 1500 B.C. have referenced the 'ā – a – ā disease', conceivably an old name for schistosomiasis, and portrayed an ailment described by release from the penis (Di Bella et al., 2018). The antiquated Egyptians were urged to maintain a strategic distance from contaminated water to stop this disease and they were also educated to wear penile sheaths made up of linen, realities which take after the course of schistosomiasis. Later, a strangely high occurrence of haematuria was detected by Prospero Alpini (1553–1616) in Egypt. Theodore Maximilian Bilharz (1825 to 1862), who conducted autopsies on infected Egyptians, became the first scientist to discover the disease after identifying schistosomal bladder worms and portal system which he described as "pointed terminal projection" and called it *Distomum (Schistosoma) haematobium* (Tan & Ahana, 2007). Interestingly, the name schistosomiasis (schistos=split and soma=body) was given by David Friedrich Weinland in 1858. However, the presence of a halfway host to support schistosomiasis's life cycle was first viewed as recently as 1902. In 1918, Robert Thomson Leiper understood *Schistosoma*'s life cycle and acknowledged sweet water snails as transitional hosts of these trematodes (Di Bella et al., 2018).

The symptoms of schistosomiasis were first described by a Chinese Medicine practitioner named Yoshinao Fujii (1818 to 1895) in 1847 (Pesigan et al., 1958). In 1904, Fujiro Katsurada, a pathologist at Okayama Medical College in Japan, discovered a worm in the portal vein of an infected cat and subsequently named it *S. japonicum*. Later, in 1907, Luigi Westenra Sambon from the London School of Tropical Medicine identified a third *Schistosoma* species and named it *Schistosoma mansoni* in honor of Patrick Manson (1824-1922), who was at the time renowned as the 'father of tropical medicine' (Inobaya et al., 2014).

In 1910, Marc Armand Ruffer diagnosed the infection of *Schistosoma haematobium* in mummies for the first time. This author reported the occurrence of countless calcified eggs of *S. haematobium*, generally surrounded by the straight tubule (Ruffer, 1910). Decades later, Deelder *et al.* (1977) used an immunodiagnostic test, ELISA, to report the circulating anodic antigen of schistosome in the cheeks, guts, and skin of the mummies. This study provides an alternate way to detect schistosomiasis using other parts of the mummy when viscera either are not kept properly or not available for direct detection of the parasite (Deelder et al., 1977). Recently, in 2014, a PCR-based method to detect DNA of the *Schistosoma* parasite in mummies was reported (Matheson et al., 2014).

1.3 Epidemiology and Distribution of *Schistosoma*

Schistosomiasis is a common disease in the tropics and sub-tropical regions of the world. This disease is also considered a neglected tropical disease (NTD) and it is rated second after malaria (Inobaya et al., 2014). Globally, more than 240 million individuals are affected by schistosomiasis causing almost 70 million disability-adjusted life years (Olveda et al., 2017; World Health Organization: WHO, 2016). Moreover, approximately 600 million people are at risk of developing the infection (Vale et al., 2017). The African region and the Eastern Mediterranean are disproportionately affected by the disease with 42 and 16 countries, respectively, being endemic for the infection. Other regions include 10 countries in America, 6 in the Western Pacific, and 3 countries in Southeast Asia. For example, Turkey and Corsica are affected by the disease in the European region, as described in Fig. 1 (World Health Organization: WHO, 2013). Particularly in Saudi Arabia, *S. mansoni* and *S. haematobium* are the major species implicated in schistosomiasis (Lotfy & Alsaqabi, 2010). The global distribution of *Schistosoma* species is shown in Figure 1, and the schistosomiasis distribution in 2010 is shown in Figure 2.

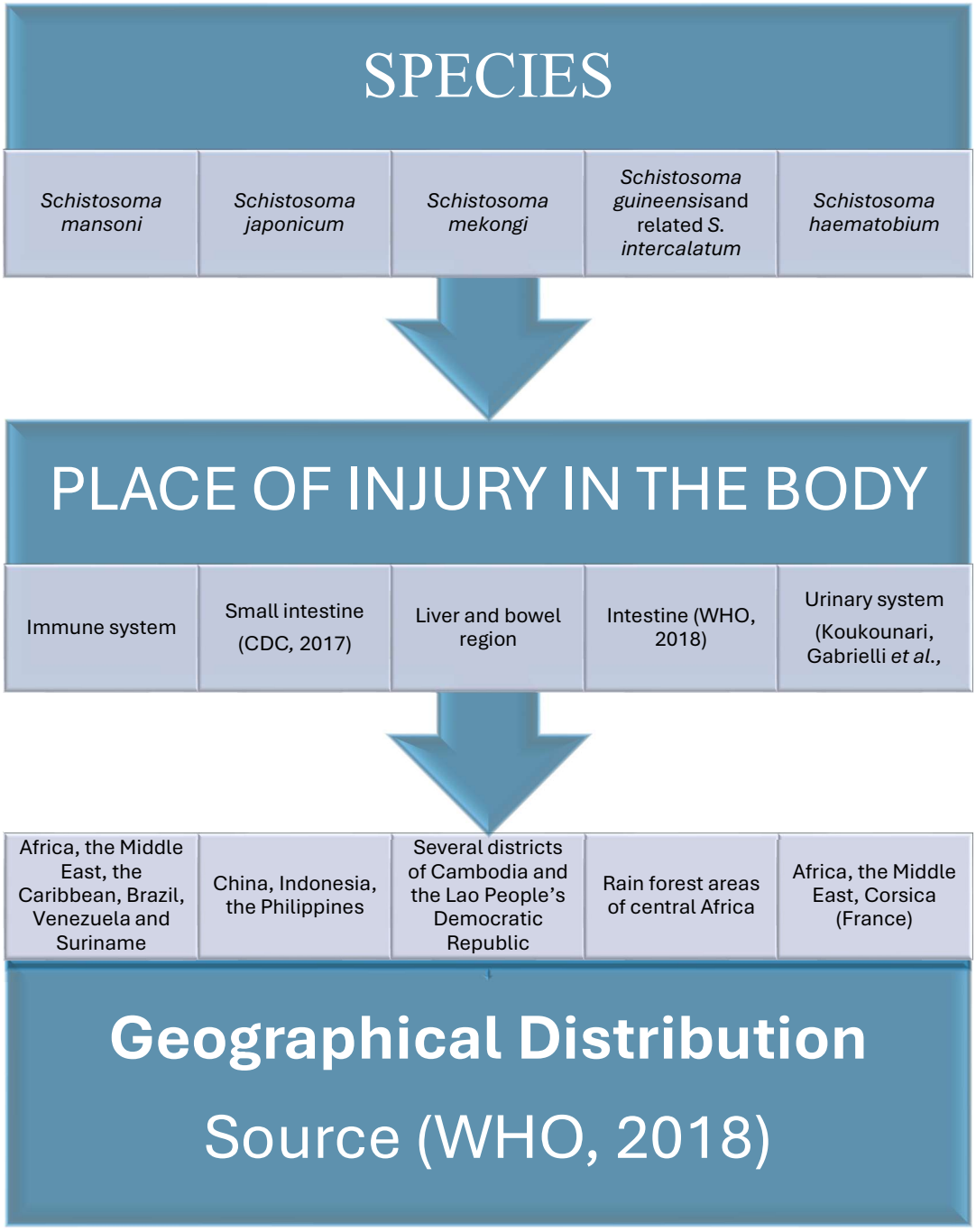


Figure 1-1: Representation of *Schistosoma* species and their geographical distribution (WHO, 2018).

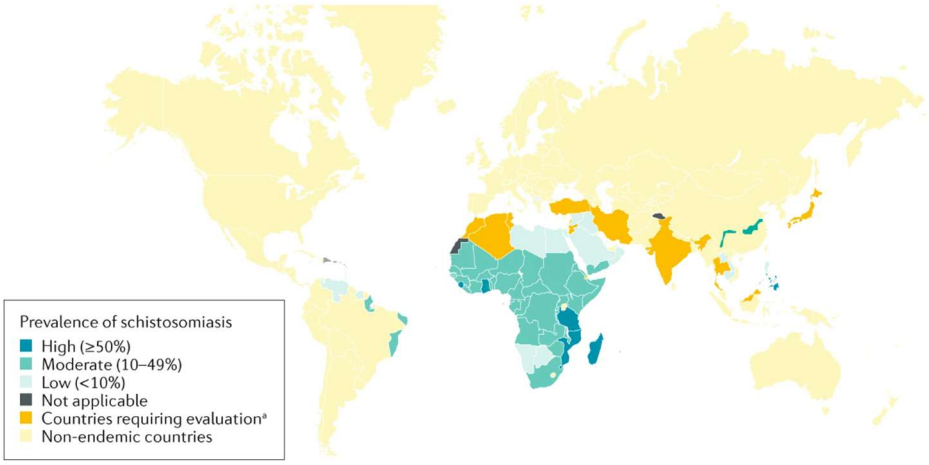


Figure 1-2: Worldwide geographical distribution of schistosomiasis in 2012 (McManus et al., 2018).

1.4 Life Cycle of *Schistosoma*

The human and the snail act as the hosts for the schistosomiasis parasites. The life cycle of the parasite begins immediately when its eggs are excreted through faeces or urine into the environment (Colley & Secor, 2014). Freshwater aids in hatching of the eggs to miracidia which remain dormant for 6-12 hours swimming using ciliary movement to find freshwater snails which are their intermediate hosts. The snails provide an ideal environment for the parasites because of the soft tissue which is easy to penetrate. To our knowledge, various species types of freshwater snails transmit specific *Schistosoma* species. These include *Biomphalaria*, *Bulinus*, and *Oncomelania* for *S. mansoni*, *S. haematobium*, and *S. japonicum*, respectively (Deribew et al., 2022).

In the next stage, after hatching and penetration of the snail, the parasites start multiplying. This process begins by losing the miracidium's cilia and then growing into a mother sporocyst which produces a daughter sporocyst through the asexual process (Parker-Manuel & Wilson, 2022). The daughter sporocyst inhabits the hepatic and gonadal tissue of the snail where it develops into cercariae after 2-4 weeks through metamorphosis (Gryseels & Strickland, 2013). After some time, the fork-tailed cercariae swims out of the snail under light stimulation (Gryseels, 2012). The cercariae swim through the water using their forked tails until they encounter a mammalian host, which they penetrate through the skin. According to Gordon et al. (2012), *S. japonicum* can infect over 40 species of mammals, which act as reservoir hosts. The parasite manages to penetrate the human or mammalian skin by secreting proteolytic enzymes and employing mechanical action (Ross et al., 2007).

The transmission cycle involves larvae-contaminated water, which, upon contact with human skin, allows the larvae to penetrate and initiate infection (McManus et al., 2018; Moreira-Filho et al., 2021; Munday et al., 2020). In the body, the larvae develop into adult schistosomes which migrate into the lumen of the intestine or bladder. It is important to note that some species infect faster than others. For instance, *S. japonicum schistosomulae* are found in the dermis within only 2 hours of exposure and faster ones reach the dermal blood vessels within the same time (Nation et al., 2020). On the other hand, *S. mansoni* and *S. haematobium* are slower in terms of penetrating the dermis and take up to 48 hours to penetrate the skin layers and another 72 hours to reach dermal blood vessels. Schistosomulae migrate to the lungs and the heart from the dermis through the lymphatic vessels (Nation et al., 2020). The parasites then leave these organs of the body and migrate to vestibule circulation or portal vessels until maturity and mating. The *Schistosoma* worms inhabit and breed in different body parts of the reservoir host. For instance, *S. japonicum*, *S. mekongi*, and *S. intercalatum* parasites stay in the mesenteric and the portal vessels while *S. haematobium* inhabits the vesical plexus (Maguire, 2010).

In this environment, the females produce eggs, which are then released from the body through urine or faeces, completing the parasite's life cycle (Silva, 2016); other eggs are trapped in the body's tissues, leading to an immune response that can result in chronic inflammation, organ damage, and a range of health issues (Zheng et al., 2023).

The adult male and female parasites mate and reproduce in these locations. The female schistosomes take about four to six weeks after penetrating the human skin to start laying eggs. Male worms embrace the female within the gynaecophoric canal during mating. *S. haematobium* worms, however, take a longer time to start laying eggs (9 weeks) (Jenkins-Holick & Kaul, 2013). According to Bustinduy and King (2013), the worms breed their entire lifetime, and it is estimated that female *S. mansoni* and *S. haematobium* worms produce about 300 eggs while *S. japonicum* produces the highest number of eggs at around 3000. Nearly half of them are excreted through the faecal and urethral process where the life cycle begins. The eggs that stay inside the human body cause inflammation, granuloma, and fibrosis. Figure 3 shows the entire life cycle of the *Schistosoma* species (McManus et al., 2020).

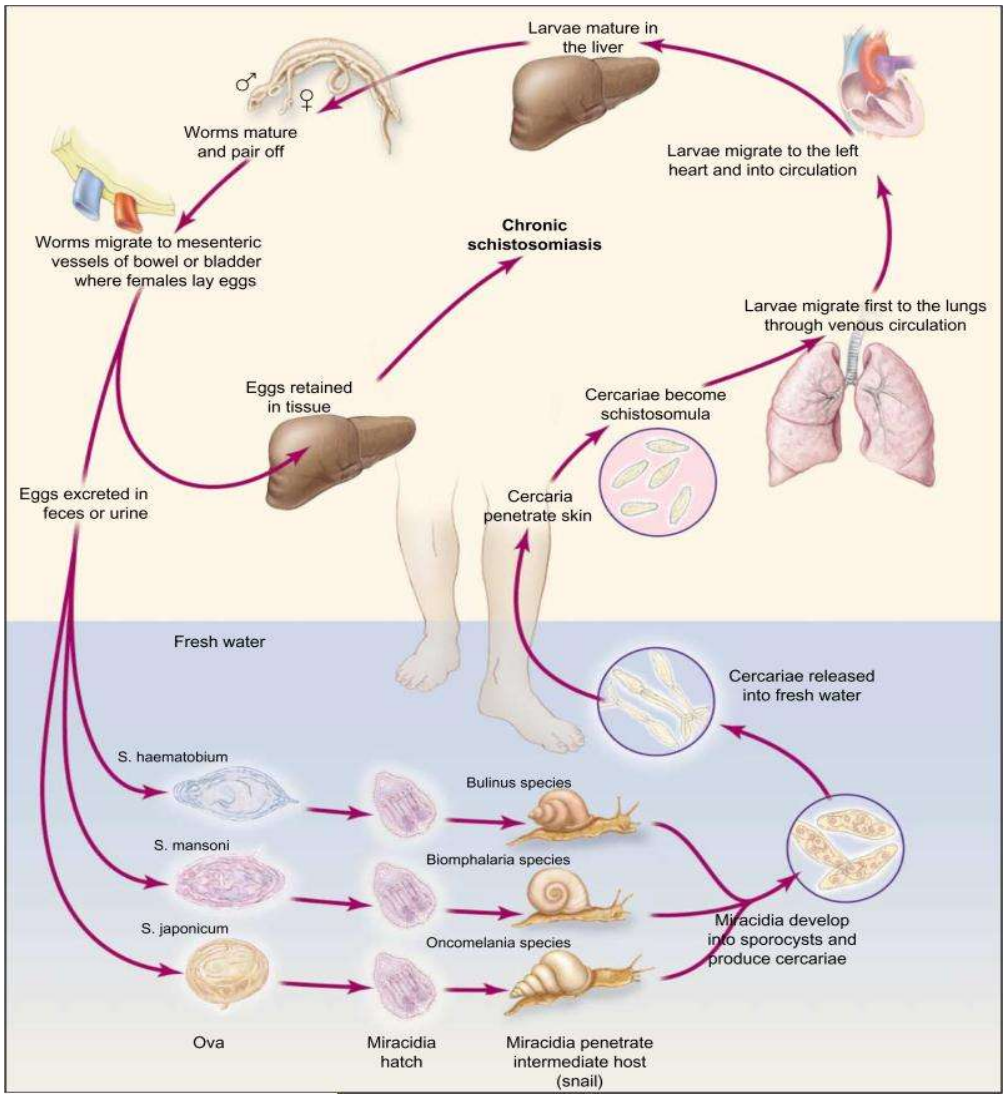


Figure 1-3: The life cycle of schistosomiasis parasites. Adapted from McManus et al., (2020) and Ross et al., (2002).

1.5 Control and Treatment of Schistosomiasis, Chemotherapy, and Vaccines for controlling schistosomiasis

1.5.1 Control and Treatment of Schistosomiasis

Despite many attempts made, praziquantel (PZQ) remains the leading treatment drug for human schistosomiasis, being used for almost all *Schistosoma* types capable of infecting humans (Munday et al., 2020; Nogueira et al., 2022; WHO, 2026).

Although it plays an essential role in controlling schistosomiasis, PZQ demonstrates some critical disadvantages. Firstly, the drug is much more efficient at killing adult forms of parasites than juveniles, limiting its efficacy and making possible transmission persist in endemic areas (Nogueira et al., 2022; CDC, 2024; WHO, 2026).

The molecular mechanism of action of praziquantel has not yet been fully resolved. Based on current evidence, this molecule uses a variety of factors to affect schistosomes' physiology. According to one of the most reliable models, PZQ immediately causes calcium overload in parasites, leading to muscular contraction, damage to teguments, paralyzation and subsequent elimination of affected parasites (Doenhoff et al., 2008; Nogueira et al., 2022).

Based on the available data, it would be inaccurate to state that there is clear evidence either of the existence of praziquantel resistance or its absence. Reduced susceptibility and drug failure cases have already been noted in certain field or laboratory settings. Besides, reduced susceptibility can be experimentally selected for. Nonetheless, recent literature reviews demonstrate that there are no cases of praziquantel resistance developed in endemic regions, and the problem remains relevant. Therefore, the most appropriate statement would be that reduced susceptibility exists but not full-fledged praziquantel resistance (Doenhoff et al., 2008; Eastham et al., 2024).

As a rule, the standard dosage of praziquantel applied within the treatment programmes against schistosomiasis is 40 mg/kg, which is typically administered in a single oral dosage. It should be stressed that in addition to species and age, various programs also use different treatment dosages. It should be mentioned that praziquantel is currently the only drug applied on a large scale in the treatment of schistosomiasis. Consequently, because of its disadvantages, the need for the identification of new drugs is obvious (WHO, 2024; WHO, 2026; Munday et al., 2020).

One of alternative agents, which is not widely applied but has proved to be successful, is oxamniquine, which has been primarily used in the treatment of S. mansoni. The activity of this molecule relies on the presence of a schistosome-specific sulfotransferase; hence, it cannot be applied in relation to other species. As a result, oxamniquine remains a relevant but non-preferred drug for schistosomiasis control (Inobaya et al., 2014; Alwan et al., 2023).

In general, current methods to fight schistosomiasis rely mainly on the use of praziquantel even though this drug has critical disadvantages. In this regard, since there are many parasite-specific physiological targets and regulatory mechanisms, the development of drugs that affect their function is highly relevant. For example, the importance of targeting phosphodiesterases that regulate cyclic nucleotide signaling is increasingly evident due to its regulatory properties (Munday et al., 2020; Zheng et al., 2023).

1.5.2 Chemotherapy

Up to 1975, schistosomiasis chemotherapy depended on the utilization of several medications focusing on the DNA synthesis and metabolism of carbohydrates and protein. After that, OXA and PZQ were applied for the treatment (Harder, 2002). The latter is utilized as the first choice of therapy and is registered by the WHO (World Health Organization: WHO, 2016). However, the drug alone is not capable of preventing schistosomiasis infection-associated lesions such as hepatic fibrosis and bladder deformities (Colley et al., 2014). So, the therapies based on the combination of anthelminthic, antimalarial, and antioxidants were investigated against schistosomiasis (Gouveia et al., 2018).

The tegument is a protective covering that is reconstructed every six hours (Shaw & Erasmus, 1988). It is critical in osmoregulation, nutrient uptake, immunity, and excretion (El-Shabasy et al., 2015). It is also very important to ensure the infection and survival of the parasite inside a human (Skelly & Wilson, 2006). Due to these factors, the tegument has been extensively studied since the late 1960s. The development of vesicles around the tubercles and loss of thistles is observed when the parasite is exposed to treatment either with a subcurative dose of PZQ or with a single vaccination of attenuated cercariae (El-Shabasy et al., 2015). Furthermore, De Oliveira *et al*. (2012) explored the medication's effects and found that both male and female worms showed tegumental changes when exposed to essential oil of the medical plant *Baccharis trimera* in vitro assays.

1.5.3 Vaccines for controlling schistosomiasis

To our knowledge, vaccines are a potent tool for controlling infectious diseases. In this line, several vaccine candidates for schistosomiasis have been evaluated by different authors during the last few years (Molehin et al., 2022; Tebeje et al., 2016). So, schistosomiasis vaccines are designed to stimulate the immune system to recognize and destroy the parasite.

Despite the development of several potential vaccines over the past few decades, none have been licensed for human use to date. (Molehin et al., 2022; Sanches et al., 2021). Only two molecules, fatty acid-binding protein (Sm14) & *S. mansoni* tetraspanin-2 (Sm-TSP-2) have entered human clinical trials (Molehin et al., 2022; Tebeje et al., 2016). According to Rehman *et al*. (2021), the development of the *Schistosoma* vaccine is complicated by the parasite's complex life cycle. Even though many vaccines focus on the early larval stages of the parasite, it would be more effective to develop a vaccine that targets adult worms. Several potential vaccine candidates for schistosomiasis have been identified, including *S. mansoni*

cercarial elastase (SmCE) which is essential for the parasite's penetration of the skin, and tetraspanins that play a crucial role in the parasite's development and immune evasion (Anisuzzaman & Tsuji, 2020; El-Faham et al., 2017). However, more research is required to better understand the immune response required to control *Schistosoma* infection.

Other potential vaccines being evaluated for schistosomiasis include glutathione S-transferases (GSTs), which are enzymes involved in the parasite's detoxification process for oxidative stress, and the schistosome egg antigen (SEA), which is released by the parasite's eggs and is responsible for the chronic inflammation and tissue damage associated with the disease (Anisuzzaman, & Tsuji, 2020; You et al., 2018).

One of the most promising approaches to vaccine development is based on the identification of antigens which are typically involved in the interaction between the parasite and its host (Langenberg et al., 2020). Moreover, the use of attenuated or genetically modified parasites is another interesting option (Fukushige et al., 2015) as they are modified to reduce their virulence while still retaining their ability to stimulate the host's immune system. This approach has the advantage of allowing the production of large quantities of antigens for use in vaccine development and can potentially improve the immune response to the parasite.

Several vaccines based on these methods have been tested in animal models and clinical trials. One of the most promising candidates is the Sm14 vaccine, which is based on the surface antigen Sm14 (Tendler et al., 2018). According to the same authors, the Sm14 vaccine has been shown to induce protective immune responses in animal models of schistosomiasis in Brazil (Tendler et al., 2018). Recently, Santini-Oliveira *et al.* (2022) developed a new promising Schistosomiasis vaccine candidate, utilizing the Sm14/GLA-SE formulation, which has demonstrated favorable outcomes in trials involving healthy young women in Brazil.

1.6 The potential of phosphodiesterases (PDE) of *S. mansoni* as drug targets

1.6.1 PDE structure and diversity

PDEs play an important role in cellular signalling by regulating the levels of intracellular cyclic nucleotides (cAMP and cGMP) (Uzair et al., 2018). These enzymes are ubiquitous and can be found in all organisms, from bacteria to humans.

PDEs are classified into 11 distinct families based on the following criteria: amino acid sequence, structure, and biochemical properties (Lee et al., 2020). These families include PDE1-11, each with a unique regulatory mechanism and specificity for cAMP and cGMP hydrolysis. Human PDE1, PDE2, and PDE3 families are selective for cGMP or cAMP, while PDE4, PDE7, and PDE8 families hydrolyse cAMP preferentially. On the other hand, PDE5, PDE6, PDE9, and PDE10 families similarly act on both cAMP and cGMP.

The structure of PDEs is characterized by a conserved catalytic domain, which modulates PDE activity through interactions with regulatory molecules, such as protein kinases and phosphatases, and other signalling molecules (Lim et al., 2019). The regulatory domain is important for the modulation of enzyme activity by external signals. Its composition can vary depending on the type of enzyme. For example, the PDE7 family has an additional domain, the glo domain, that is responsible for the binding of cAMP and regulating its hydrolysis.

Several studies have investigated the potential of PDE inhibitors as antischistosomal agents. For example, Botros et al. (2020) showed that the PDE inhibitor rolipram had a significant inhibitory effect on the motility of adult *S. mansoni* in vitro.

While the potential of targeting cAMP and PDEs as drug targets for the treatment of schistosomiasis is promising, several challenges and limitations exist. One major challenge is the identification of selective and potent inhibitors that target specific PDE isoforms in *S. mansoni* without affecting host PDEs. This is crucial to minimize off-target effects and potential toxicity. Furthermore, the use of PDE inhibitors may also affect other signalling pathways that are regulated by cAMP, leading to unintended consequences.

Cyclic nucleotide phosphodiesterases hydrolyse cAMP and cGMP to produce 5’-AMP and 5’-GMP, respectively (Conti & Beavo, 2007). cAMP and cGMP are second messenger signalling molecules responsible for influencing signalling pathways and managing intracellular calcium concentration in both diseased and healthy individuals (Francis et al., 2011; Kametani & Haga, 2015). In this line, the function of these PDEs is to harmonise the cellular concentration of cAMP and cGMPb (Kametani & Haga, 2015).

Several reasons make PDEs a good drug target. First, the architecture of the PDE enzymes is unique at the active sites, making it possible to develop selective inhibitors. Secondly, in contrast to kinases, the endogenous levels of cAMP and cGMP substrates for PDEs are

relatively low, typically ranging from less than 1 to 10 μM, making them susceptible to inhibition by PDE inhibitors at low concentrations. Additionally, PDE has various N-terminal control domains and shares a C-terminal catalytic domain (Omori & Kotera, 2007). There are several drugs, for various medical conditions, on the market that are based on the inhibition of mammalian PDE, including cilostazol (Gupta et al., 2020) and sildenafil (Huang, 2011) that specifically inhibit PDE3A subtype and cGMP-specific PDE type 5, respectively.

The most studied PDE is one that exclusively hydrolyses cAMP molecules (Klussmann, 2016). Human PDE4 has been considered a potential drug target because it has extensive significance in forming new blood vessels, brain function, and responses to stress and inflammation. PDE4 has shown promising results as a target for selective inhibitors, particularly in vitro and in vivo models of depression, asthma, Alzheimer's, and Parkinson's diseases (Eskandari et al., 2015; Klussmann, 2016). Currently, clinical investigations and studies with animal models are trying to reverse a decline in cognitive function and Alzheimer's disease (Heckman et al., 2015).

Various PDEs including PDE4 have been widely investigated, particularly for their potential therapeutic functions in parasitic protozoa (Shakur et al., 2011). For instance, *Trypanosoma brucei* expresses five PDEs, some of which have been confirmed as druggable targets, specifically TbrPDEB1 and TbrPDEB2 (Bland et al., 2011). The chemical confirmation and validation of the two targets were performed with the use of phenylpyridazinones and a series of catechol pyrazolinones (Orrling *et al*., 2012). In *Trypanosoma cruzi*, TcrPDE4 (TcrPDEB1), an ortholog of HsPDE4 (*Homo sapiens* PDE4), showed a characteristic inhibition profile of the PDE4 subfamily and specificity for cAMP over cGMP (Li et al., 2018). In some cases, PDE has been shown to contain valuable therapeutic targets including in *Leishmania* (Seebeck et al., 2011). The validation of *trypanosomal* PDEs as drug targets revealed several powerful hits that are utilized in selectivity over human PDE4 (Blaazer et al., 2018; de Heuvel et al., 2019). Thus, the *T. brucei* PDE4-equivalent TbrPDEB is essential in the compartmentalization and regulation of cAMP signalling. These processes are vital in the transformation, proliferation, and differentiation of the parasite (de Koning et al., 2012).

In *S. mansoni*, cAMP and cAMP-dependent protein kinases influence the movement of cilia in the miracidia stage (Matsuyama et al., 2004). Moreover, adenylyl cyclase modulators hindered the conversion of miracidia into mother sporocytes, and the transformation gets delayed when the miracidia are exposed to general PDE inhibitors (Long et al., 2017). This

study also revealed that the inhibition of *S. mansoni* PDE4 (SmPDE4) is related to the anti-parasitic activity. In this line, PDE inhibitors showed promising potential against schistosomiasis, mainly targeting parasite ovipositing.

Moreover, it has been reported that a series of oxaborole inhibitors of human PDE4 also inhibited schistosomal PDE4, causing hyper-motility and degradation of *S. mansoni* in vitro (Long et al., 2017; Zheng et al., 2023). Expressing this enzyme in the *Caenorhabditis elegans* PDE4-mutants restored their sensitivity to these inhibitors (Long et al., 2017). According to the same authors, four orthologs of SmPDE4 were identified as well as a link between recombinant SmPDE4A inhibitory EC50 values and the anti-schistosomal implications of benzoxaboroles's series. They reported that human PDE4 inhibitor roflumilast also inhibits SmPDE4A at a sub-nanomolar concentration (Long et al., 2017).

Another class of therapeutic targets used in the treatment of parasitic diseases such as schistosomiasis are the protein kinases (Doerig, 2004). They play an important role in eukaryotic organisms through phosphorylation of substrate proteins. The protein is involved in various biological processes which include gene expression, metabolism, apoptosis, and cellular proliferation (Hanks, 2003). Excessive and unregulated levels of protein kinases have been associated with chronic diseases such as inflammation, autoimmune diseases, and cancer (Colley & Secor, 2014). There is strong evidence that protein kinases can be used to develop anti-parasitic drugs since eukaryotes, protozoa, and helminths use them to control cellular functions (Bahia et al., 2007; LoVerde et al., 2009). Some of the potential therapeutic targets in protein kinases include cyclic guanosine monophosphate- (cGMP-) dependent protein kinases (PKGs) of *Toxoplasma* (Donald et al., 2002). While several protein kinases have been viewed as effective drug targets for years, only 40 protein kinases of *S. mansoni* have experimental functional evidence (Gava et al., 2019). However, the same authors mentioned that there is still a need for much further research.

A significant anti-parasitic effect has been observed in vivo when protein kinases were inhibited, specifically *Plasmodium* (Diaz et al., 2006) and a *Plasmodium* cAMP-dependent PKA (Knockaert et al., 2000). Cyclic nucleotide second messengers are produced by purine nucleotide cyclases and regulate cyclic nucleotide-dependent protein kinases among other proteins. Furthermore, Taylor et al. (2008) note that the activity of the kinases can be manipulated by regulatory cyclic nucleotide-binding (CNB) domains with cyclic nucleotide analogs.

cAMP-dependent protein kinases have been studied in both male and female *Schistosoma* parasites and have localized integument, somatic musculature, seminal vesicle, and gynaecologic muscles consistently. Polo-like Kinases (PLK1) and sak kinases, in *S. mansoni*, have been found as the proteins controlling gametogenesis and involved in gonad development, respectively (Dissous et al., 2011). Some other proteins like ERK kinases are also approved drug targets as they are involved in vital processes like worm coupling, reduction in egg release, and homeostasis (Ressurreição et al., 2015). Recently, the characterisation of various cytosolic and receptor protein tyrosine kinases in *S. mansoni* has proposed a new outlook for the development of novel chemotherapy for the disease.

The c-Jun N-terminal kinase (JNK) is another important signalling pathway, which is involved in the regulation of developmental processes in various organisms (Gava et al., 2019). Its role in egg and embryo development has already been demonstrated by Bagowski *et al*. (2001). These protein kinases have also been found to be involved in assembling the spindle during meiosis mouse egg development (Huang, 2011). In *S. mansoni*, JNK protein (SmJNK) (Smp_172240) was characterized by de Andrade et al. (2014). Knockdown of SmJNK damages the tegument of adult worms and disrupts the maturing of vitellin organs. As a result, the oviposition was lowered, and the worm became more susceptible to the host immune system. In *S. haematobium*, the SmJNK ortholog has been reported as a fundamental druggable kinase target (Gava et al., 2019). This finding was based on its essentiality for the survival of the parasite as shown by knock-out or lethal gene knockdown attributes in different species (Stroehlein et al., 2015).

Schistosomes have a well-developed central nervous system (CNS) which includes serotonin, the most widely distributed neurotransmitter in the *schistosoma*l nervous system (Boyle et al., 2000). In some studies, serotonin has been implicated in the parasite's motility and in the sexual differentiation of schistosomes, regulating the processes that lead to the development of male and female schistosomes (Mair et al., 2000).

1.6.2 PDE Signalling pathways

Signal transduction is the process of transmitting molecular signals from the exterior of cells to the interior of cells. Molecular signal transmission is important because it provides physiological balance. The *Schistosoma* parasite has been reported to possess various signal transduction mechanisms during its life cycle (Inobaya et al., 2014; Nation et al.,

2020). Figure 4 represents signal transduction mechanisms, particularly protein kinases, that are known to be involved during the life cycle of a typical schistosome.

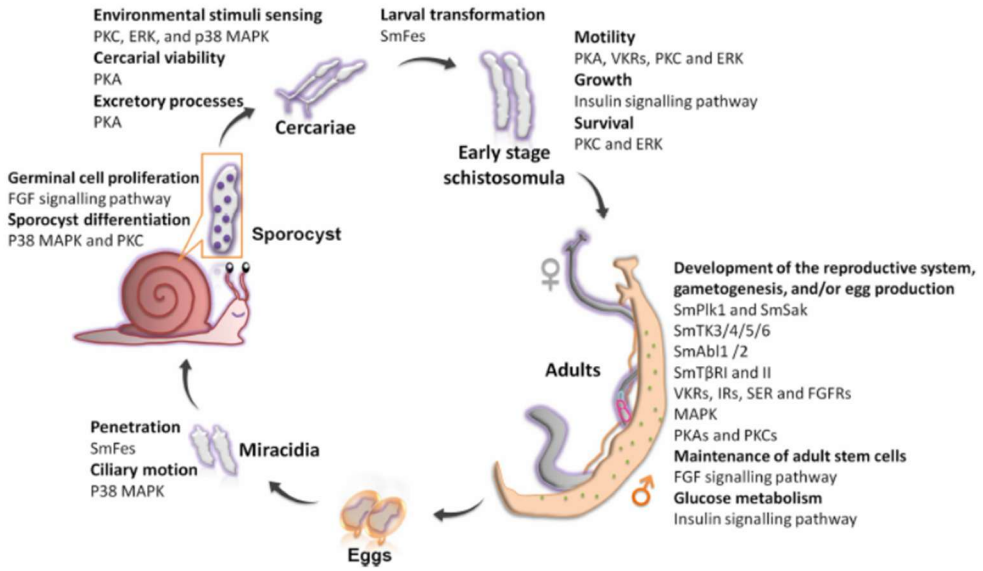


Figure 1-4: Signal Transduction mechanisms in various life functions of a schistosome. Adapted from Du et al. (2017).

The germinal and adult stem cell functions are maintained by the fibroblast growth factor (FGF) signalling pathway in schistosomes. In the initial phases of schistosome development, the cAMP-activated Protein Kinase A (PKA) is responsible for the parasite's movement and excretion processes (Hirst et al., 2016). Moreover, the regulation of ciliary beat is orchestrated through the MAP kinase pathway, as described by (Ressurreição et al., 2015). On the other hand, the tyrosine kinase SmFes, present in the cytoplasm, is involved in the penetration of larvae into the snail host (Bahia et al., 2007). The homeostatic factors of the schistosome including phenotype and motility are regulated by the extracellular signal-regulated kinase (ERK) signalling pathway and Protein Kinase C (PKC) (Ressurreição et al., 2015). Glucose metabolism has been associated with Insulin-like peptides in both *S. mansoni* and *S. japonicum* (Du et al., 2017).

It is important to note that SmRas plays an important role in the development and proliferation of the parasite, mainly in females. In this line, the development and reproduction of female worms are dependent on the presence of male parasites (LoVerde et al., 2009). There are various genes encoding signal transduction functions in a localized tegument. These include the transforming growth factor (TGF)-β family receptors (Davies et al., 1998), epidermal growth factor receptors (Shoemaker et al., 1992), Ras pathway components (Santos et al., 2002), and receptor serine/threonine kinases. The signal transmission in the schistosome goes through intracellular Smad proteins (Beall et al., 2000; Osman et al., 2004) to nucleic receptor sites (Bertin et al., 2005; Freebern et al., 1999). The research on the sexual reproduction and development of schistosomes is pivotal in understanding its signal transduction pathways.

Our body's G-protein-coupled receptors (GPCRs) are fundamental in controlling our reactions to hormones, neurotransmitters, and external stimuli, influencing key physiological functions (Rosenbaum et al., 2009). According to the same authors, these receptors have significant potential as therapeutic targets for treating a wide range of diseases.

Once activated, these receptors couple with a G-protein and initiate a cascade of events that leads to the formation of cyclic AMP (cAMP) and cyclic GMP (cGMP). These molecules then diffuse through the cytoplasm and bind to one of the 11 enzymes within the phosphodiesterase family. Once bound to a PDE enzyme, the cAMP or cGMP molecules are broken down, resulting in the production of AMP and GMP (Calamera et al., 2022). This process is essential to properly regulate various cellular processes and responses, such as apoptosis, cell division, migration, and differentiation. Some of these processes, PDE inhibitors are illustrated in Figure 5.

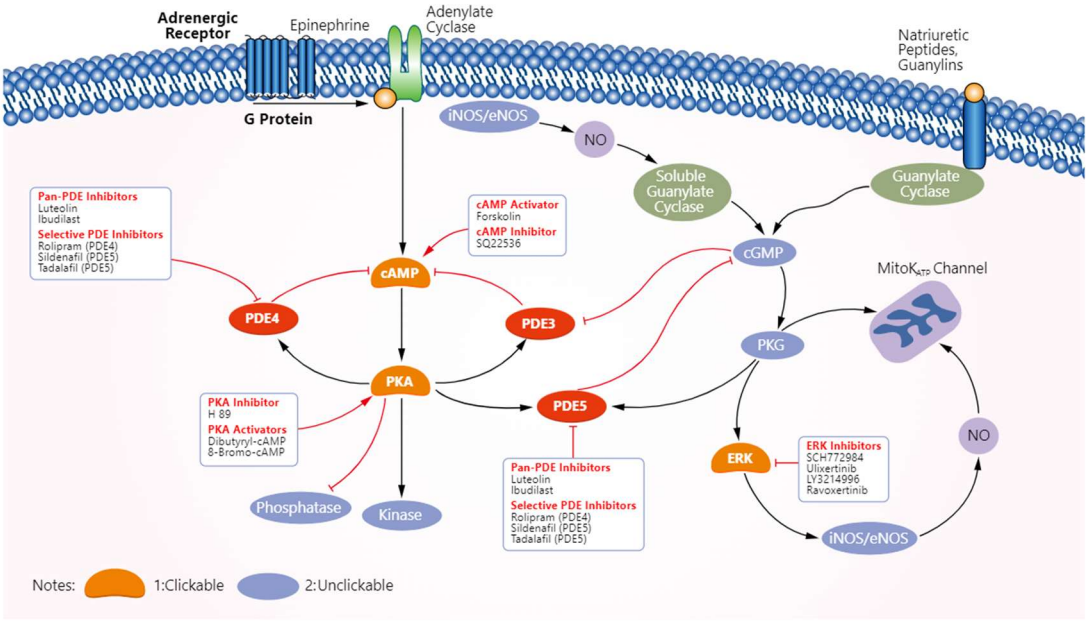


Figure 1-5: PDE signalling. Adapted from (Lim et al., 2019).

1.6.3 Other signalling components

As highlighted previously, schistosomes possess multiple AC isoforms that are differentially expressed and regulated during different stages of their life cycle (Halls & Cooper, 2017). Thus, targeting ACs could provide an alternative approach for disrupting the cAMP signalling pathway and interfering with the parasite's physiology.

Schistosomes possess a wide range of protein kinases that are involved in various biological processes; some of them are related to the cAMP signalling pathway including PKA, PKC, and MAPKs. PKA is a major downstream effector of cAMP, and its activation leads to the phosphorylation of various target proteins, including ion channels, transcription factors, and enzymes (Ressurreição et al., 2015). PKC, on the other hand, is activated by diacylglycerol (DAG) and calcium ions and is involved in regulating membrane trafficking, cell proliferation, and apoptosis.

MAPKs are a family of serine/threonine kinases that are activated by various extracellular signals, such as growth factors, cytokines, and stress stimuli, and are implicated in mediating cellular responses to these signals (Hirst et al., 2016). Some of them that are expressed in schistosomes including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK.

Sm5-HT7 (serotonin type 7) appears to be involved in the regulation of smooth muscle contraction in *S. mansoni*, which is important for the parasite's movement within the host (Patocka et al., 2014). It is noteworthy that these researchers were the first to deliver molecular evidence demonstrating the existence of a functional serotonin receptor (Sm5HTR) in *S. mansoni*. Interestingly, serotonin (5-HT) stimulates movement via this important neuronal protein, which plays a significant role in the motor control apparatus of *S. mansoni*. According to Kim et al. (2013), it plays an important role in the modulation of immune responses and reduction of the severity of intestinal inflammation. This situation is related to the parasite's ability to move within the host, making it a potential drug target for chemotherapy.

1.7 The potential of cAMP phosphodiesterases of *S. mansoni* as drug targets

To our knowledge, previous studies have demonstrated the potential of targeting smAC (*S. mansoni* adenylate cyclase*)*. According to Taft *et al.* (2010), increased cAMP levels promote by the adenylate cyclase activator forskolin or with the non-selective PDE inhibitor isobutylmethylxanthine (IBMX) affects miracidia, blocking/delaying larval transformation during the life cycle of *S. mansoni*. RNAi-mediated knockdown of smAC expression led to impaired glucose uptake and reduced viability of adult parasites (Hunter et al., 2021; Tedla et al., 2019). However, a recent study performed in other organisms has shown that inhibition of tmAC can lead to decreased cAMP levels and impaired intracellular signalling (Nguyen et al., 2021). For this reason, this enzyme showed to be a promising target for drug development. Overall, targeting ACs in *S. mansoni* represents a promising approach for developing new chemotherapy agents against schistosomiasis. In this line, further research in this field is required.

In recent years, PDE inhibitors have also gained attention as potential therapeutic agents against parasitic diseases. The identification of the PDE enzymes involved in cAMP signalling in *S. mansoni* provides an opportunity to develop novel drugs specifically affecting the parasite based on 11 PDEs (SmPDE1-11). Several studies have investigated the impacts of PDE inhibitors on the viability and development of *S. mansoni*. For example, the PDE5 inhibitor, sildenafil, has been shown to inhibit the development of this parasite by reducing the parasite's ability to invade the host (Zheng et al., 2023). Similarly, the PDE4 inhibitor, rolipram, has been shown to reduce the viability of *S. mansoni* larvae by increasing the level of cAMP in the parasite (Cheuka, 2022).

Long et al. (2017) worked on the characterization of four sequences of SmPDE4 (*Schistosoma mansoni* PDE4), namely SmPDE4A, SmPDE4B, SmPDE4C, and SmPDE4D, present in the genome and transcriptome of *S. mansoni*. They tested 1085 benzoxaborole compounds and two catechol-inhibitor drugs for activity against huPDE4 variants. These authors reported that some of those compounds inhibited PDE4 variants by causing hypermotility and degeneration of the parasite (Long et al., 2017).

In another experiment, performed by Botros *et al.* (2019), 265 compounds were tested for antischistosomal activity, and assessed in vitro. They reported that 64% of these compounds were effective against the disease and led to hypermotility and impairment of worm activities. Two compounds having promising in vitro activity were then tested for in vivo activity. When combined with praziquantel (PZQ), these compounds demonstrated increased effectiveness compared to PZQ alone, as evidenced by the elimination of immature eggs and a higher number of dead eggs. Moreover, the combination of PDE inhibitors with other drugs has been shown to enhance their efficacy against *S. mansoni* (Botros et al., 2020). As an example, the coadministration of sildenafil with PZQ has demonstrated enhanced efficacy against the immature stages of the parasite (Ferrari et al., 2003). Similarly, the combination of the PDE3 inhibitor, cilostamide, with artemether has been implicated in increasing the antiparasitic activity of artemether against *S. mansoni* (Abay et al., 2013).

Drug screening is associated with the systematic testing of potential drug candidates or existing compounds to determine their safety, efficacy, and therapeutic properties (Damiati et al., 2018; Lin et al., 2020). The first step is to identify compounds that exhibit desirable pharmacological activity. The process of drug screening typically begins with in vitro assays, where the candidate compounds are tested using isolated cells, tissues, or enzymes. This initial screening allows researchers to assess the compounds' interactions with specific molecular targets and determine their potential therapeutic effects. Promising compounds identified during this stage can then proceed to in vivo studies (Wang et al., 2020), using animal models to evaluate compounds' behaviour in complex biological systems.

In addition to in vitro and in vivo testing, drug screening also includes various preclinical and clinical trials. Preclinical trials involve further evaluation of the compounds' safety and efficacy in animals before advancing to human trials. Clinical trials, which involve human subjects, are conducted in different phases to assess the compounds' safety, dosage, effectiveness, and potential side effects. These trials are essential for obtaining regulatory approval and determining the overall benefit-risk profile of the drugs.

In Chapter 6 of this thesis, we will describe in detail the studied drugs (quinazoline, furan, iminothiadiazole, sulfide, piperidine, and imidazole-containing compounds) and their effects on *Schistosoma*.

1.8 Previous Functional Complementation Work on *Schistosoma mansoni* PDEs

One of the important components of the current research was based on Munday et al.'s (2020) paper on the first systematic cloning and functional characterization of *Schistosoma mansoni* phosphodiesterases (SmPDEs) in heterologous expression systems. Specifically, in this experiment ten out of eleven predicted SmPDE genes were cloned, classified based on homology to human PDE families, and expression profiling was performed for various mammalian host developmental stages. As a result, it was possible to establish an initial panel of core SmPDE genes, name them, and create a foundation for further exploration of their functions (Munday et al., 2020).

It is also worth mentioning that several of the described enzymes were proved to act as cyclic nucleotide phosphodiesterases using yeast complementation studies. Also, a new complementation model in *Trypanosoma brucei* based on tetracycline-induced expression of a heterologous PDE has been introduced in this study. Using this approach, it was shown that SmPDE4A could replace the function of essential TbrPDEB1/B2 locus in *T. brucei* but other SmPDEs tested in this initial version of the assay could not. Furthermore, SmPDE11 was shown to preferentially hydrolyse cGMP rather than cAMP, which explains why it failed to complement the cAMP-dependent *T. brucei* assay (Munday et al., 2020).

Nonetheless, some questions remained unanswered. To be more precise, only the role of SmPDE4A in the *T. brucei* complementation model has been confirmed; it was unknown whether other SmPDEs could function as complements in this model. Moreover, it was uncertain whether the inability of those enzymes to complement this particular test relied on the fact that it was impossible due to substrate preferences or catalytic activity, or if it could have something to do with subcellular localisation, expression levels, and other technical issues connected with the tagging used in the experiment. Despite success in inhibitor

screening of SmPDE4A, the pharmacology screen of other SmPDE family members had not yet been completed (Munday et al., 2020).

So, the primary purpose of this study is the extension of the mentioned functional analysis of SmPDEs and testing their potential for being applied in the *T. brucei* complementation model under modified experimental conditions and the development of a platform for broad inhibitor screening. In this way, the current project aims to fill the gap left open in the study by Munday et al. (2020).

1.9 Genomic Features

Genome-scale tools available for *Schistosoma mansoni* have been extremely beneficial for parasite gene discovery and evaluation. Indeed, the first genome sequencing results indicated that this organism possesses a large and complicated nuclear genome. Later annotation updates contributed to improving contiguity, correct chromosome assignment, and accuracy of gene modeling, thereby allowing more precise identification of the genes, exon/intron annotation, and comparative assessment of parasite enzymes compared to their counterparts in mammals (Berriman et al., 2009; Chevalier et al., 2024).

Schistosoma mansoni is characterized by a diploid genome composed of eight pairs of chromosomes (seven pairs of autosomes and one pair of sex chromosomes). While earlier cytogenetic analyses provided an initial understanding of genome organization, genomic investigation needs to be primarily based on contemporary reference assembly, rather than on size estimation only. Contemporary improvements in genome revision made such genome better applicable for the gene discovery, annotation, and function exploration (Oliveira and Johnston, 2001; Chevalier et al., 2024).

In this context, the work of Munday et al. (2020), focused on discovering PDE-encoding genes in the organism under discussion, has shown that *S. mansoni* contains eleven PDE family members representing various classes known in humans. Importantly, these authors also noted that the annotation of the genes in question needs additional confirmation through experiments, since some inconsistencies were detected between experimental PDE sequences and those predicted from genome. Such discrepancies are connected to variations in exon structure, coding sequence length, and domain composition in specific SmPDEs (Munday et al., 2020).

Importantly, the genome significance of discovered PDE-encoding genes is reflected not only in the total number of found SmPDEs but also in sequence features distinguishing some of them from their mammalian counterparts. Thus, Munday et al. (2020) discovered that several SmPDEs are characterized by the presence of atypical exon usage pattern and inserts in or near catalytic domains, which implies possible impact on their biological activity and properties (including sensitivity to inhibitors), which is critical for target validation purposes (Munday et al., 2020).

Therefore, genome of *S. mansoni* can be used for complete annotation of its PDE family, naming, and classifying individual PDE genes. However, even if a particular gene was found in genome and classified accordingly, this does not mean that it encodes an active PDE. Therefore, while the current project relies heavily on genomic information, it combines it with other methods, including complementation experiments and analysis of pharmacological activity, which helps to link genomic annotation with functional assessment (Munday et al., 2020; Chevalier et al., 2024).

1.9.1 Systematic Naming of SmPDEs

A systematic nomenclature of *S. mansoni* phosphodiesterases (SmPDEs) was first proposed by Munday et al. (2020), based on phylogenetic clustering of schistosome genes against representative members of human PDE families. The researchers identified eleven PDEs encoded by genes in the *S. mansoni* genome and classified them according to their evolutionary proximity to particular human PDE families. This allowed for a rational nomenclature of schistosome PDEs that facilitated comparative analyses with the corresponding mammalian genes (Munday et al., 2020).

Specifically, the schistosome PDEs were named SmPDE1, SmPDE2, SmPDE4A, SmPDE4B, SmPDE4C, SmPDE7var, SmPDE8, SmPDE9A, SmPDE9B, SmPDE9C, and SmPDE11. Of those, three genes fell into the PDE4 cluster and were classified accordingly: SmPDE4A–C. The genes that fell within clusters of human PDE1, PDE2, and PDE9 were classified as SmPDE1, SmPDE2, and SmPDE9A–C, respectively. Finally, the genes that were reclassified to other clusters were designated as SmPDE8 and SmPDE11 and SmPDE7var, respectively (Munday et al., 2020).

This naming convention is justified by the evolutionary history rather than the mere number-based nomenclature, and as such, allows for better interpretation of their potential function

and structure. At the same time, Munday et al. (2020) showed that caution is warranted when applying the phylogenetic classification of PDEs since it does not allow for the prediction of their function and pharmacological significance. Accordingly, the systematic naming provides an adequate classification scheme, although it does not determine substrate preference, localization, and activity of particular proteins (Munday et al., 2020).

Figure 1-6 shows phylogenetic relationships between schistosome and human PDEs and the rationale behind the nomenclature of SmPDEs.

Gene ID	PDE4NPD Name
Smp_134140	Sm4A
Smp_141980	Sm4B
Smp_129270	Sm4C
Smp_134500	Sm1
Smp_197150	Sm9A
Smp_146120	Sm9B
Smp_124250	Sm9C
Smp_135500	Sm2
Smp_044060	Sm8
Smp_179590	Sm11
Smp_153640	SM7?

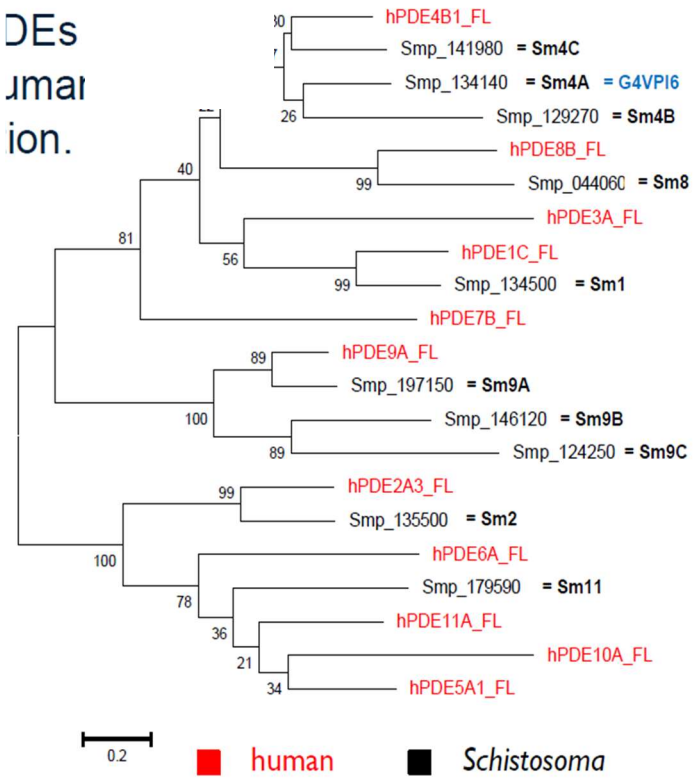


Figure 1-6: The Phylogenetic tree of human and parasitic PDEs (Munday et al., 2020).

1.9.2 Analysis of the SmPDE Sequences

Sequences analysis of SmPDE enzymes has provided essential information concerning the variability of structural organization in the family of enzymes in *S. mansoni*. As indicated by Munday et al. (2020), despite the presence of common catalytic motifs, some of the enzymes in the family have unique structural characteristics distinct from their mammalian orthologs in terms of coding sequence, exon structure, and domain organization. The implication of these findings is that while the PDE family in schistosomes possesses the

conserved catalytic mechanism, there are unique structural elements that should be considered during selective drug design (Munday et al., 2020).

First, one of the significant findings of the sequence analysis was that the experimentally validated sequences of the family do not match the genome annotation in many cases. There were differences in the structural organization of the enzymes in terms of exon structure, length, and domain arrangement in contrast with reference sequences obtained from genomic databases. Therefore, genome sequencing alone is insufficient for determining the structure of the enzymes accurately, and the sequence needs to be validated before making functional conclusions (Munday et al., 2020).

Second, the structural variations in the enzymes of interest are associated with unusual insertions in the sequence structure or unique exon arrangements close to the catalytic domain. Such anomalies can be associated with altered substrate specificity, intracellular localization, and sensitivity to inhibitors. Therefore, in addition to providing insight into the structural variation in schistosome PDE enzymes, the sequence analysis can be used in designing drugs against parasites (Munday et al., 2020).

In summary, the sequence analysis is critical for moving forward from genomic annotation to functional studies. The findings provide evidence that can inform the selection of SmPDE candidates for complementation and pharmacological experiments, and that homology alone does not ensure correct prediction of enzymatic behavior (Munday et al., 2020).

1.10 Aims of the study

Although recent progress has been made toward the identification and initial characterization of the phosphodiesterase (PDE) gene family of *S. mansoni* (SmPDEs), there remain important gaps in our understanding of their functions and activities. Previous research from the De Koning laboratory characterized the SmPDE gene family and demonstrated the principle of heterologous complementation; however, functional complementation in the *T. brucei* system was accomplished for SmPDE4A only in its original design. It was thus unclear whether any other SmPDE proteins would be capable of substituting for essential PDE functions in trypanosomes or whether their inability to do so was due to substrate specificity, low catalytic activity, localization requirements, or other limitations intrinsic to the initial expression strategy. A lack of pharmacological evaluation of SmPDEs as targets

in a complementary trypanosome cellular system was also apparent. These specific aims were addressed as part of the current study (Munday et al., 2020).

The overall goal of this project was to characterize selected *S. mansoni* phosphodiesterases through cloning and expression in heterologous complementation systems followed by pharmacological investigation.

Specific aims included:

a) to isolate and clone the full-length, untagged open reading frames for SmPDE7var, SmPDE1, SmPDE8, and SmPDE9C into tetracycline-inducible vector plasmids for subsequent introduction into the *T. brucei* complementation system;

b) to determine whether induction of these SmPDEs would lead to functional complementation of the loss of the essential TbrPDEB1/B2 locus and allow for the continued survival of the complemented cell line;

c) to examine the functional contributions of catalytic domains through cloning and expression in the tetracycline-inducible expression system based on TbrPDEB1 in appropriate knockouts; and

d) to perform inhibitor sensitivity testing for SmPDE expressing complementation cell lines to evaluate their suitability as pharmacological models for schistosome PDEs.

Chapter 2: Materials and Methods

2.1 Bacterial strains and molecular cloning procedures

2.1.1 Bacterial strains and culturing

To conduct bacterial experiments, we utilized competent *E. coli* cells that were cultured either on LB agar or in LB broth at 37 ºC using a shaking incubator. The preparation of competent *E. coli* cells involved transforming XL1 *E. coli* cells by subjecting them to heat shock for 45 seconds at 42 ºC, after which they were incubated on ice for two minutes. Subsequently, 50 µl of XL1 cells defrosted on ice were mixed with 100 µl of LB broth and incubated at 37 ºC for 30 to 45 minutes with shaking. Finally, 75 µl of the transformed mixture was spread on an ampicillin-containing agar plate (100 µg/ml Ampicillin (Sigma)) and incubated overnight at 37 ºC.

2.1.2 Preparation of plasmid DNA from *E. coli*

The NucleoSpin Plasmid Kit (Macherey-Nagel) was employed to perform a miniprep of plasmid from *E. coli* cells according to the manufacturer's instructions. To extract the plasmid of low copy number, a 10 ml culture was used, which was first centrifuged at 3400 × *g* for 10 minutes, and the supernatant was then discarded. The pellet was then resuspended with buffer A1 containing RNase A, lysed with Buffer A2, and gently inverted (9 to 10 times) without vortexing to avoid shearing of the gDNA. Buffer A3 was added, mixed by inverting the tubes and then centrifuged at 11,000 × *g* for 10 minutes to separate the DNA from the cell debris. The entire supernatant was loaded onto the NucleoSpin Plasmid column to bind the plasmid DNA to the column. The column was then washed with buffer AW and buffer A4 (which contained ethanol to increase DNA binding) before being centrifuged for 2 minutes at 11,000 × *g* to dry. elution buffer was added to the column, and the plasmid DNA was eluted from the column by centrifuging at the same speed for 1 minute. The concentration and purity of the plasmid DNA were analysed using a NanoDrop spectrophotometer (Thermo Scientific).

2.1.3 Amplification of genomic DNA

In order to amplify SmPDEs, primers flanking the open reading frames and with required restriction sites were used. To determine appropriate annealing temperatures, gradient PCR

was performed at temperatures ranging from 50 ºC to 70 ºC. High-Fidelity Phusion polymerase (NEB) was used for PCR amplification, and the PCR program used can be found in Appendix 1. The resulting amplicons were A-tailed and then ligated into the pGEMT Easy vector (Promega) before being transformed into XL1 *E. coli* cells. Colonies were PCR-screened using M13F and M13R primers, and positive clones were isolated and sent for Sanger sequencing to confirm the nucleotide sequence of the PDE genes. To ensure the fidelity of the clones, four independent clones from the same PCR screen were sent for sequencing.

2.1.4 Cloning of PCR products into cloning vectors (pGEMT easy vector)

PCR amplification was used to amplify full-length PDE from gDNA, and the resulting product was A-tailed using 2 µl of dATP, 10 µl of GoTaq reaction buffer, and 0.2 µl of GoTaq polymerase (Promega) by incubating at 72 ºC for 15 min. After incubation, loading dye was added to the reaction mix, and the samples were run on a 1% agarose gel. The correct band was identified under UV and cut out. The purified product was then ligated into the pGEMT Easy vector (Promega) after being cleaned up using the NucleoSpin Gel and PCR clean-up Kit (Macherey-Nagel).

2.1.5 Ligation

Ligation of the A-tailed ORF or digest from pGEMT to their respective plasmids (pGEMT or parasite vectors respectively) was usually performed in a 10 µl ligation reaction containing: vector backbone and insert DNA (in a 1:3 concentration ratio), 1× ligase buffer and T4 DNA ligase (1:10 total volume), in a sterile microcentrifuge tube. The reaction was either incubated for at least three hours at room temperature or overnight at 4 ºC.

2.1.6 Transformation of *E. coli* with plasmid DNA

Upon inserting the constructs into the appropriate plasmids (either pGEMT or the final vector), the vector was transformed into XL1 *E. coli* through the following procedure: Firstly, a 50 µl quantity of XL1 cells was added to 5 µl of the ligation reaction in a sterile microcentrifuge tube, which had been defrosted on ice. The mixture was then placed on ice for 30 minutes, heat-shocked at 42 ºC for 45 seconds, and further incubated on ice for 2 minutes. Afterwards, 100 µl of LB broth was introduced and the mixture was incubated at 37 ºC, while being shaken, for 30 to 45 minutes. Finally, 75 µl of the transformation reaction was spread onto an ampicillin-containing agar plate (with 100 µg/ml Ampicillin (Sigma)) and then left to incubate at 37 ºC overnight. This entire process was performed for each of

the obtained constructs in the XL1 *E. coli* cells to obtain a sufficient number of copies (concentration) for further experimentation, such as transfection.

2.1.7 Restriction digestion and gel electrophoresis

To confirm the concentration of vector DNA obtained from bacterial cultures through miniprep, the Nanodrop machine was used to obtain the required concentration and purity of DNA (with a λ260/280 ratio of 1.8 to 2 for pure DNA samples). The necessary restriction enzyme was used to digest the vectors at 37 °C overnight. This reaction was carried out in a total volume of 100 µl, containing more than 25 µg of purified DNA, 1x restriction buffer, 2 µl of the restriction enzyme, and ddH_2O. After overnight incubation, the entire digest product was loaded onto a 1% agarose gel (prepared by dissolving 1 g of agarose in 100 ml of 1% TAE buffer using the microwave and allowing it to set for over 30 minutes) and run at 110 V for 40 min. Following electrophoresis, the gel was observed under UV light, the desired band was excised, and DNA was extracted using the NucleoSpin gel extraction kit.

2.1.8 Gel extraction and PCR clean-up

Clean-up of PCR products or extraction of DNA from the gel was performed using the NucleoSpin PCR and Gel extraction kit (Macherey-Nagel). For a PCR product or digest product, the binding condition was adjusted by the addition of 2× volume of NT1 buffer per sample volume. For DNA in gel, 2x volume of NT1 per 1 mg of gel was added and the gel slice was left in the solution at 50 ºC for 5-10 min or until the gel was completely dissolved. The DNA was then bound to the column by centrifuging samples loaded to the column at 11,000 × *g* for 30 s. After all the dissolved samples had passed through the column, the silica membrane was washed twice with NT3 (supplemented with ethanol in order to increase binding; 200 ml ethanol to 50 ml NT3 buffer) and then dried by centrifuging at 11,000 × *g* for 1 min. The DNA was eluted by adding 40 µl of warm elution buffer (placed in the heat block at 70 °C) and centrifuging again at 11,000 × *g* for 1 min. The DNA concentration was assessed using NanoDrop and stored at -20 ºC in the elution buffer.

2.1.9 Colony PCR

After an overnight incubation, PCR screening was performed on the bacterial colonies. Colonies were selected from the agar plate using sterile 200 µl pipette tips, streaked on a new LB agar plate, and numbered. Each tip was then dipped into a corresponding PCR tube containing the GoTaq PCR master mix and amplified using the GoTaq PCR screen protocol (Appendix 1). The new agar plate was then incubated at 37 ºC overnight. Electrophoresis on

a 1% agarose gel was performed following PCR screening using a forward primer of the gene and a reverse primer of the 3' flanking plasmid sequence. Positive clones were identified as colonies by the presence of bands on the agarose gel that matched the expected molecular weight. These clones were then used to prepare a 10 ml LB/Amp culture from the overnight agar plate. The plasmid was isolated from the 10 ml culture using the NucleoSpin Plasmid kit (Macherey-Nagel) and was sent to Source BioScience for Sanger DNA sequencing.

2.1.10 Sample preparation for Sanger sequencing

Source Bioscience performed sequencing on all samples using their Sanger sequencing services. Before sequencing, the DNA samples were purified, and their concentrations were measured using Nanodrop. For sequencing of plasmid DNA, 5 µl per reaction of both primer and DNA at a concentration of 100 ng/µl for plasmid DNA and 3.2 pmol/µl for primers were utilized. For PCR product sequence confirmation, 5 µl per reaction of both primer and DNA was also submitted, with a concentration of 3.2 pmol/µl for primers and 10 ng/µl per 500 bp of the PCR product.

2.2 Generation of constructs: Cloning of digested inserts into destination plasmids (final parasite vector)

2.2.1 Generation of pRPai210 plasmids for expression in TbrPDEB1/2 single knockout

The ORF of the SmPDE1 gene in plasmid pHDK241 were amplified by PCR with primers which have *Bam*HI and *Bgl*II restriction sites attached (Table 2.1). These ORFs were then A-tailed and ligated into the pGEMT-Easy vector (Figure 2.1), transformed into *E. coli* XL1 cells, grown up in LB broth, isolated by miniprep and sent for sequencing. After sequence confirmation, the respective SmPDE1 gene was digested out of pGEMT using *Bam*HI and *Bgl*II and ligated to the pRPa$^{i\mathbf{210}}$ vector (also digested with *Bam*HI and *Bgl*II) from David Horn's lab (Alsford et al., 2005). Ligation reactions were transformed into *E. coli* XL1 cells and colonies were PCR screened using primer HDK528 (5'-TTGAAGACTTCAATTACACC-3'; present in the plasmid) and a specific reverse primer of each gene (Table 2.1), in order to confirm insertion. Positive clones were grown up in 10 ml LB broth, and plasmids were extracted by miniprep and sent for sequencing to confirm correct integration in the vector.

SmPDE7var in plasmid pHDK246 was double digested with the enzymes *Bam*HI and *Bgl*II. The pRPai[210] vector plasmid backbone was digested using *Bgl*II and *Bam*HI restriction enzymes (NEB, Hitchin, UK) overnight at 37 ºC to remove the gene it carried. The digested products were separated by agarose gel electrophoresis at 120 V for 45 min. The correct-sized band was identified under UV light and cut out from the agarose gel. DNA was extracted from the gel fragment containing the gene band and purified using the NucleoSpin gel extraction kit. The ligation reaction was carried out with a T4 DNA ligase kit using an insert: vector ratio of 3:1, followed by the transformation of ligation products into *E. coli* XL1 cells to screen colonies that have grown after overnight incubation. PCR screening used forward primer HDK903 of the gene and reverse primer MB302 of the 3' flanking plasmid sequence (Table 2.11).

SmPDE8full in plasmid pHDK211 and SmPDE9C in plasmid pHDK196 were amplified by PCR with forward and reverse of each gene primers restriction sites included (Table 2.1). The PCR product was run on a gel and the band was cut out and cleaned. DNA concentration was measured with nanodrop (concentrations will be low [10-100 ng/µl]. The pRPai[210] vector plasmid backbone was digested using *Bgl*II and *Bam*HI restriction enzymes (NEB, Hitchin, UK) overnight at 37 ºC to remove the gene it carried. Insertion of the gene into the vector was using the NEBuilder HiFi DNA assembly cloning kit (New England Biolabs Inc). Screening colonies that grew after overnight incubation were screened by restriction digest or PCR using a forward primer of the gene and a reverse primer of the 3' flanking plasmid sequence. Positive clones were selected and cultured in 10 ml LB broth at 37 ºC under agitation overnight. The generated plasmid was then extracted by miniprep (Macherey-Nagel) and sent for sequencing to confirm the correct integration of the gene in the vector.

Table 2-1: Primers for amplification of SmPDEs ORF into pRPai210 vector

Gene	Primer name	Position	Res. site	Sequence 5' – 3'
SmPDE1	**HDK1415**	Forward	*Bgl*II	AGATCTGATGGGCTCCTGTGCATCGAC

	HDK1468	Reverse	*Bam*HI	GGATCCTTAATACAAACAGATAATTGAGT
SmPDE8full	**HDK1791**	Forward	*Bgl*II	AGATCTGGTCGGAGACGCTGTCGAAC
	HDK1792	Reverse	*Bam*HI	GGATCC TTATTCCTGATGTATGGTTG
SmPDE9C	**HDK1786**	Forward	*Bgl*II	AGATCT ATGATGTTCAAACGCCTGATCAG
	Kdk1798	Reverse	*Bam*HI	GGATCC TTAGTTAAACAGAAGAAGCAGG

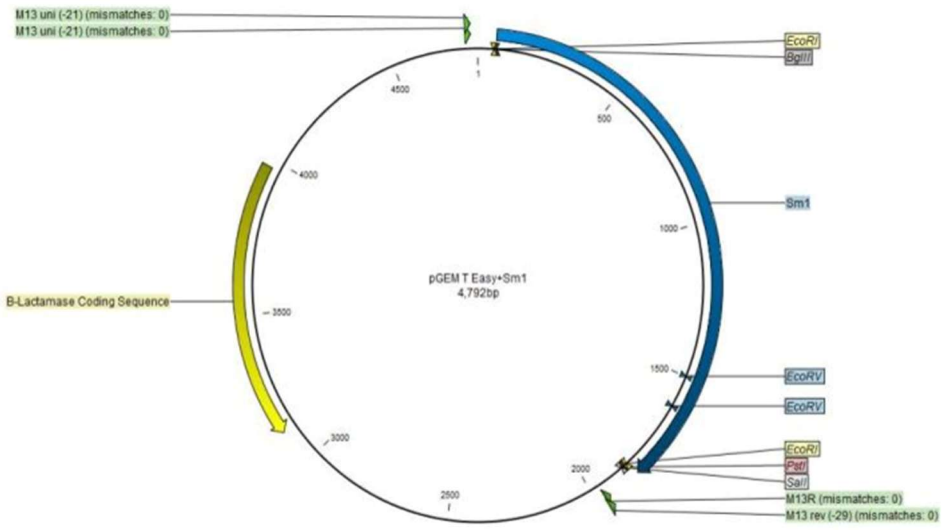


Figure 2-1: SmPDE1 Ligation with pGEMT Easy vector.

2.2.2 Generation of pRPaiB1-cat dom-6Myc plasmids for expression in TbrPDEB1/2 single knockout

2.2.2.1 Preparation of the pRPaiB1-cat dom-6Myc plasmid

This TbrPDEB1 fragment was synthesized from the PvuII site all the way to the C-terminal end, followed by an XbaI site in order to reconstruct the TbrPDEB1 lacking the catalytic

domain. The fragment was inserted into *Escherichia coli*, extracted via miniprep, and released using the PvuII-HF and XbaI restriction enzymes. Simultaneously, the plasmid containing TbrPDEB1 with the catalytic domain (pRPai+TbrPDEB1-6myc; pHDK79) was introduced into *E. coli*, isolated via miniprep, and cut using the same restriction enzymes in order to obtain the portion without the catalytic domain but retaining the rest of TbrPDEB1 along with the 6×Myc tag. After ligation of these fragments, a TbrPDEB1-based inducible expression construct, which is now called pRPaiB1-cat dom-6Myc acceptor plasmid, that can accommodate different PDE catalytic domains in the future, was obtained. Positive recombinant colonies were selected through PCR amplification using primers HDK656 and HDK657 and were sequenced by the use of primers HDK528, MB302

Sending for sequencing: 3 reactions to cover the entire gene, using primers HDK528, MB302 and HDK432.

2.2.2.2 Preparation of the catalytic domain inserts:

The catalytic domains of SmPDE4A in plasmid pHDK133, SmPDE7var in plasmid pHDK245 and SmPDE11 in plasmid pHDK232 were amplified using a forward primer with a 5' *Mfe*I and a reverse primer with 3' *Bgl*II restriction site (Table 2.2).

Table 2-2: Primers for amplification of the catalytic domain SmPDEs into pRPaiB1-cat dom-6Myc vector

Gene	Primer name	Position	Res. site	Sequence 5' – 3'
SmPDE4A	HDK1525	Forward	*MfeI*	CAATTGGAGTCCACTTACCATGCGGAT
	HDK1526	Reverse	*Bgl*II	AGATCTCGGCCTTGCTAATTGTGTGA
SmPDE7var	HDK1527	Forward	*MfeI*	CAATTGGAGGCTGCCTACCACAACTTCA
	HDK1528	Reverse	*BglII*	AGATCTGGATTCGGATGCATTCATTGT
SmPDE11	HDK1529	Forward	*Mfe*I	CAATTGAGGAAGAACTACCGAGAGGT
	HDK1530	Reverse	*Bgl*II	AGATCTCGACTTTGCGATTCGTTCCGCA

The process of amplification was carried out using the Phusion polymerase and 58 °C as a starting temperature and the other temperature conditions of amplification. This step was

followed by double digestion of the amplified products of PCR reactions with *Mfe*I-HF and *Bgl*II.

2.2.2.3 Ligation of catalytic-domain inserts into pRPaiB1-cat dom-6Myc

The ligation of the cleaved catalytic domain inserts into the pRPaiB1-cat dom-6Myc construct was carried out, after which positive colonies were screened using the corresponding forward primer for each insert and primer MB302. Sequencing confirmed positive clones, which were further linearized using AscI and used for transfection of TbrPDEB1/2 knockout cell lines.

2.3 Parasite lines and culture

2.3.1 *T. brucei* cell line and cultures

Trypanosoma brucei 2T1 cells, as described by Alsford et al., 2005, and our complemented cell lines were used for this purpose. Bloodstream forms of these cell lines were grown in HMI-9 medium (Invitrogen) containing 10% heat-inactivated foetal bovine serum gold (FBS; Gibco) in culture flasks, at 37 ºC, in a 5% CO_2 atmosphere. Different cell lines (2T1 cells and derived cell lines, our complemented cell lines and TbrPDEB1/2 sKO cells, illustrated in Figure 2.2) were cultured in the presence of different selective antibiotics to retain their antibiotic resistance. 2T1 cells and the cell lines derived from them were grown under selective pressure with antibiotic puromycin (0.2 µg/ml) and phleomycin (0.5 µg/ml). Our complemented cell lines were cultured under their respective antibiotic selection marker while the TbrPDEB1/2 sKO cells (generated by Dr. Jane Munday) were cultured under 0.2 µg/ml puromycin, 5 µg/ml blasticidin and 0.5 µg/ml phleomycin.

2.3.2 Sub-culturing / passage of the cells

T. brucei cells were passaged every third day by either resuspending with a 10 ml pipette to dissociate clumps of cells. For passaging, 5 µl of the old culture was used to inoculate 5 ml of fresh HMI-9 media supplemented with 10% FBS in a new flask, that is, a 1:1000 dilution was made. This was incubated at the temperature of 37 ºC, in a 5% CO_2 atmosphere.

2.3.3 Preparation and recovery of stability

To create stability, cells in the logarithmic phase were utilized. For *T. brucei*, 0.5 ml of log phase culture was combined with 0.5 ml of HMI-9 and 10% FBS, and 30% glycerol was added to each cryovial. This resulted in a final glycerol concentration of 15%. The cryovials

were then positioned in a thermally insulated container, or alternatively, in a box containing cotton wool for insulation. These were then placed in a -80 ºC freezer for 24-48 hours. The insulated container and cotton wool allowed for a gradual decrease in temperature to ensure the survival of more cells during the freezing process. After freezing, the tubes were moved to liquid nitrogen for long-term storage.

To recover the stability, cryovial tubes were removed from the liquid nitrogen and the cells were defrosted quickly. For *T. brucei*, the stabilate cells were initially transferred to 5 ml pre-warmed media in a tube and centrifuged at 1300 x *g* at 4 ºC for 5 minutes to eliminate the glycerol, which may have had an impact on cell recovery. The supernatant was then cautiously decanted off, and the pellet was resuspended and moved to a vented flask containing 5 ml of fresh HMI-9 with 10% FBS. The flask was then incubated at 37 ºC in an environment with 5% CO_2 for cell growth.

2.4 Parasite in vitro work

2.4.1 Transfection of pRPai210 -Hygro and pRPaiB1-cat dom-6Myc Hygro in TbrPDEB1/2 single knockout cells

To produce the desired strains, constructs containing PDEB1 or PDEB2 of *T. brucei* were developed and placed under tetracycline control in either pRPa^{i210}or pRPa$^{iB1\text{-}cat\ dom\text{-}6Myc}$ vectors, which include a hygromycin resistance cassette. These constructs were digested overnight with *Asc*I from New England BioLabs to linearize the plasmid. Bloodstream forms of *T. b. brucei* 2T1 with one PDEB1/2 allele knocked out (TbrPDEB1/2 sKO cells) were cultivated until the density of the culture reached 1×10^7 cells ml^{-1}. Samples of 10 ml were then transferred to a 15 ml centrifuge tube from Corning and centrifuged at 1300 x *g* for 10 min at 4 ºC in a Heraeus Biofuge centrifuge.

The pellet was resuspended in the remaining fluid and then combined with 100 µl Human T-Cell solution to be transfected with 10 µg ethanol-precipitated DNA from the generated construct containing the heterologous PDE using an Amaxa Nucleofector II electroporator from Amaxa Biosystems with program X-001.

After transfection, the transfectants were placed in pre-warmed HMI-9 with 10% FBS medium and allowed to recover for 8 – 16 hours at 37 ºC and 5% CO_2. The relevant antibiotics such as hygromycin (2 µg/ml) (used for selection of the pRPa cassette), phleomycin (0.5 µg/ml) (antibiotic pressure for 2T1 cells), and blasticidin (5 µg/ml) (used

for selection of the first knockout cassette) were added to the cells following recovery. Then, limiting dilutions (1:20, 1:100, 1:200) were used to clone out the cells. Put 200 µl of dilution 1 (26.6 ml HMI-9 +26.6 µl of drugs) into every well of one 96-well plate, 200 µl of dilution 2 (25.6 ml HMI-9 +25.6 µl of drugs) into all wells of a second plate and 200 µl of dilution 3 (11 ml HMI-9 +11 µl of drugs) into a third. End up with plates of 1/20, 1/100 and 1/200 dilutions. Expect to get clones from 1/100 or 1/200 dilution plates, but it depends on the efficiency of the transfection.

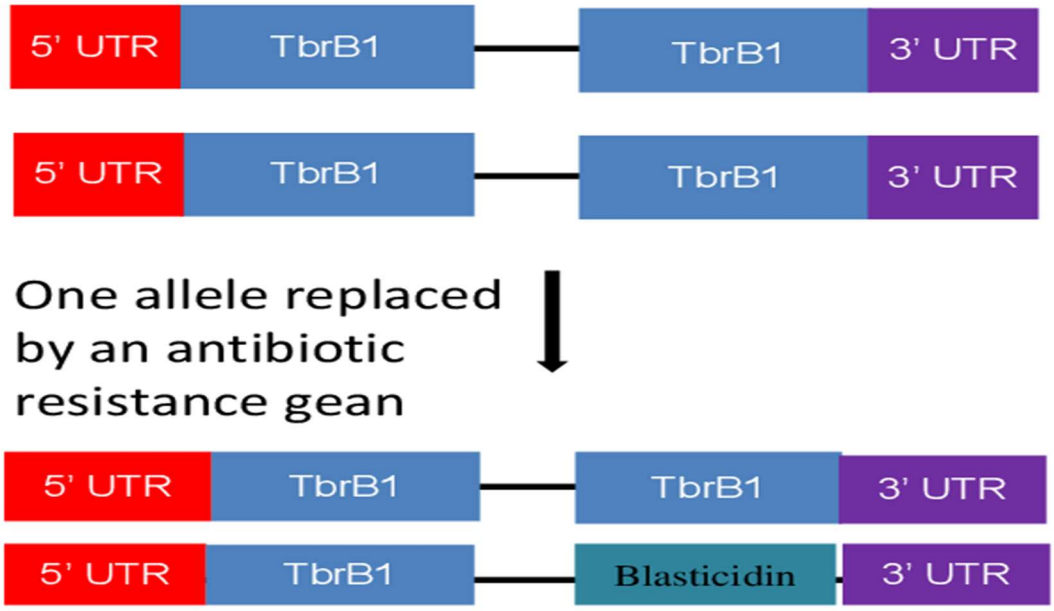


Figure 2-2: Construction of a conditional PDE knockout and expression system, making TbrPDEB1/2 sKO cells. Starting from the T. b. brucei 2T1 cell line, one tandem allele was replaced by a blasticidin antibiotic resistance gene (Construct made by Dr. Jane Munday).

The second round of transfection was performed to delete the second allele of TbrPDEB1/2. This second knockout was done after verification of the successful insertion of the respective PDEB1 or B2 gene in the TbrPDEB1 sKO cells. To delete the second TbrPDEB1/2 allele, the bacteria with the plasmid for puromycin knockout construct were grown up. A map of this construct is shown in Figure 2.3. This Puromycin-resistance gene knockout plasmid was extracted from the lysed bacteria cells and the DNA concentration was measured using NanoDrop and stored at -20 ºC in the elution buffer. The plasmid was digested overnight at 37 ºC using the *Pvu*II-HF, *Sbf*I-HF, and *Acl*I restriction enzymes. To separate, the digest was run on a 1% agarose gel, the appropriate band of ~ 2.9 kb was extracted from the other bands of 400 bp, 1.3 kb, and 1.2 kb . Digestion with two enzymes (PvuII-HF and SbfI-HF) would

only yield two bands of 2.9 kb and ~ 2.85 kb which is problematic to separated properly on the agarose gel. Clean up of the 2.9 kb band was performed according to the manufacturer's instructions, mentioned in 2.1.8.

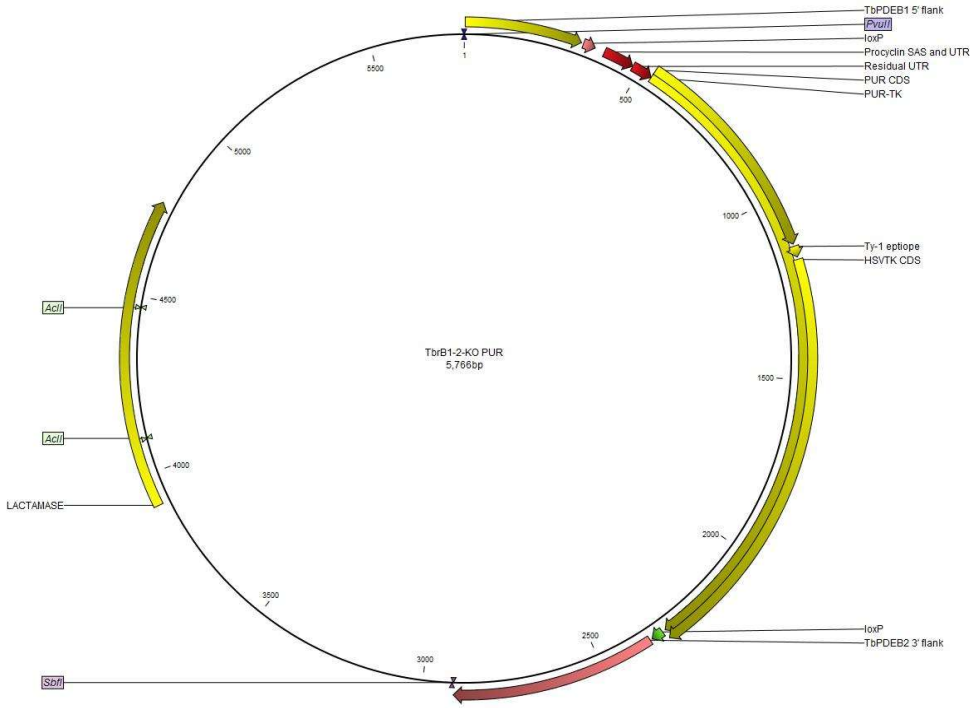


Figure 2-3: Plasmid map showing important sites for enzyme restriction digestion of the constructs for the knockout of the TbrPDEB1/2 locus.

The above structure shows the key ORF integration region and restriction sites. It is suitable to be digested prior to transfection so that the desired fragment can be purified from the 1% agarose gel and be transfected into the *T. brucei* cells.

2.4.2 Preparation of genomic DNA from parasites

Genomic DNA from wild-type and generated cell lines of parasites (*T. brucei*) was prepared using the NucleoSpin Tissue kit (Macherey-Nagel) following the manufacturer's instructions. Firstly, the cells were pelleted. For *T. brucei*, 10^7 cells were centrifuged at 1300 × *g* for 10 minutes. The pellets were then resuspended with 200 µl buffer T1 (resuspension buffer), to which 25 µl Proteinase K solution (to digest protein) and 200 µl Buffer B3 (Lysis buffer) were added, and samples were mixed vigorously by vortexing and incubated at 70

ºC for 10-15 min. After incubation, DNA binding conditions were adjusted by adding 210 µl of ethanol and the sample was vortexed vigorously. Samples were then loaded onto the column and centrifuged at 11,000 × *g* for 1 min to allow the DNA to bind to the column. Membrane silica containing the gDNA was washed with Buffer BW and Buffer B5 (this contains ethanol and increases the binding of the DNA) and at each step centrifuged for a minute. Silica was once again dried by centrifuging for 1 min and the highly pure gDNA was eluted by adding 100 µl elution buffer (a higher concentration of DNA was obtained when the elution buffer was first incubated at 70 ºC prior to adding it onto the column) and centrifuged for 1 min. The final DNA concentration was determined using the NanoDrop machine.

2.4.3 Ethanol precipitation

Vectors digested or linearized were gel extracted from 1% agarose gel using the NucleoSpin Gel extraction kit (Macherey-Nagel) to a final volume of 100 µl. This was then precipitated in 2 volumes of 100% (200 µl) cold ethanol on ice and 1:10 (30 µl) volume of 3 M sodium acetate (pH 5) at -80 ºC for over 1 h. This was centrifuged at high speed, 17,000 × *g* for 20 min and washed with 70% cold ethanol on ice then centrifuged at 17,000 × *g* for 10 min. The pellet was left to air-dry in a sterile hood for over 30 min and then resuspended in 10 µl sterile water. Samples were stored at -20 ºC.

2.4.4 Selection of *T. brucei*-positive clones

Generally, after incubation of transfectants for ~7 days, positive colonies presented as yellow cultures in the wells, indicating dense growth. These were then confirmed by PCR after growth in a flask containing the respective antibiotics and gDNA extraction. After transfection with the pRPa constructs, colonies that turned yellow from dense growth were first screened using antibiotic-resistant markers. Since the pRPa cassette replaced the puromycin cassette in the 2T1, true positive clones were expected to die when cultured with puromycin and hygromycin and to only survive in the presence of Phleomycin/ Hygromycin media. For the pRPa^{i210} or the pRPa$^{iB1\text{-}cat\ dom\text{-}6Myc}$ clones, first-round knockout of the endogenous TbrB1/B2 genes was done using a Hygromycin-resistance knockout cassette and second allele knockout was done using a Puromycin-resistance cassette (this could now be used as the cells died in the presence of Puromycin before transfection).

In order to confirm the correct integration of the pRPa^{i210} and pRPa$^{iB1\text{-}cat\ dom\text{-}6Myc}$ cassette; genomic DNA was extracted as described in Section 2.4.2 and PCR was carried out using

HDK528 (forward primer within the pRPa plasmid sequence) and reverse primers for each gene.

After the second-round transfection, the removal of TbrPDEB1/2 and the presence of Puromycin and Blasticidin cassettes were confirmed. From the positive clones obtained for all SmPDEs gDNA was extracted as described in §2.3.2 and a confirmatory PCR using GoTaq polymerase was carried out (protocol in appendix 1) using primer pairs A to D. Primer pair A is HDK707 forward primer upstream of TbrPDEB1and HDK683 is the reverse primer for blasticidin; primer pair B is HDK707, a forward primer upstream of TbrPDEB1, and MB419 (reverse primer of the puromycin cassette); primer pair C is HDK40, a forward primer 5' upstream of TbrPDEB1 and HDK541 a reverse primer of TbrPDEB1; and primer pair D is HDK543, a forward primer of TbrPDEB2 and HDK545, a reverse primer of TbrPDEB2 with stop codon.

2.5 Growth analysis

To find whether the genes are crucial for cell survival in control and complementing cell lines, a growth analysis was conducted with and without tetracycline in the medium. Briefly, in 24-well plates, 2×10^4 cells ml^{-1} of bloodstream parasites were seeded in a duplicate set and incubated with 5% CO_2 at 37 ^oC. After every 12 h, cells were counted using a haemocytometer. When cell densities exceeded 1×10^6, the cultures were diluted 1:100 in media (+/- tet) at every 48 h; they remained undiluted if the concentration was lower than 1×10^6. To further check the growth of the cell in tetracycline-free media, a further growth analysis experiment was conducted without diluting the cells over the phases of the experiment.

2.5.1 Delayed tetracycline re-addition assay

For distinguishing between cytotoxicity that is irreparable and cytostasis that is reversible, the complementation strains chosen for study were grown without tetracycline until they stopped growing. Then tetracycline was added to the cultures that did not grow anymore, and growth counts were determined over time using the same method that was utilized during the normal growth curve experiment. Growth failure upon tetracycline re-addition indicated that cell death was induced irreversibly by PDE deficiency.

2.6 Alamar blue drug sensitivity assays

2.6.1 T. b. brucei drug sensitivity assay

To evaluate the effectiveness of standard trypanocides and test compounds against parasites, a resazurin-based drug sensitivity assay (Alamar Blue) was conducted. The aim of the test was to determine their potential for cross-resistance to other drugs and examine their toxicity to mammalian cells, in comparison to the parasites, using previously established protocols (Coustou et al., 2010; Eze et al., 2019; Ebiloma et al., 2017). The assay works by measuring the ability of viable cells to convert the blue-coloured resazurin into the pink-coloured resorufin. This conversion allows for the calculation of the 50% effective concentration (EC_{50}) of the drugs and test compounds (Räz et al., 1997). The test compounds were prepared in two-fold serial dilutions in 96-well white opaque flat-bottomed plates (Cell Star, Greiner Bio-one, Frickenhausen, Germany), along with drug-free medium and controls. The number of viable cells was determined from the strength of fluorescence, which is proportional to the number of viable cells (Gould et al., 2008). Analysis of fluorescence readings was done using GraphPad Prism with the help of non-linear regression to a variable slope 4 parameter dose-response model. Values of EC50 were obtained from the curves and, if used, provided along with their standard error and 95% confidence interval. The representative raw dose-response curves were saved for inclusion in the results section of the study. No comparisons between cell lines using student's t-test was done, however, any differences between cell lines would be described if another statistic was applicable. *T. b. brucei* cells were counted and adjusted to 2×10^4 cells/ml, and 100 µL of the adjusted cells was added to each well. Pentamidine was used as a positive control, while drug-free incubations served as the negative control (well 24). The plate was incubated for 48 h, after which resazurin dye was added, and further incubated for 24 h. Finally, the fluorescence in each well of the plate was measured using a FLUOstar Optima plate reader (BMG Labtech, Durham, NC, USA) with filters at wavelengths of 544 nm for excitation and 590 nm for emission.

Table 2-3: List of primers used, their sequences and purposes.

Primer	Sequence	Use	Restriction site

HDK528	TTGAAGACTTCAATTACACC	F- pRPa sequencing	
HDK903	AAGCTTATGTTCATGAACAAGCCCTTTG	F-First 210 bp TbrB1	HindIII
MB302	TAACCAACCTGCAGGCG	R	
HDK656	TTAATTAAATGTTCATGAACAAGCCCTTT	F- full CDS TbrB1	*Pac*I
HDK657	TCTAGAACGAGTACTGCTGTTGTTGCCAGAA	R- full CDS TbrB1	XbaI
HDK432	GCCATCATTCCCATCTCTCTAC		
HDK707	GCTGGTGTTCGGCTGTTAG	F- upstream TbrB1-B2 locus	
HDK683	GGATCCTTAGCCCTCCCACACATAACC	R- mid-Blasticidin gene	
HDK541	AGATCTACGAGTACTGCTGTTGTTGCCA	R- TbrB1 ORF	*Bgl*II
MB419	TGAGGAAGAGTTCTTGCAGC	R- mid-puromycin gene	
HDK540	GGCCGGCCATGTTCATGAACAAGCCCT	F-TbPDEB1 ORF	FseI
HDK543	GGCCGGCCATGACACACAACGGTGGTCGT	F-TbPDEB2 ORF	FseI
HDK545	AGATCTCTAAGACGAAGCCCCAGTACT	R-TbPDEB2 ORF	*Bgl*II

HDK713	ATGCAAGCTAGGCCACACCT	F-check for circular plasmid	
HDK535	CGGACAGGTATCCGGGTAAGC	R-check for circular plasmid	

Chapter 3: Generation of Untagged SmPDE Expression Constructs and Functional Complementation in a Conditional *Trypanosoma brucei* PDEB1/B2 Knockout Line

3.1 Introduction

PDEs have been shown to play an integral role in cyclic nucleotide signaling as they mediate the hydrolysis of both hydrolysis of cyclic AMP and cyclic GMP and thus regulate the intensity and longevity of these signaling cascades. With respect to *S. mansoni*, it has been identified that PDEs might serve as attractive therapeutic targets due to the vital importance of cyclic nucleotide signaling in parasite biology. Furthermore, numerous SmPDEs show considerable differences from their mammalian counterparts, making pharmacological exploration worthwhile (Munday et al., 2020; Zheng et al., 2023).

Previous research conducted in the De Koning lab provided the first broad understanding of the *S. mansoni* phosphodiesterase family through the cloning and characterization of multiple *S. mansoni* PDEs in heterologous systems. This previous work demonstrated that several *S. mansoni* phosphodiesterase homologues served as cyclic nucleotide phosphodiesterases in yeast-based assay systems and that SmPDE4A complemented TbrPDEB1/B2 knockout strains in *Trypanosoma brucei*. However, despite these initial findings, all *T. brucei* complementation constructs were tagged at the C-terminus with GFP, raising uncertainty as to whether the inability of other SmPDEs to complement represented a biochemical limitation inherent to the protein itself or rather stemmed from difficulties associated with heterologous expression, such as protein misfolding or mislocalization (Munday et al., 2020).

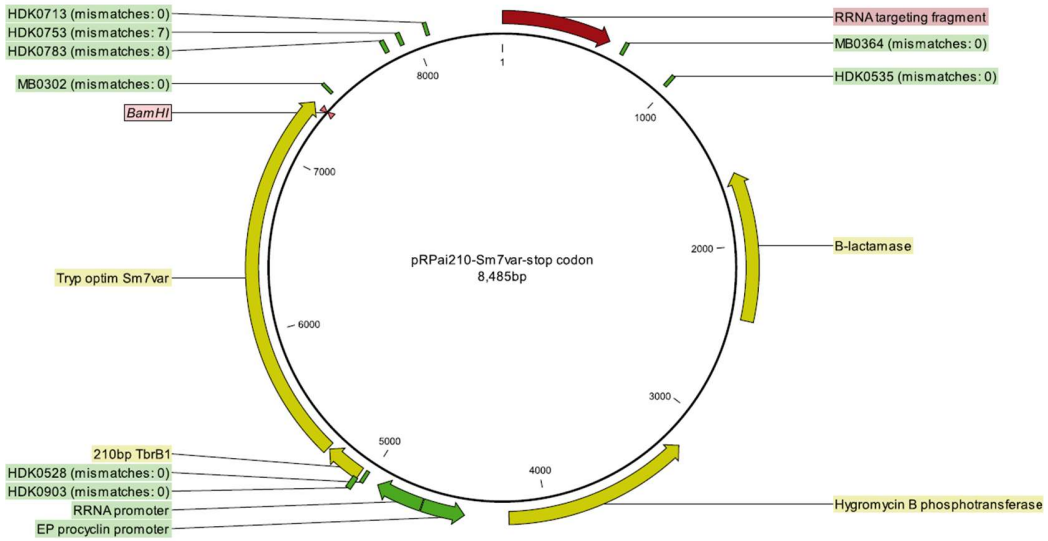
Use of the *T. brucei* complementation system is of particular interest to the problem since tandem genes TbrPDEB1 and TbrPDEB2 are crucial for survival of bloodstream-form trypanosomes. To test for complementation, one allele pair of TbrPDEB1/B2 genes is knocked out, and then heterologous PDE is expressed from the rRNA locus in a tetracycline inducible manner. If the heterologous PDE provides sufficient cAMP degradation in the right cellular

environment, the second allele pair of endogenous TbrPDEB1/B2 can be knocked out. Thus, the resulting line is now dependent on the induction of tetracycline-responsive PDE expression for survival; thus, growth following the induction of transcription of heterologous gene and death following its removal serves as a measure of complementation (Oberholzer et al., 2006; Luginbuehl et al., 2010; Munday et al., 2020).

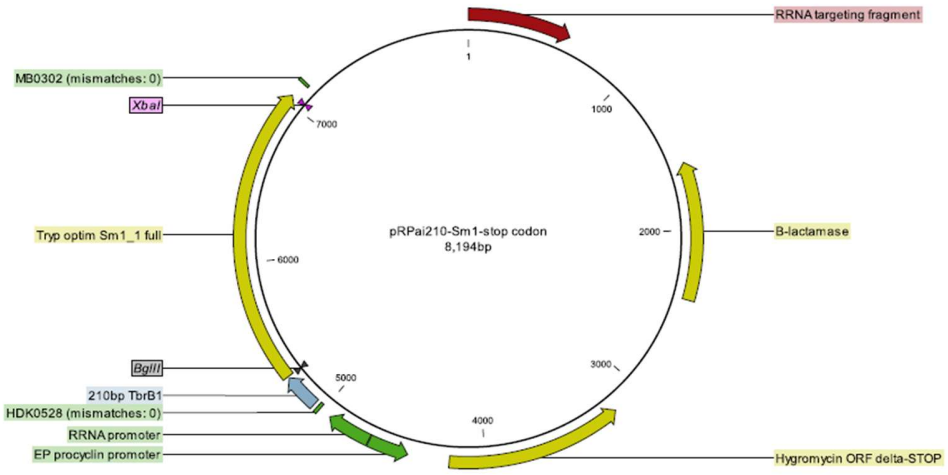
With the aim of addressing aforementioned concerns, this study sought to re-examine the complementation platform using full-length versions of SmPDEs devoid of any tags. Four phosphodiesterase candidates have been chosen for this experiment: SmPDE7var, SmPDE1, SmPDE8, and SmPDE9C. The selected genes were cloned into tetracycline-inducible expression plasmids and tested for their ability to support growth of the conditional TbrPDEB1/B2 knockout strain. In doing so, this research attempts to address not only construction of an expression system but also its biological validation.

Figure 3-1. Diagrams of four different stop codon constructs. A, pRPaSmPDE7var; B, SmPDE1; C, SmPDE9Cfull; and D, SmPDE8full.

A



B


RRNA targeting fragment
MB0302 (mismatches: 0)
XbaI
Tryp optim Sm1_1 full
BglII
210bp TbrB1
HDK0528 (mismatches: 0)
RRNA promoter
EP procyclin promoter
pRPai210-Sm1-stop codon
8,194bp
B-lactamase
Hygromycin ORF delta-STOP
1
1000
2000
3000
4000
5000
6000
7000

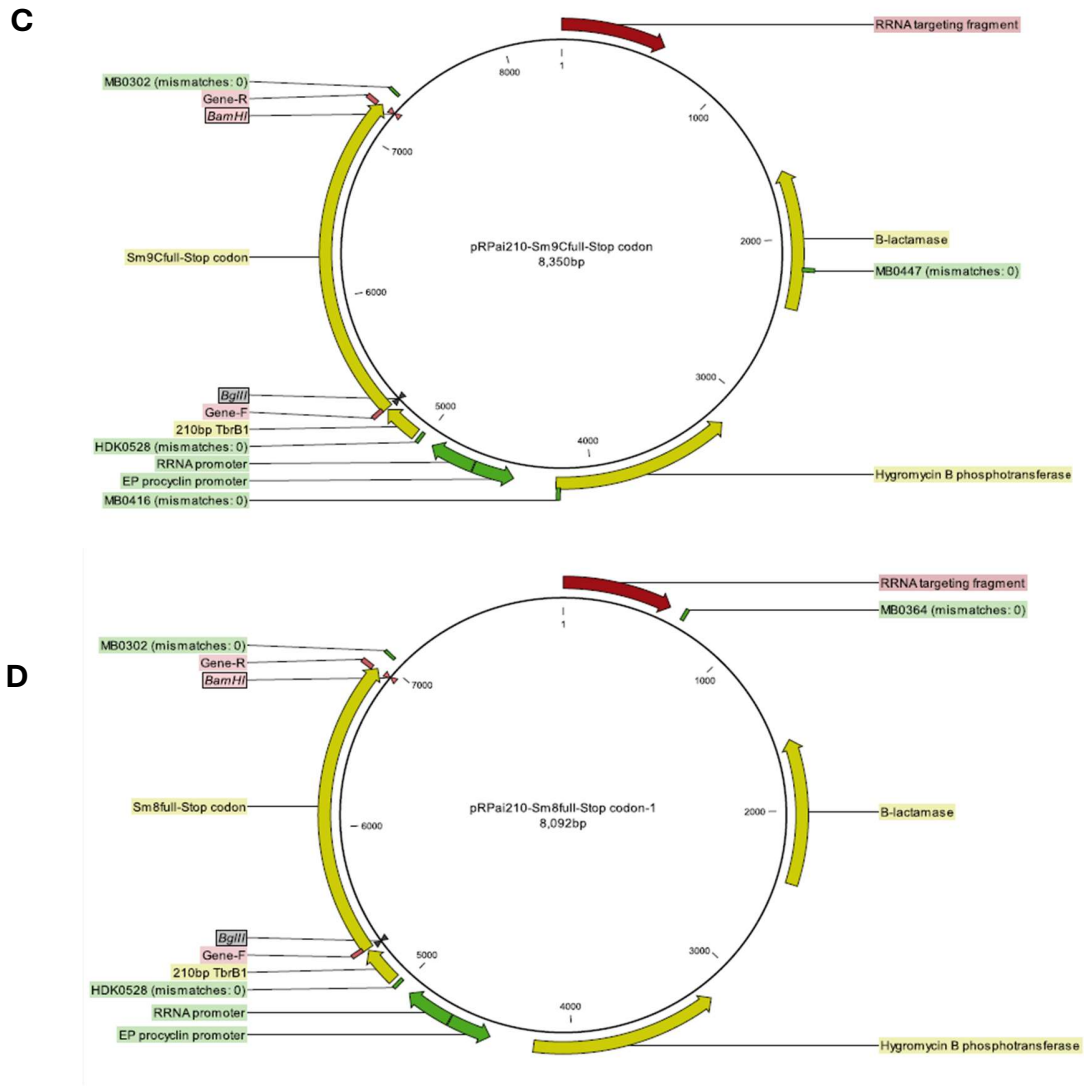


Figure 3-1: Plasmid maps of four different stop codons analised showing important restriction enzyme sites for digestion. A - the pRPaSmPDE7var; B - SmPDE1; C - SmPDE9Cfull and D - SmPDE8full.

3.2 Aims

In this chapter, it was aimed to prepare the untagged versions of selected SmPDEs, and the functionality of these enzymes in replacing the essential role of the TbrPDEB1/B2 locus in *T. brucei* was to be determined. The aims of this chapter were:

(a) untagged versions of tetracycline-inducible expression constructs containing full-length SmPDE7var, SmPDE1, SmPDE8, and SmPDE9C would be prepared;

(b) these constructs would be introduced into *T. brucei* PDEB1/B2 knockout cells which had been genetically modified conditionally;

(c) it would be tested if the resulting lines could survive after the deletion of the remaining endogenous TbrPDEB1/B2 locus; and

3.3 Results

3.3.1 Generation of untagged full-length SmPDE expression constructs

Full-length gene expression cassettes were made for SmPDE7var, SmPDE1, SmPDE8, and SmPDE9C, and a stop codon was added after the termination of the coding region to avoid fusion with the GFP coding region. This technique was used to determine if the deletion of the GFP tag from the C-terminal end of the SmPDE genes could improve their functionality within the *T. brucei* complementation system. These constructs retained the inducible expression nature of the pRPai210 vector but produced untagged versions of the SmPDE proteins.

The SmPDE1 gene was successfully amplified and sub-cloned into the final inducible expression vector. Positive clones of the anticipated size were observed in each relevant phase, confirming the success in obtaining the untagged construct.

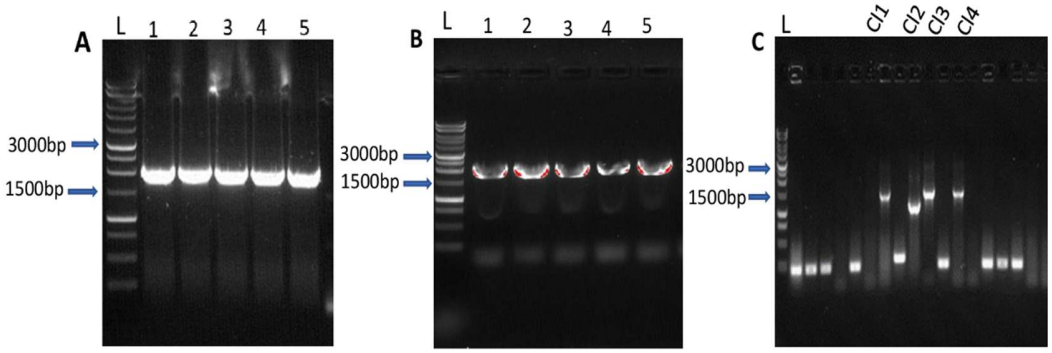


Figure 3-2: Re-amplifying and cloning of SmPDE1 with STOP codon. A) demonstrates the re-amplification of SmPDE1 with STOP codon using a proofreading polymerase at the size of 1800bp 1-5 positive clones. B) Illustrates the ligation of the PCR product into the pGEM-T Easy vector in 1800 bp. Lanes 1-5 all show positive clones. C) The litigation of the PCR product into the vector pRPai210 was successful ligation of the gene sequence and got 1,3, and 4 positive clones. L: 1kb DNA ladder (Promega).

Figure 3-2. Re-amplification and Cloning of SmPDE1 with Stop Codon. A) Re-amplification of SmPDE1 with Stop codon employing proof-reading enzyme with an anticipated amplicon size of 1800 bp. B) Ligation of PCR amplified fragments with a size of 1800 bp to pGEM-T Easy vector; numbers 1–5 are positive colonies. C) Sub-cloning PCR fragments into vector pRPai210; positive colonies 1, 3 and 4; L=1 Kb ladder (Promega).

SmPDE7var was also cloned out of the intermediate construct and ligated in the backbone of pRPai210. Positive clones have been obtained through colony screening, which indicates success in obtaining the final untagged construct.

Figure 3-3. Schematic showing sequential digestion of vectors for the ligation and subcloning of SmPDE7var to create the final vector pRPai210. A) Digestion of vector pHDK246 to isolate the gene of interest using BamHI digestion, creating a fragment size expected at 2061 bp. B) Digestion of vector pRPai210 with BamHI and BglII, creating a backbone fragment expected at 6417 bp. C) Subcloning of SmPDE7var into the final vector pRPai210 and isolation of positive clones using PCR, as shown by the expected fragment size at 2394 bp. L: 1 kb DNA ladder (Promega). The same general strategy was applied to clone SmPDE8 and SmPDE9C genes as well. Each gene was amplified successfully and isolated at the expected sizes, after which the gene was inserted into the final vector pRPai210. The successful cloning at the expected sizes of each SmPDE indicates that all four SmPDEs have been successfully converted into untagged inducible constructs.

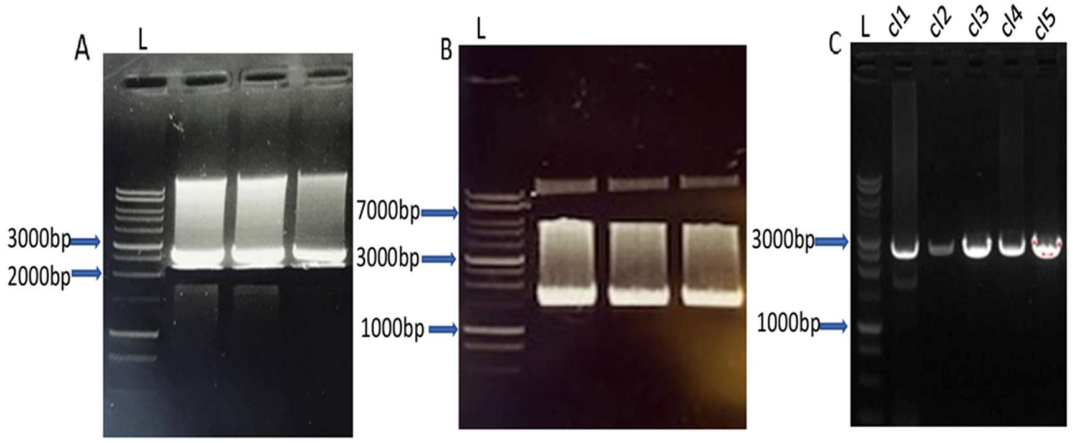


Figure 3-3: Various steps involved in the ligation and sub-cloning of SmPDE7var into the final vector pRPai210. A) Digest of vector pHDK246 to release the gene of interest, with *Bam*H1; samples were run on agarose gel and showed the correct bands at 2061 bp. B) The vector pRPai210 is digested with the restriction enzymes *Bam*H1 and *Bgl*II and the correct bands at 6417 bp were cut out. C) sub-cloning SmPDE7var into final vector pRPai210 and selecting the correct bands for ligation at 2394 bp (clone 1-5) L: 1 kb DNA ladder (Promega).

Fig. 3-4. Amplification of full-length SmPDE8 and SmPDE9C with stop codons. A) Amplification of full-length SmPDE8 with expected size amplicon of 1668 bp. B) Amplification of SmPDE9C with expected size amplicon of 1926 bp. L: 1 kb DNA ladder (Promega)

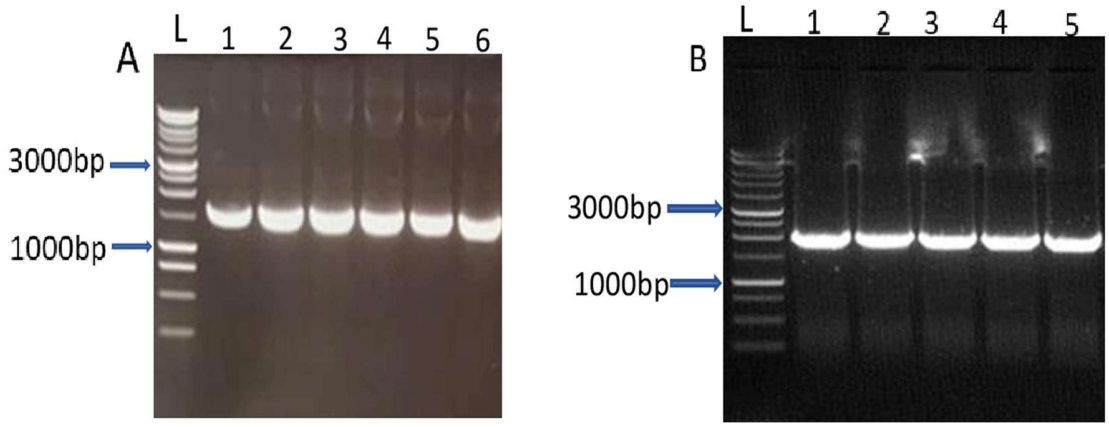


Figure 3-4: Amplification of SmPDE8 full and SmPDE9C with stop codons. A) Amplification of SmPDE8 full length and the band of 1668 bp was cut out 6 positive clones. B) The SmPDE9C was amplified and got 5 positive clones at 1926bp. L: 1kb DNA ladder (Promega).

Fig. 3-5. Sub-cloning of SmPDE8 and SmPDE9C into vector pRPai210. A) Ligating SmPDE8 (1668 bp) with the final backbone vector (6425 bp), digesting ligated vector using restriction enzymes BglII and BamHI, giving expected sizes of insert and backbone. B) SmPDE9C recombinants digested with BglII and BamHI and giving expected banding pattern. L: 1 kb DNA ladder (Promega).

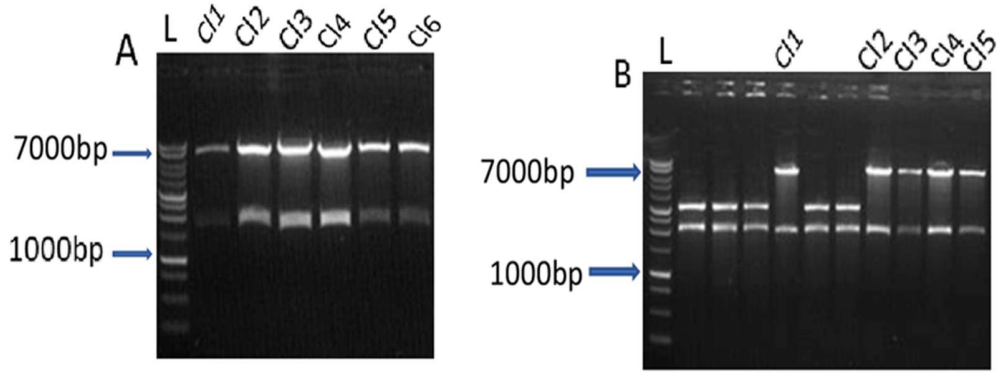


Figure 3-5: Sub-cloning of SmPDE8 and SmPDE9C into the vector pRPai210. A) Ligation SmPDE8 (1668 bp) with the final vector (6425 bp) by NEBuilder HiFi DNA, digestion with enzymes BglII and BamH1 resulting in two bands of 1600 bp and 6425 bp, respectively, for 6 positive clones. B) digestion of SmPDE9C with the backbone by BglII and BamH1 enzymes identified 5 positive clones, each of them containing 2 bands. L: 1kb DNA ladder (Promega).

3.3.2 Complementation strategy in the conditional TbrPDEB1/B2 knockout system

The untagged SmPDE genes previously created were then tested under the conditional *Trypanosoma brucei* complementation assay. In this approach, sequential knock-out of the natural TbrPDEB1/B2 locus is performed along with tet inducible expression of a foreign PDE gene integrated at the rRNA locus. The idea behind this approach is that viability following the elimination of both natural copies will depend on whether the newly engineered SmPDE can provide sufficient PDE activity for survival.

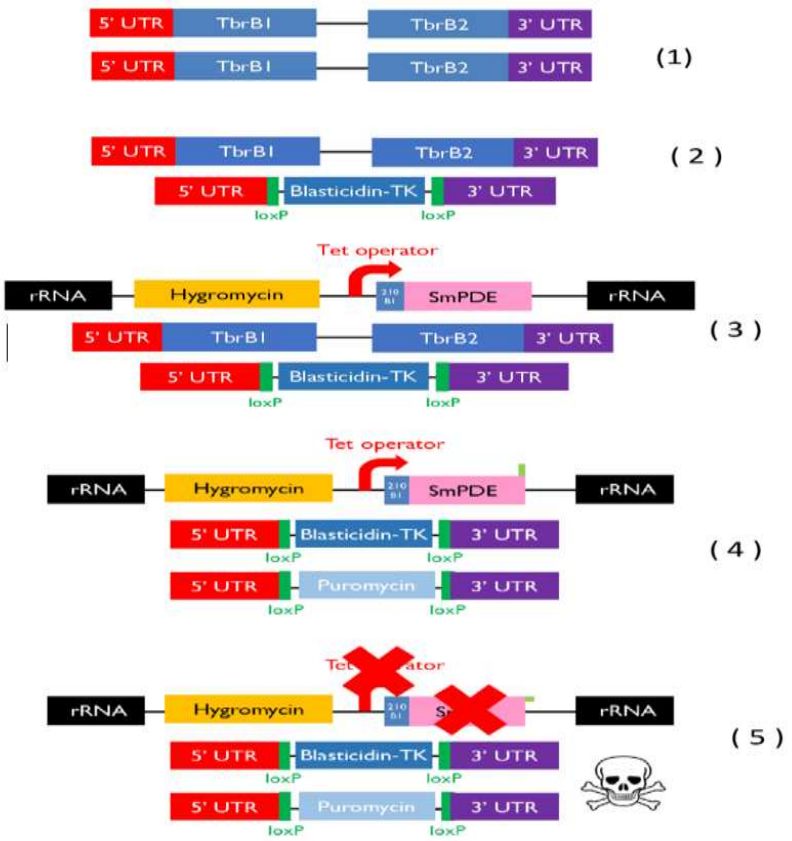


Figure 3-6: Complementation strategy for *S. mansoni* PDEs in *T. brucei* (Kim et al., 2013).

3.3.3 Introduction of the complementation constructs into the single-knockout background

Before transfecting these constructs into the *Trypanosoma brucei*, they had to be linearized to facilitate targeted integration of the constructs at the designated locus of rRNA gene. The linearized products were of the anticipated sizes and were used to transfect the TbrPDEB1/B2 single knockout line.

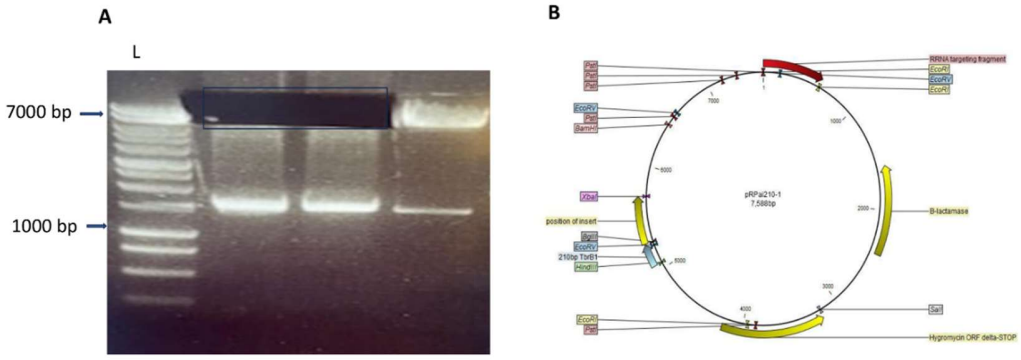


Figure 3-7: Transfection of pRPai210 -Hygro in TbrPDEB1/2 single knockout cells; (A) Agarose gel electrophoresis result showing discrete bands representing the smaller bands and the top bands (7588 bp), was cut out and extracted from the gel; (B) The plasmid map of the pRPai210 vector showing important restriction enzyme sites for digestion. L: 1kb DNA ladder (Promega).

Integration of the pRPai210 complementation constructs into single knockout lines was confirmed by PCR using genomic DNA from the transfected lines. Four positive clones were identified for SmPDE1, SmPDE8, SmPDE9C, and SmPDE7var, suggesting that all four SmPDE constructs without the tag have been successfully transfected into the sKO lineages and can be used for complementing the other alleles.

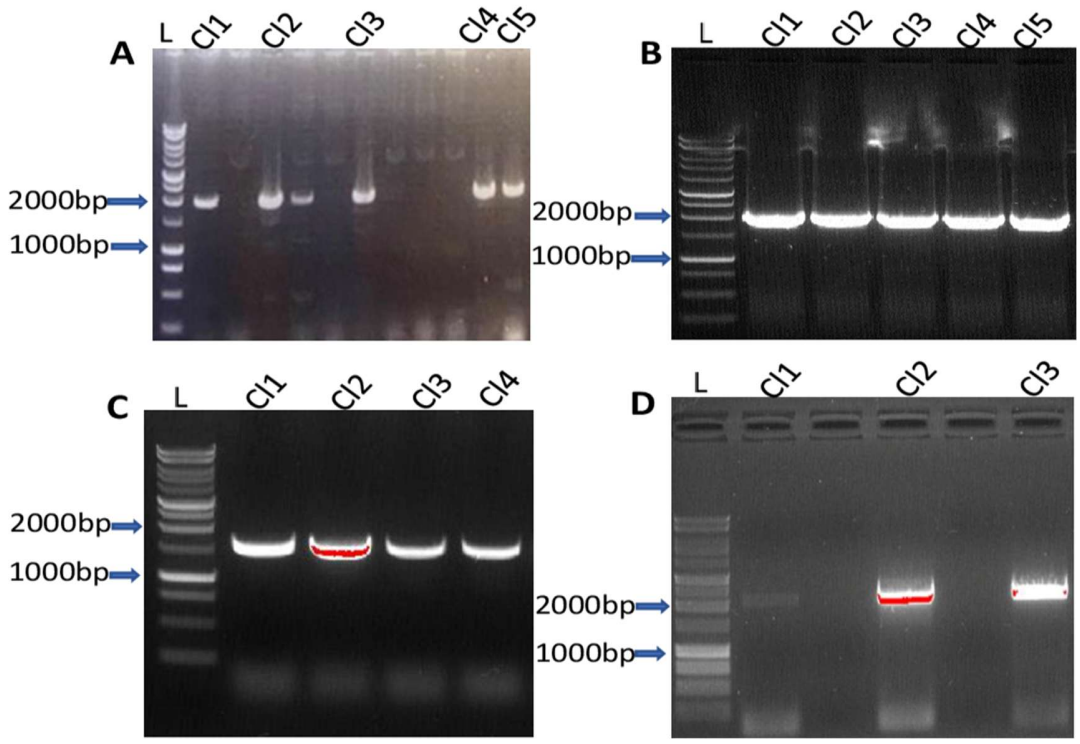


Figure 3-8: PCR results and integration of different PDEs into plasmids. A) Integration was confirmed for sKO+SmPDE1 and 5 positive clones were identified from the correct size band in 1996 bp. B) sKO+SmPDE8full was correctly integrated into the plasmids and 5 positive clones with correct band size in 1800 bp were obtained. C) Transfection with sKO+SmPDE9C obtained several positive clones at the correct size of 2140 bp. D) sKO+SmPDE7var was correctly integrated into the plasmids and 2 positive clones with correct band size in 2287 bp were obtained. L: 1kb DNA ladder (Promega).

3.3.4 Generation and molecular confirmation of double-knockout complemented lines

After the successful generation of the single knockout strains carrying the inducible SmPDE genes, the remaining endogenous alleles for the TbrPDEB1/B2 pair were deleted. The expected result was to produce double knockout strains whose survival hinged solely on the induction of the SmPDE through tetracycline.

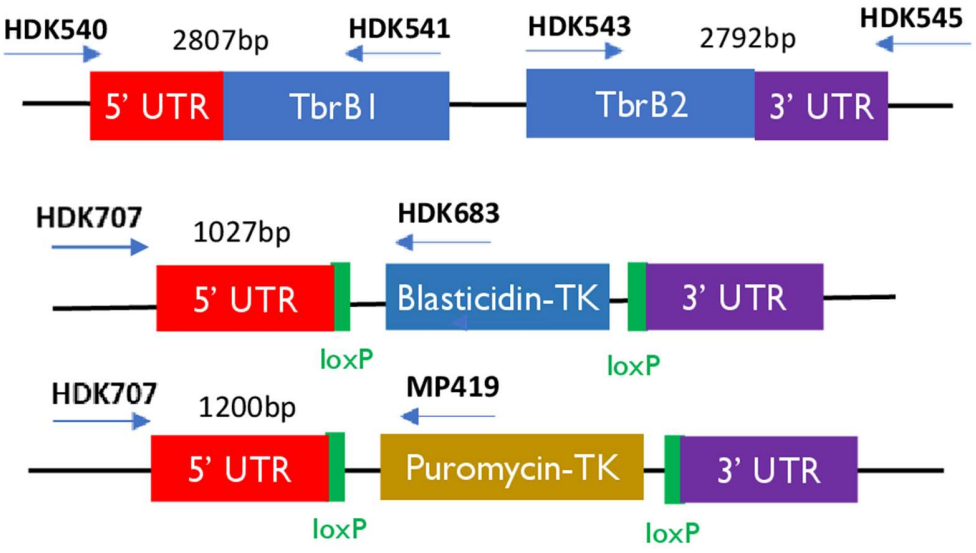


Figure 3-9: Complementation strategy for *S. mansoni*PDEs in *T. brucei*. Adapted from Monday et al., 2020.

The PCR analysis technique was used to confirm the genotype prediction for the proposed double knockout mutants. In the case of SmPDE1, the chosen clones showed the predicted banding pattern, which indicated the successful knockout of both native TbrPDEB1/B2 alleles while retaining the inducible SmPDE1 gene cassette.

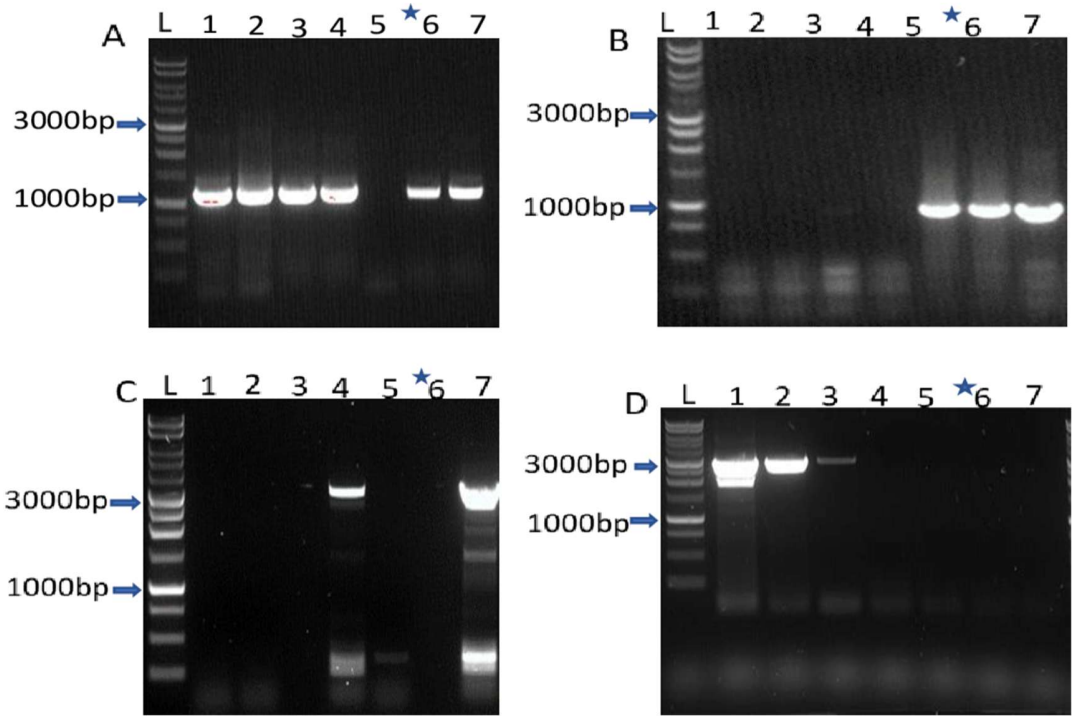


Figure 3-10: PCR analysis of the SmPDE1 complementation in *T. brucei*.A) is HDK707 the Forward Primer (FP) Upstream TbrB1-B2 locus to HDK 683 the reverse primer (RP) mid-Blasticidin gene, resulting in a fragment of approximately 1027 bp. B) PCR analysis use HDK707 the FP Upstream TbrB1-B2 locus to MB419 RF mdi Puromycin gene at size ~1200 bp. C) PCR amplification from HDK540 the FP TbrB1 ORF to HDK541 RP TbrB1 ORF no stop at size 2807 bp. D) PCR amplification from HDK543the FP TbrB2 ORF to HDK545 RP TbrB2 ORF no stop at size 2792 bp. Legend: 1kb DNA ladder (Promega); wells 1-7 represent different gDNA samples; 1 = B1/2 Sko , 2 = sKO + SmPDE1, 3 = B1/2 dKO+SmPDE1 cl.1, 4 = B1/2 dKO + SmPDE1 cl. 2, 5 = B1/2 dKO+ SmPDE1 cl. 3, 6 = B1/2 dKO + SmPDE1 cl.4, 7= B1/2 dKO + SmPDE1 cl.5; Blue Star indicates expected clone for SmPDE1 complementation.

Similarly, PCR verification was performed for SmPDE7var, SmPDE8full, and SmPDE9C. All selected clones showed diagnostic bands for their respective genes, confirming the generation of complemented double-knockout strains expressing the full-length protein of SmPDE under tetracycline regulation.

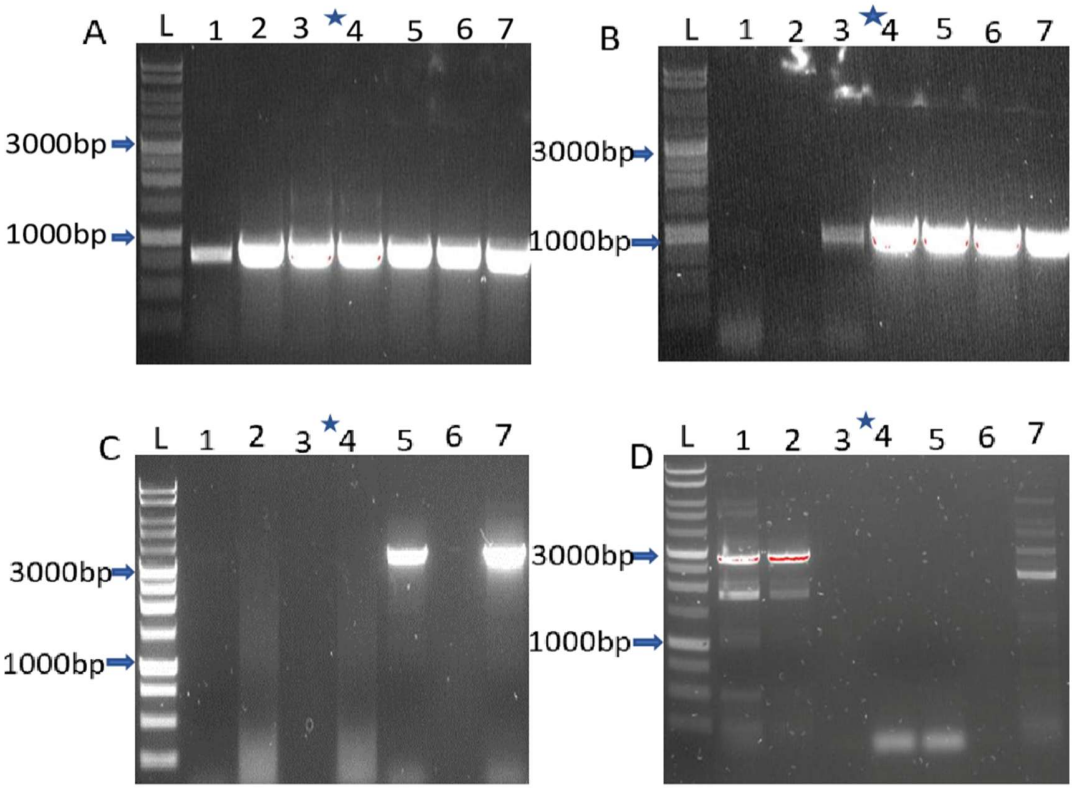


Figure 3-11: SmPDE7var PCR complementation in *T. brucei*. A) PCR from the FP Upstream TbrB1-B2 locus to RF mid-Blasticidin gene at size ~1027 bp. B) FP Upstream TbrB1-B2 locus to RF mid-Puromycin gene at size ~1200 bp. C) FP TbrB1 ORF to RP TbrB1 ORF no stop at size 2807 bp. D) FP TbrB2 ORF to RP TbrB2 ORF no stop at size 2792 bp. Legend: 1kb DNA ladder (Promega); wells 1-7 are different gDNA samples; 1 = B1/2 Sko , 2 = B1/2 sKO + SmPDE7var, 3 = B1/2 dKO+SmPDE7var cl.1, 4 = B1/2 dKO + SmPDE7var cl. 2, 5 = B1/2 dKO+ SmPDE7var cl. 3, 6 = B1/2 dKO + SmPDE7var cl.4, 7= B1/2 dKO + SmPDE7var cl.5; Blue Star indicates expected clone for SmPDE7var complementation.

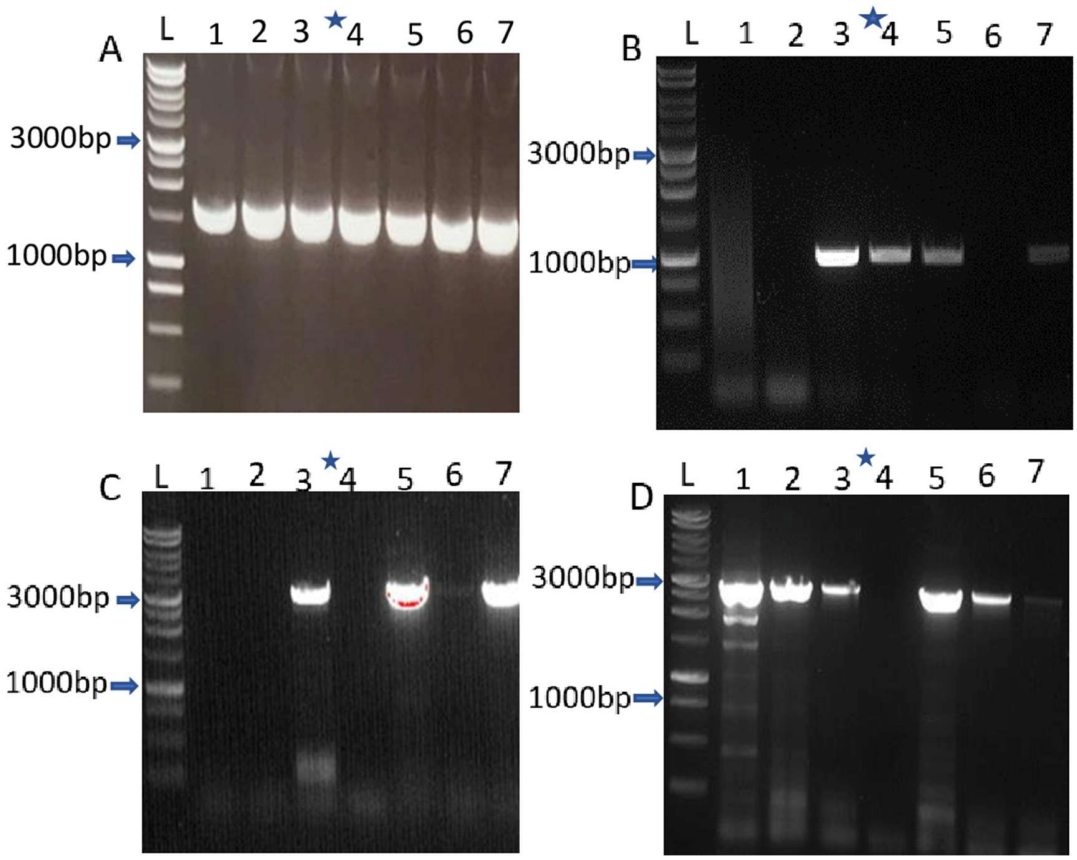


Figure 3-12: SmPDE8full PCR complementation in *T. brucei*. A) PCR use FP Upstream TbrB1-B2 locus to RF mid-Blasticidin gene at size ~1027 bp. B) FP Upstream TbrB1-B2 locus to RF mdi Puromycin gene at size ~1200 bp. C) FP TbrB1 ORF to RP TbrB1 ORF no stop at size 2807 bp. D) FP TbrB2 ORF to RP TbrB2 ORF no stop at size 2792 bp. Legend: 1kb DNA ladder (Promega); wells 1-7 are different gDNA samples; 1 = B1/2 Sko , 2 = B1/2 sKO + SmPDE7var, 3 = B1/2 dKO+SmPDE8full cl.1, 4 = B1/2 dKO + SmPDE8full cl. 2, 5 = B1/2 dKO+ SmPDE8full cl. 3, 6 = B1/2 dKO + SmPDE8full cl.4, 7= B1/2 dKO + SmPDE8full cl.5; Blue Star indicates expected clone for SmPDE8full complementation.

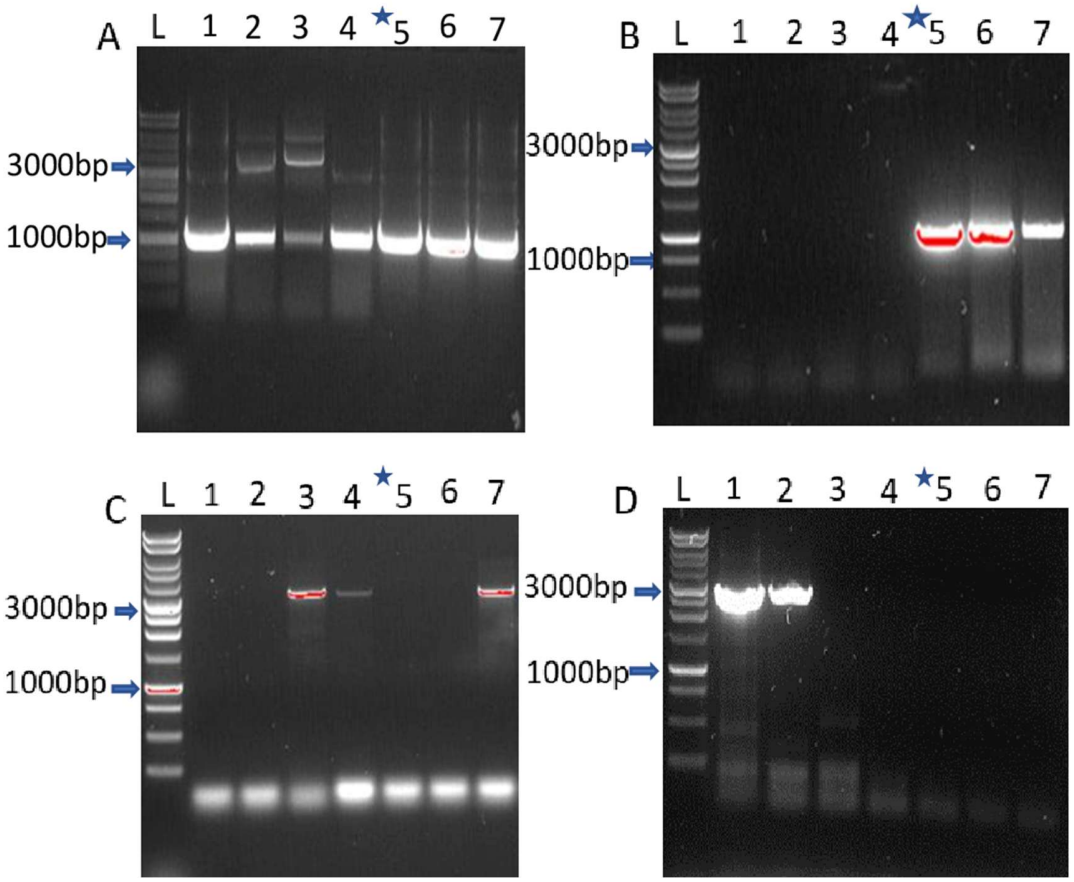


Figure 3-13: SmPDE9C PCR complementation in *T. brucei*. A) FP Upstream TbrB1-B2 locus to RF mid Blasticidin gene at size ~1027 bp. B) FP Upstream TbrB1-B2 locus to RF mdi Puromycin gene at size ~1200 bp. C) FP TbrB1 ORF to RP TbrB1 ORF no stop at size 2807 bp. D) is FP TbrB2 ORF to RP TbrB2 ORF no stop at size 2792 bp. L: 1kb DNA ladder (Promega); wells 1-7 are different gDNA samples; 1 = B1/2 Sko , 2 = B1/2 sKO + SmPDE9C, 3 = B1/2 dKO+SmPDE9C cl.1, 4 = B1/2 dKO + SmPDE9C cl. 2, 5 = B1/2 dKO+ SmPDE9C cl. 3, 6 = B1/2 dKO + SmPDE9C cl.4, 7= B1/2 dKO + SmPDE9C cl.5; Blue Star indicates expected clone for SmPDE9C complementation.

3.3.5 Growth analysis

The effect on cell growth was tested in order to determine the ability of the full-length, untagged SmPDEs to sustain life in the induced lines of *T. brucei*. It was found that in tetracycline conditions, the complemented strains containing SmPDE7var, SmPDE1, SmPDE8full, and SmPDE9C were viable and showed continuous growth, while some lines grew more slowly than the wild type line. On the other hand, without tetracycline, the lines stopped growing, which suggests that their viability depended on the inducible expression of the heterologous gene.

The obtained results correspond to the successful functionality of the full-length, untagged SmPDE genes in the conditional TbrPDEB1/B2 knockout background. They also indicate that the absence of a GFP tag did not affect the ability of the lines to express functional PDE activity and, for tested SmPDEs, made it possible to obtain viable inducible lines ready for pharmacological testing.

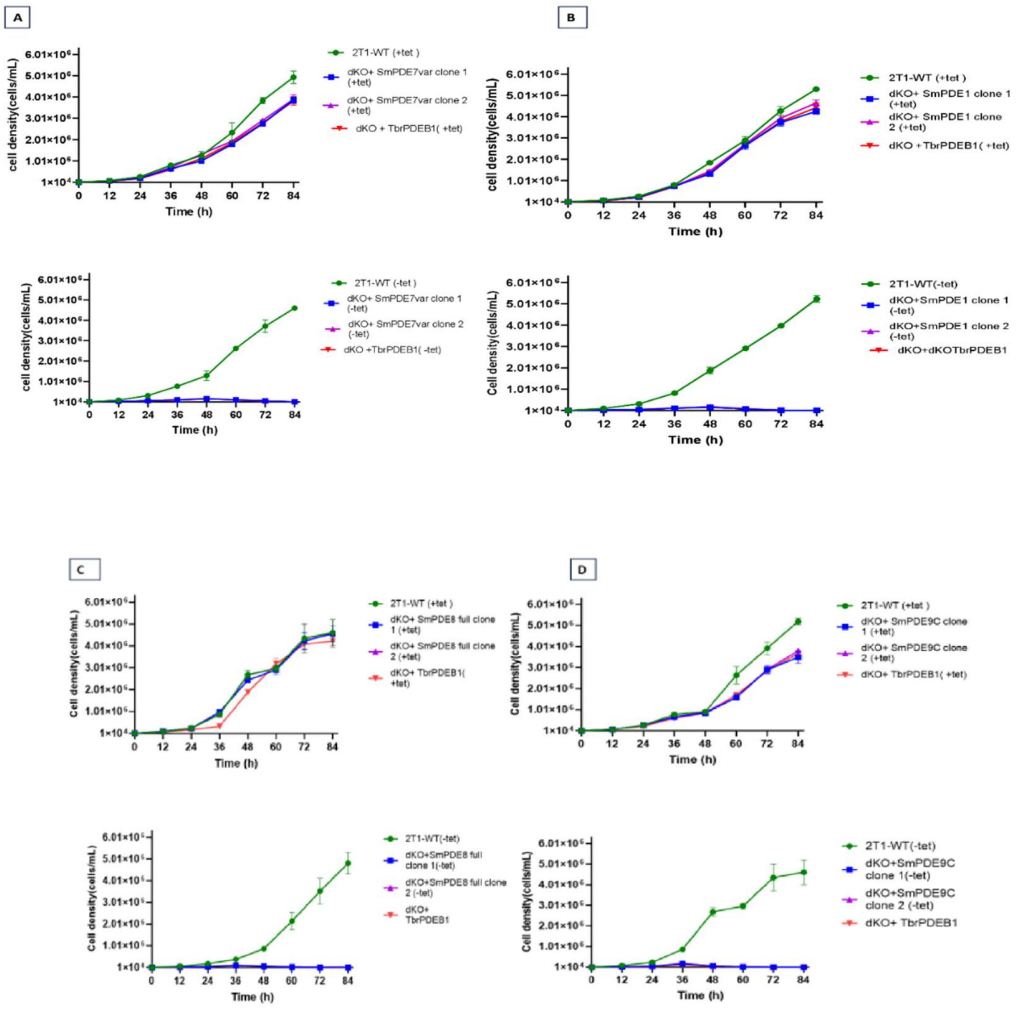


Figure 3-14: Growth assay of complementation cell lines in *T. brucei* system. (A) Growth analysis of control cell lines and the dko +SmPDE7va (B) Growth analysis of control cell lines and the dko +SmPDE1 (C) Growth analysis of control cell lines and the dko +SmPDE8full and (D) Growth analysis of control cell lines and the dKO +SmPDE9C.

3.3.6 Delayed tetracycline re-addition experiment

To differentiate cytotoxicity from mere arrest of growth without induction, tetracycline was re-administered to cells after a period of growth inhibition without induction. It was observed that the administration of tetracycline did not induce growth in the growth-arrested cells, suggesting that the failure to induce SmPDE production had resulted in cytotoxicity and not in mere cytostasis. This result is significant for future experiments involving inhibitors, since it shows that the failure to induce essential PDE production may be interpreted as cytotoxic rather than cytostatic.

3.4 Discussion

In this chapter, the concept of the complementation assay is combined with the construction of full-length cDNA constructs to circumvent one of the shortcomings of the *T. brucei* SmPDE complementation system described above. Four full-length cDNAs encoding inducible untagged SmPDEs were generated and used to complement the conditional knock-out line lacking TbrPDEB1 and B2 in *T. brucei*. Molecular confirmation confirmed that all designed constructs were successfully integrated into the cell line and yielded complemented double knock-out lines of SmPDE7var, SmPDE1, SmPDE8, and SmPDE9C.

The key advantage of the new strategy was the disconnection between fluorescence and complementation assay. As opposed to tagged constructs in which any disruption of complementation might arise from changes in protein folding, activity, interactions with other proteins, or intracellular localisation, untagged constructs showed their ability to provide functional PDE activity in the host organism, which was the main purpose of complementation experiments. Indeed, while only one full-length inducible SmPDE has been described before (Munday et al., 2020), the successful integration of additional genes proves their viability and ability to perform as an auxiliary source of PDE activity under the experimental conditions applied.

Growth assays provided further evidence for the biological significance of the complementation. All four SmPDEs, once induced with tetracycline, proved to be viable; the absence of tetracycline in the medium resulted in growth loss. Such results indicate a dependency of viability on the expression of the inserted gene as soon as the endogenous locus is knocked out.

Additionally, some of the constructed lines showed lower growth rate compared to the WT parasite, indicating that SmPDEs may not possess identical functions to the native enzymes in some aspects. This difference may involve the expression level, catalytic properties, interaction network or even sub-cellular location.

Therefore, this chapter yields a collection of SmPDEs that can be utilised as controls for the next chapter in which a pharmacological analysis of the obtained compounds is carried out. At the same time, this result allows to draw further conclusions about the nature of the work – the removal of the GFP tag resulted in the emergence of more SmPDE-dependent strains. The delay in the addition of tetracycline further confirms the explanation of the complementation system, where it is clear that shutting down the inducible SmPDE gene leads to cell death rather than growth arrest. It is evident that this conclusion is vital when carrying out future inhibitor experiments.

Chapter 4 – Replacement of the catalytic domain of TbrPDEB1 with the corresponding domain of SmPDEs (SmPDE7var, SmPDE4A, and SmPDE11).

4.1 Introduction

Phosphodiesterases (PDEs) represent primary regulators of cyclic nucleotide signal transduction via the degradation of cAMP and cGMP, thus regulating the intensity and duration of signal responses. In *T. brucei*, cAMP signal transduction is important for various biological processes, such as motility, differentiation, and survival, making PDE function necessary for the parasite's survival (Makin & Gluenz, 2015; Oberholzer et al., 2015). In humans, PDE activity is encoded by many genes grouped into eleven distinct families, depending on sequence features, substrate specificity, and inhibitor selectivity (Bolger, 2021).

T. brucei possesses five genes coding for phosphodiesterases, namely PDEA, PDEB1, PDEB2, PDEC, and PDED. The closest relative to mammalian PDEs are *T. brucei* PDEB1 and *T. brucei* PDEB2, both being cAMP-specific phosphodiesterases with two N-terminal GAF domains, but different subcellular localizations: TbrPDEB1 is localized in the flagellum, while TbrPDEB2 is localized in the flagellum and cytoplasm. Due to the necessity of the TbrPDEB1/B2 system in the survival of bloodstream forms, it provides an adequate background for analyzing the possibility of replacement of endogenous functions of phosphodiesterases by heterologous PDEs (Oberholzer et al., 2006; Oberholzer et al., 2015).

Based on the results presented above, complementation experiments with *S. mansoni* full-length PDEs can be affected by more aspects than just catalytic activity, such as subcellular localization and interaction with the cellular environment. Thus, in this experiment, substitution of the TbrPDEB1 catalytic domain was attempted using only the catalytic domain of certain SmPDEs, keeping the remaining parts of the protein N-terminal and C-terminal to the catalytic domain. The approach allows to preserve the regulation and localization abilities of the endogenous trypanosome PDE while evaluating whether the imported catalytic domain may substitute the endogenous one to provide the needed catalytic activity.

For this purpose, catalytic domains of SmPDE4A, SmPDE7var, and SmPDE11 were integrated into a mutant TbrPDEB1 vector construct and analyzed in the knockout system of *T. brucei* parasites deprived of PDEB1/B2. Thus, the approach serves not only as another step towards the refinement of the complementation system but also determines if catalytic-domain replacement can lead to obtaining more appropriate experimental lines suitable for pharmacological evaluation. Figure 4-1 shows the cloning strategy used for generation of catalytic-domain swapped constructs.

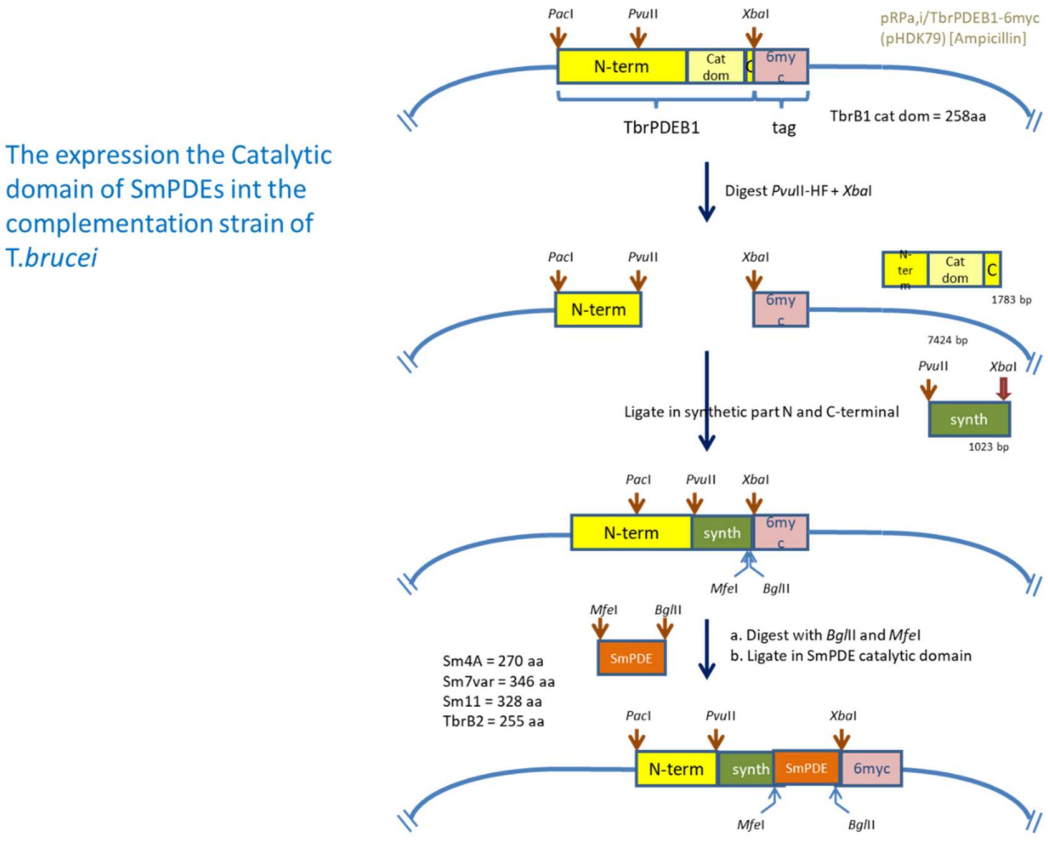


Figure 4-1: The diagram shows Strategy cloning steps to introduce TbrPDEB1 with SmPDEs and expression of the catalytic domain of SmPDEs.

4.2 Aims

The goal of this chapter was to examine whether the catalytic domains of the phosphodiesterases of certain *Schistosoma mansoni* strains could serve as functional replacements for the catalytic domain of TbrPDEB1 in the context of *Trypanosoma brucei* complementation experiments. In

particular, the aim was to create recombinant constructs containing the catalytic domains of SmPDE4A, SmPDE7var, and SmPDE11 in the TbrPDEB1 expression system, to express such constructs in the knockout strain of TbrPDEB1/B2 under inducible growth conditions, and to evaluate their viability.

4.3 Results

4.3.1 Construction of an inducible TbrPDEB1 cassette lacking the catalytic domain

The inducible expression cassette was created whereby the catalytic domain of TbrPDEB1 was deleted while the rest of the TbrPDEB1 framework and the 6×Myc tag remained intact. The strategy used in creating this construct involved designing it in such a way that the cassette serves as the backbone that can accommodate any foreign PDE catalytic domains. The strategy involved in the generation of this cassette is presented in Figure 4-2.

A synthetic fragment of 1023 bp representing the C-terminal part of TbrPDEB1 excluding the catalytic domain was obtained at the expected size following digestion. Simultaneously, digestion of the original pRPa,i+TbrPDEB1-6myc plasmid generated a larger backbone fragment of approximately 7424 bp after the deletion of the native catalytic domain part. Thus, the expected outcome confirmed successful synthesis of both DNA fragments needed for making the inducible expression cassette.

Digestion and ligation of the synthetic fragment to the backbone of the vector resulted in the creation of an inducible expression cassette for TbrPDEB1 without the catalytic domain but having the rest of the TbrPDEB1 frame work along with the 6×Myc tag. Screening of the colonies through PCR identified positive clones producing the product with the expected size of 2034 bp. Therefore, it is clear that an inducible expression cassette had been successfully made.

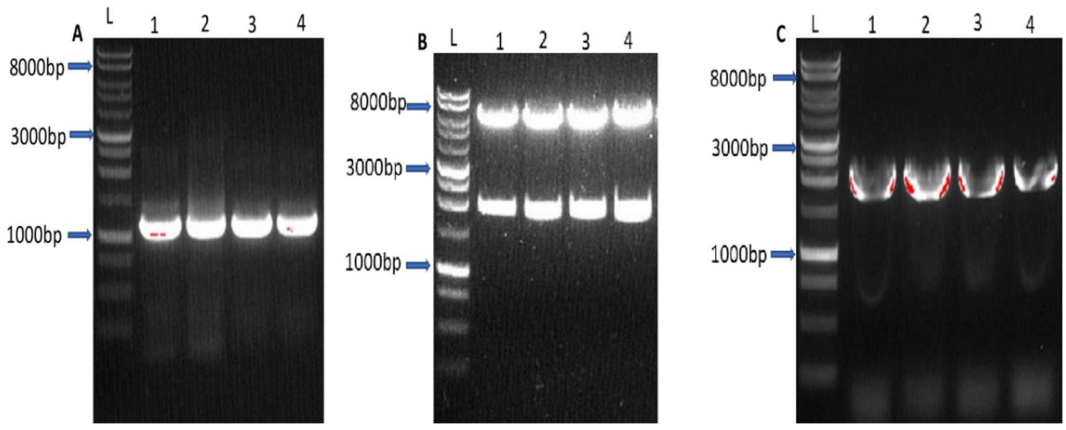


Figure 4-2: The molecular part of the catalytic domain swap strategy, Processes to make the pRPaiB1-cat dom-6Myc plasmid. A) Gel confirmation of the drop-out after *Pvu*II / *Xba*I digestion of the synthetic TbrPDEB1 fragment at 1023 bp. B) Gel confirmation of digestion of the pRPai+TbrPDEB1-6myc and cut out at 7400bp. (C) Screening of the colonies by PCR using primers for TbrB1 and get bands at 2034 bp; 1-4 positive colonies. L, 1 kb Ladder (Promega).

4.3.1.2 Insertion of SmPDE catalytic domains into the TbrPDEB1 backbone

The amplification of the catalytic domains of SmPDE4A, SmPDE7var, and SmPDE11 resulted in the generation of amplicons of 822 bp, 1050 bp, and 996 bp, respectively, in accordance with expectations. These amplicons were further cloned into the inducible TbrPDEB1 vector construct created in the previous subsection, producing a series of hybrid constructs where the catalytic domain of the TbrPDEB1 gene is replaced with that of the respective SmPDE.

Screening for recombinants through PCR produced positive colonies of each catalytic-domain construct, suggesting that they have been successfully introduced into the plasmid backbone. The DNA isolated from the positive colonies was sequenced, and the sequencing results showed the presence of catalytic-domain inserts. All of these findings suggest that catalytic-domain swap constructs have been successfully engineered for SmPDE4A, SmPDE7var, and SmPDE11.

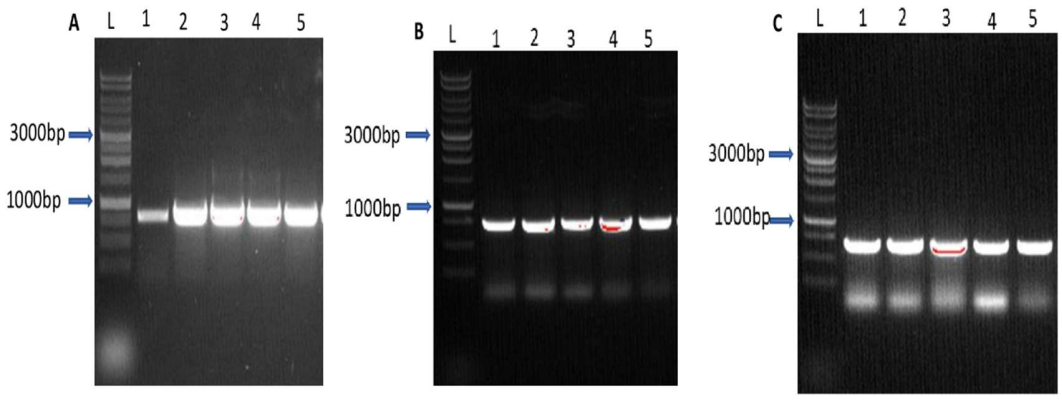


Figure 4-3: Gel confirmation of amplification of the catalytic domains of SmPDEs. (A) amplification of SmPDE4A using the primers HDK1525 & 1526 and the size of the PCR product is 822 bp; (B) amplification of SmPDE7var using the primers HDK1527 & 1528 and the size of the PCR product is 1050 bp; (C) amplification of SmPDE11 using the primers HDK1529 & 1530 and the size of the PCR product is 996 bp;5 positive colonies. L, 1 kb Ladder (Promega).

4.3.2 Sub-cloning of SmPDE catalytic domains into the TbrPDEB1 acceptor construct:

Upon ligating the inserts of the catalytic domain into the TbrPDEB1 inducible acceptor vector, the positive clones were detected through colony screening. Plasmid extraction and sequencing further validated the formation of domain-swapped constructs comprising the catalytic domain of the chosen SmPDEs in the TbrPDEB1 scaffold. Consequently, these constructed plasmids became the required expression constructs for transfecting the *T. brucei* complementation system.

4.3.3 Transfection of catalytic-domain swap constructs into TbrPDEB1/B2 single-knockout cells:

The confirmed domain-swap vectors were then introduced into the TbrPDEB1/B2 knockout strain to test whether the heterologous proteins would be capable of complementation after swapping out the last of the endogenous PDE enzymes. The resistance of the vectors to hygromycin allowed for the selection of cells under drug selection pressure, allowing for the acquisition of clones with integrated vectors.

Genomic DNA from the selected clones was analyzed via PCR, with expected-size products generated for each construct, thus confirming that the catalytic-domain swap vectors had been successfully integrated into the TbrPDEB1/B2 knockout strain. This confirms that vectors with the catalytic domains of SmPDE4A, SmPDE7var, and SmPDE11 have been successfully introduced into the *T. brucei* cell line and are suitable for further complementation testing.

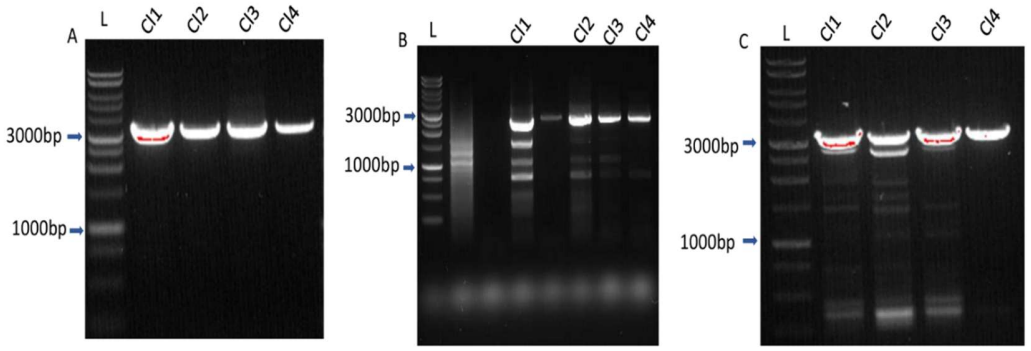


Figure 4-4: PCR confirmation of plasmid integration into TbrPDEB1/2 single knockout. Band sizes correspond to sizes of the respective catalytic domains of SmPDEs. A) SmPDE11CD using forward primer HDK528 and reverse primer HDK1530 to obtain a band size of 3016bp; B) SmPDE7varCD using forward primer HDK528 and reverse primer HDK1528 (SmPDE7var) to generate a band with a size of 3070 bp; C) SmPSE4ACD using forward primer HDK528 and reverse primer HDK1526 (SmPDE4A), generating a band with a size of2842 bp; 4 positive colonies. L, 1 kb Ladder (Promega).

4.3.4 Selection of positive *T. brucei* clones and confirmation of second-round knockout of catalytic-domain swap constructs:

Choice of candidate complementation strains containing the catalytic domain of either SmPDE4A, SmPDE7var, and/or SmPDE11 was based on genetic proof of the correct genotype achieved by the second round of knockout. The following four factors were considered necessary for successful complementation of *T. brucei* PDE activity: presence of the blasticidin-resistant cassette, presence of the puromycin-resistant cassette, and absence of the TbrPDEB1 and TbrPDEB2 bands. Overall, these criteria reflect the completion of endogenous TbrPDEB1/B2 gene replacement and absolute dependency on the construct for any PDE activity.

Results of the PCR analysis showed that both catalytic domains of SmPDE4A and SmPDE7var possessed the required features needed for complementation. Clones positive for both antibiotic-resistance cassettes were obtained in which the presence of both endogenous TbrPDEB1 and TbrPDEB2 bands is not detected. In conclusion, the catalytic domain of SmPDE4A and SmPDE7var has been proven to provide functional complementation of PDEB1/B2 knockout in the TbrPDEB1 gene context.

Conversely, clones with the inserted SmPDE11 catalytic domain were not able to satisfy all the required molecular markers of complementation. Integration of the construct into the genomic DNA at a later time compared with the SmPDE4A and SmPDE7var clones was found; however, all the selected lines were not positive for the required molecular profile of a complementation strain. Thus, under the described conditions, the catalytic domain of SmPDE11 is not able to support the functionality of the PDEB1/B2 null mutant.

So, catalytic domain exchange was found to be successfully performed for the case of SmPDE4A and SmPDE7var, but not for the SmPDE11 enzyme. Therefore, two clones for growth and pharmacology analysis have been obtained.

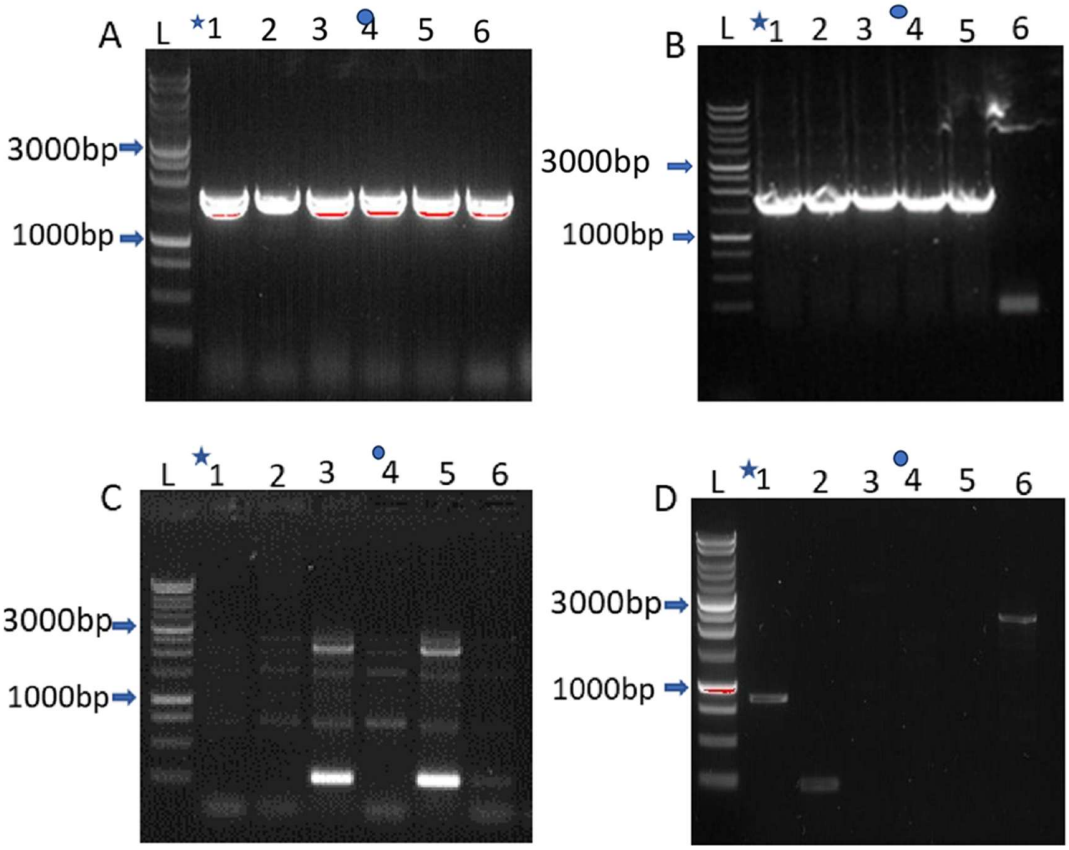


Figure 4-5: PCR analysis of the catalytic domain of SmPDEs 4A and SmPDE7var complementation in *T. brucei*. A) uses a forward primer upstream of the TbrB1-B2 locus and a reverse primer from the middle of the blasticidin gene, generating a band with a size of ~1027bp. B) PCR using a forward primer upstream of the TbrB1-B2 locus and a reverse primer in the middle of the puromycin gene, generating a band with the size of ~1200bp. C) PCR with a forward primer for the 5' end of the TbrB1 ORF and a reverse primer for the 3' end of the TbrB1 ORF yielding a band with a size of 2807 bp. D) PCR with a forward primer for the 5'-end of the TbrB2 ORF and reverse primer for the 3'-end of the TbrB2 ORF, giving a band with a size of 2792bp. L: 1 kb DNA ladder (Promega); wells 1-6 are different gDNA samples; 1 = dKO+SmPDE4ACD cl, 1; 2= dKO+SmPDE4ACD cl, 2; 3 = dKO+SmPDE4ACD cl, 3 4 = dKO+SmPDE7varCD cl, 1, 5 = dKO+SmPDE7varCD cl, 2, 6 = B1/2 sKO (Control). Blue Star clone number 1 indicates selected clone for SmPDE4A complementation and Blue circle clone number 4 indicates selected clone for SmPDE7var complementation.

4.3.5 Growth analysis of the catalytic domain of SmPDEs 4A or SmPDE7var

The growth analyses were performed to determine if the constructs carrying the catalytic domain of SmPDE4A or SmPDE7var can provide adequate survival for the *T. brucei* PDEB1/B2 knockout background strain in an inducible condition using tetracycline. The strains carrying the catalytic domain of SmPDE4A or SmPDE7var survived and displayed growth similar to the

wild-type strain under the presence of tetracycline, which shows that the fusion protein is able to provide adequate PDE activity for the survival of the parasitic cell. These results show that the catalytic domain of SmPDE4A or SmPDE7var can be used successfully with TbrPDEB1 as the scaffold in the complementation test.

On the other hand, when tetracycline was not supplied, there was an immediate drop in growth for the catalytic domain complementation strains, but the wild-type strain did not suffer any loss in growth even without induction. From these results, it is clear that the survival of the catalytic domain strains relies on the continued expression of the fusion protein via tetracycline induction.

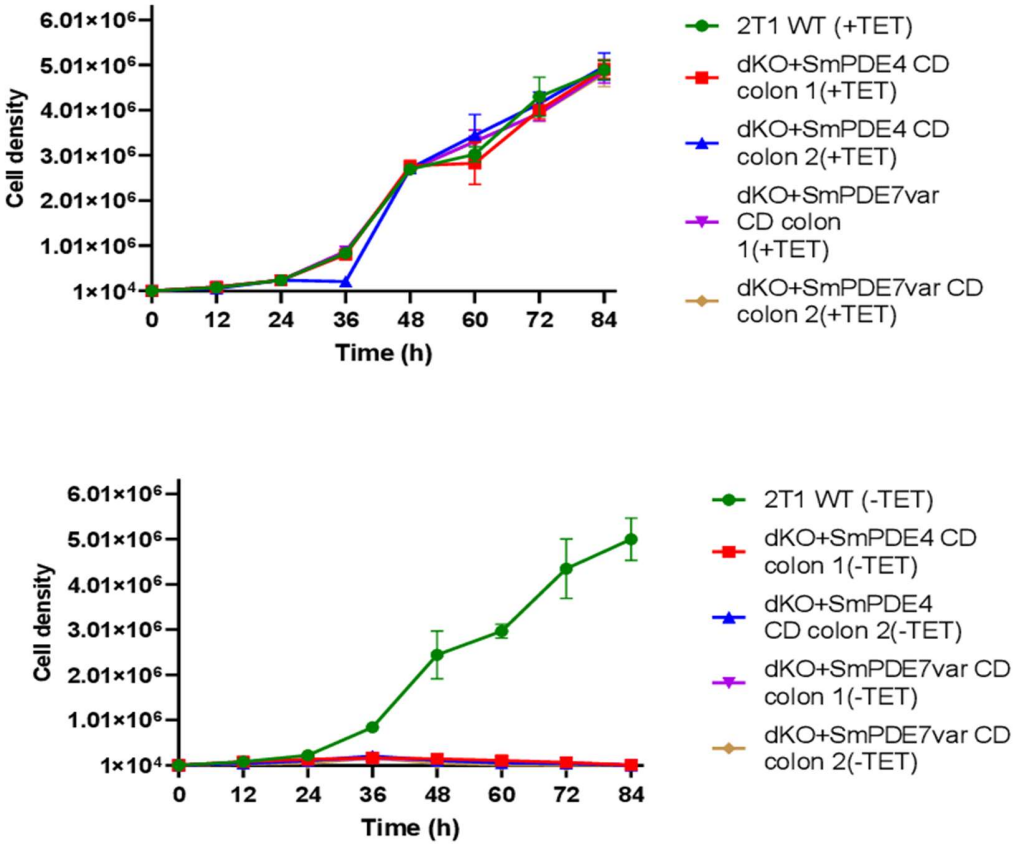


Figure 4-6: Growth analysis of catalytic-domain complementation lines carrying SmPDE4A or SmPDE7var in the *T. brucei* PDEB1/B2 null background. The wild-type 2T1 strain served as the control. Growth was assessed in the presence and absence of tetracycline. Mean values from three independent experiments are shown. Log10 number of trypanosomes × 10^4 cells/ml is plotted against time in days. Error bars represent ± standard error.

4.4 Discussion

This chapter sought to determine whether exchange of catalytic domains between TbrPDEB1 and selected *Schistosoma mansoni* PDEs would result in improved complementation compared to full-length protein-based approaches. The assumption underlying the approach suggests that the complementation by full-length proteins could be limited not only by catalytic function but also by protein localization, stabilization, and regulation in *Trypanosoma brucei*. By replacing catalytic domains with retaining the native protein framework in TbrPDEB1, it becomes possible to evaluate whether the introduced catalytic domain can restore the necessary phosphodiesterase activity without disrupting the regulation that is specific to the particular host cell.

Accordingly, experiments revealed that the catalytic-domain swap approach worked for SmPDE4A and SmPDE7var, but failed in relation to SmPDE11. The fact that SmPDE4A and SmPDE7var yielded viable *T. brucei* with all required molecular markers proves that these catalytic domains were capable of functioning in the TbrPDEB1 context in a way consistent with complementation. Conversely, the inability of SmPDE11 to create a double knock-out clone with requisite characteristics indicates that the catalytic domain of SmPDE11 could not replace the activity of the native phosphodiesterase in the host cell. Hence, the chapter provides proof that some *Schistosoma mansoni* phosphodiesterase catalytic domains could be transferred effectively to a new host.

One of the chief advantages of such an approach is the lower dependency on full-length protein localization in the process of complementation. For instance, in the case of full-length protein complementation, failures may occur due to incorrect protein localization or other protein-specific issues unrelated to catalytic activity. By introducing catalytic domains into TbrPDEB1, the approach retains all regulation-related factors (including GAF domains which are involved in the cAMP detection and PDE regulation in kinetoplastids). Nevertheless, the complementation can be ensured only if the transplanted catalytic domain can perform the cAMP hydrolysis efficiently enough for the cell.

In addition to providing functional confirmation, the created *T. brucei* strains have some utility on their own. Indeed, the fact that the *T. brucei* lines created by this approach depend solely on

one catalytic domain for growth means that the cells represent a good platform for further studies. As opposed to direct schistosome screening, this kind of approach reduces the ambiguity of the results as it allows for the analysis of compounds in biological context, yet avoids the complexity of working with parasites themselves. Despite that, the effectiveness of each drug needs to be confirmed independently for parasites since there may be significant differences in compound transportation in different organisms.

Overall, the chapter provides convincing evidence that substitution of the catalytic domain in TbrPDEB1 works as an improvement of complementation methodology. Accordingly, two out of three studied phosphodiesterases successfully complemented the loss of the catalytic domain in the trypanosome. Therefore, it became clear that the respective catalytic domains can indeed function as cAMP-hydrolysing enzymes in *T. brucei*.

Chapter 5: Pharmacological Profiling of Full-Length and Catalytic-Domain Complementation Cell Lines

5.1 Introduction

The current chapter describes the pharmacological characterization of both the full-length and catalytic domain complementation cell lines of *Schistosoma mansoni* phosphodiesterase. Dose–response curves for drug sensitivity were conducted using cell-based assays, from which EC_{50} values were calculated based on the non-linear fitting of the curve. The EC_{50} values obtained are expressed in terms of mean values, alongside their corresponding standard error and 95% confidence interval. As per the recommendations made by the examiner, t-test comparisons between EC_{50} values were not performed.

5.2 Drug sensitivity of full-length complementation cell lines

This part explains the pharmacological behavior of the complementation cells expressing the full-length SmPDEs with respect to the drugs being studied. The experiment intends to establish whether there is an association between the expression of different full-length SmPDEs and drug sensitivity in the complementation background of *T. brucei*. The EC50 values are expressed as mean ± standard error of the mean with 95% confidence interval. The data are presented comparatively without establishing their significance using the Student's t-test, as suggested by the examiner.

5.2.1 Augustyn group compounds

This sub-section highlights the corrected EC50 values of the Augustyn compounds that were evaluated against the full-length complementation cell lines. The objective here is to provide an illustration of the sensitivity level of all the cell lines to the individual drugs. Table 6.1 includes the output of all the corrected EC50 data, and Figure 6.1 and 6.2 give illustrations of NPD2023 and Pentamidine controls, respectively.

Table 5-1: Revised EC50 for Augustyn's group compounds tested in full-length *Schistosoma* complementation cell lines. EC50 values are reported in μM as mean, SE, and 95% CI from the verified replicate fitted outputs.

Compound	Cell line	n	Mean EC_{50} (μM)	SE	95% CI lower	95% CI upper
Pentamidine	WT	3	0.002	0.000780	-0.001000	0.005000
Pentamidine	Sm7var	3	0.002	0.000686	-0.000511	0.005000
Pentamidine	Sm1	3	0.003	0.000760	-0.000470	0.006000
Pentamidine	Sm8full	3	0.004	0.002000	-0.006000	0.013000
Pentamidine	Sm9C	3	0.003	0.001000	-0.002000	0.009000
Pentamidine	Sm4A	3	0.004	0.001000	-0.000776	0.008000
Pentamidine	TbrPDEB1/B2	3	0.007	0.002000	-0.002000	0.016000
NPD2023	WT	3	1.639	0.098	1.217	2.061
NPD2023	Sm7var	3	1.621	0.024	1.519	1.724
NPD2023	Sm1	3	1.405	0.051	1.184	1.626
NPD2023	Sm8full	3	1.665	0.026	1.553	1.777
NPD2023	Sm9C	3	1.649	0.083	1.293	2.005
NPD2023	Sm4A	3	1.556	0.093	1.156	1.955
NPD2023	TbrPDEB1/B2	3	1.587	0.119	1.074	2.100
NPD3024	WT	3	4.493	0.534	2.194	6.793
NPD3024	Sm7var	3	4.865	0.470	2.845	6.885
NPD3024	Sm1	3	5.152	0.268	3.998	6.306
NPD3024	Sm8full	3	5.030	0.375	3.415	6.644
NPD3024	Sm9C	3	5.113	0.203	4.239	5.987
NPD3024	Sm4A	3	4.407	0.211	3.499	5.315
NPD3024	TbrPDEB1/B2	3	4.998	0.304	3.688	6.307
NPD306	WT	3	6.508	0.142	5.896	7.120

NPD306	Sm7var	3	6.485	0.145	5.862	7.108
NPD306	Sm1	3	5.962	0.088	5.584	6.340
NPD306	Sm8full	3	5.352	0.210	4.449	6.254
NPD306	Sm9C	3	5.639	0.104	5.190	6.088
NPD306	Sm4A	3	6.046	0.114	5.556	6.537
NPD306	TbrPDEB1/B2	3	6.086	0.217	5.151	7.022
NPD311	WT	3	5.469	0.208	4.573	6.366
NPD311	Sm7var	3	5.652	0.110	5.180	6.124
NPD311	Sm1	3	5.962	0.273	4.788	7.136
NPD311	Sm8full	3	5.010	0.269	3.851	6.169
NPD311	Sm9C	3	5.644	0.071	5.340	5.947
NPD311	Sm4A	3	4.917	0.225	3.949	5.886
NPD311	TbrPDEB1/B2	3	5.050	0.560	2.639	7.460
NPD305	WT	3	9.655	0.435	7.785	11.525
NPD305	Sm7var	3	8.502	0.189	7.688	9.316
NPD305	Sm1	3	8.302	0.440	6.410	10.194
NPD305	Sm8full	3	8.874	0.821	5.342	12.406
NPD305	Sm9C	3	8.222	0.472	6.192	10.253
NPD305	Sm4A	3	8.249	0.273	7.075	9.423
NPD305	TbrPDEB1/B2	3	8.641	0.668	5.765	11.516
NPD3265	WT	3	9.720	0.834	6.130	13.309
NPD3265	Sm7var	3	9.531	0.688	6.569	12.493
NPD3265	Sm1	3	8.876	0.666	6.012	11.740
NPD3265	Sm8full	3	8.938	0.791	5.533	12.342

NPD3265	Sm9C	3	10.250	0.099	9.826	10.674
NPD3265	Sm4A	3	8.355	0.221	7.405	9.305
NPD3265	TbrPDEB1/B2	3	8.286	0.559	5.880	10.693
NPD264	WT	3	14.370	0.585	11.852	16.888
NPD264	Sm7var	3	10.154	0.595	7.592	12.716
NPD264	Sm1	3	14.360	0.771	11.041	17.679
NPD264	Sm8full	3	10.397	0.071	10.093	10.700
NPD264	Sm9C	3	11.053	0.459	9.077	13.029
NPD264	Sm4A	3	8.611	0.628	5.908	11.313
NPD264	TbrPDEB1/B2	3	11.920	1.414	5.835	18.005
NPD1246	WT	3	18.807	0.443	16.899	20.714
NPD1246	Sm7var	3	8.085	0.309	6.757	9.414
NPD1246	Sm1	3	7.583	0.502	5.424	9.741
NPD1246	Sm8full	3	10.365	0.466	8.359	12.371
NPD1246	Sm9C	3	8.260	0.528	5.987	10.533
NPD1246	Sm4A	3	8.505	0.213	7.587	9.423
NPD1246	TbrPDEB1/B2	3	15.740	0.850	12.082	19.398
NPD274	WT	3	11.031	0.713	7.962	14.101
NPD274	Sm7var	3	8.869	0.391	7.186	10.552
NPD274	Sm1	3	8.613	0.720	5.516	11.711
NPD274	Sm8full	3	9.194	0.195	8.356	10.032
NPD274	Sm9C	3	7.851	0.390	6.174	9.528
NPD274	Sm4A	3	7.952	0.191	7.130	8.774
NPD274	TbrPDEB1/B2	3	8.836	0.370	7.243	10.428

Corrected EC_{50} values in Table 6.1 show the results obtained for the Augustyn-group compounds in the full-length complementation lines. The table shows the mean EC_{50} value, its standard error, and 95% confidence interval for each combination of a drug and line, allowing one to assess the drug activity with greater rigor. Pentamidine is characterized by extremely low EC_{50} values in all lines, meaning this compound has the highest apparent activity in this dataset, while NPD compounds are characterized by increasing EC_{50} values depending on both the drug and the line. On average, the NPD2023, NPD3024, NPD306, and NPD311 drugs have comparatively lower EC_{50} values, whereas the NPD305, NPD3265, NPD264, NPD1246, and NPD274 drugs have higher values.

There is an additional fact presented under Table 6.1: the difference between EC_{50} values in different full-length complementation lines is considerable for most compounds. Thus, some lines provide consistently high and some consistently low EC_{50} estimates, which can be considered when considering a drug. However, as per the examiner's instructions, we cannot discuss the results in terms of significance but must note only that there seem to be differences in EC_{50}s in some cases, e.g., for NPD1246 and NPD264.

Finally, it may be worth mentioning that a small number of lower bounds for pentamidine estimates are negative. Such results should not be considered biologically meaningful because of low values at those concentration levels but should be included in the output regardless.

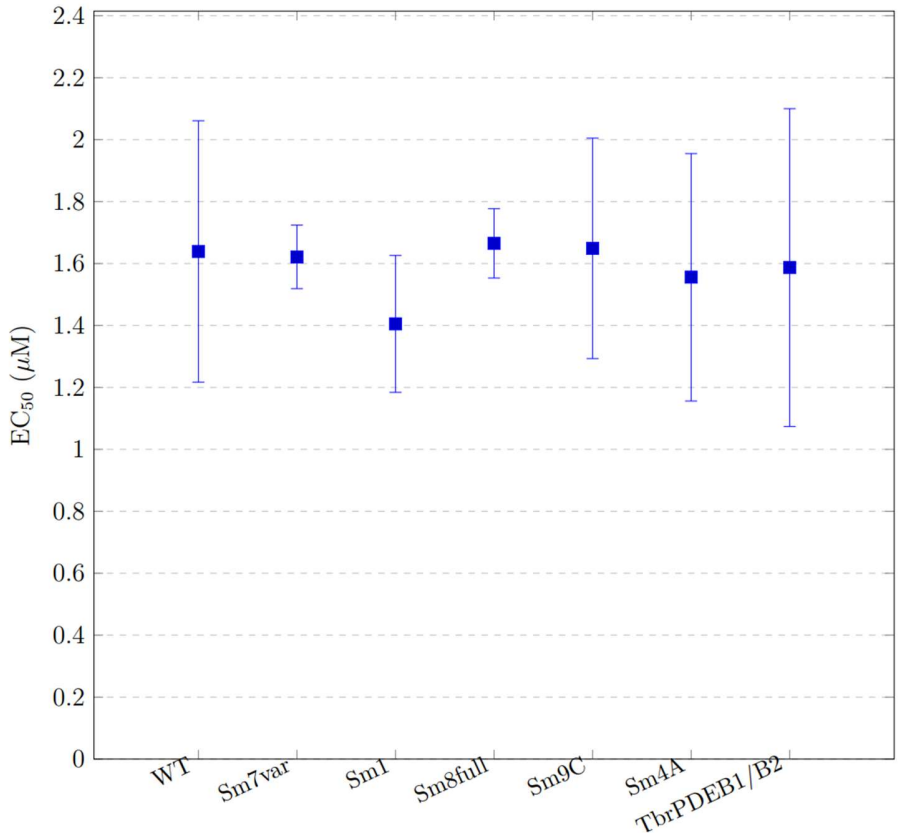


Figure 5-1: Representative EC50 for Augustyn's group compound NPD2023.

The distribution of EC50 values for NPD2023 in the complementation cell lines is illustrated in Figure 5-1. It is observed that the EC50 values for NPD2023 lie in a relatively small range, from 1.4 to 1.7 µM. Thus, it can be said that this compound produces a similar effect on the cell lines used in the experiment. Among these, the Sm1 cell line has the smallest mean value of EC50, whereas Sm8full and Sm9C cell lines have some of the largest values of EC50, but still fall into a relatively similar range.

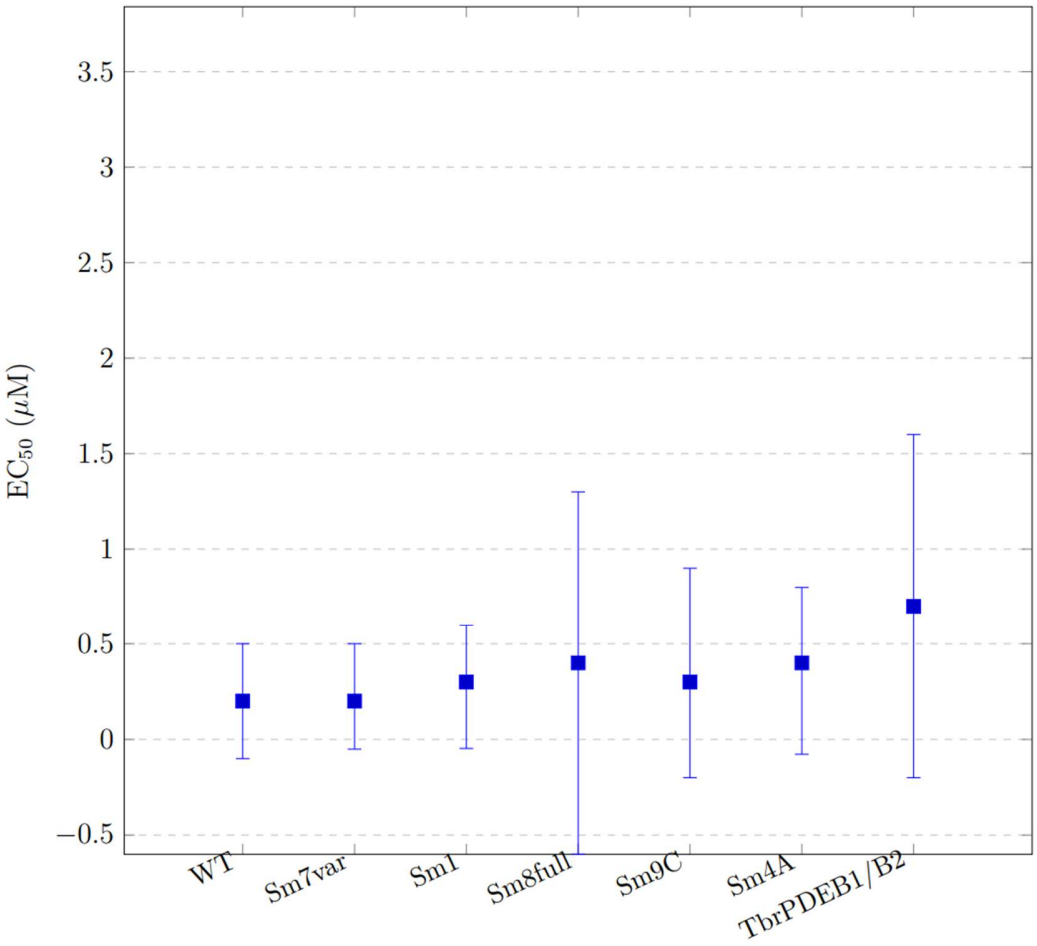


Figure 5-2: Representative EC50 for Augustyn's group pentamidine control.

Figure 5-2 represents an example of the EC50 summary for pentamidine as a control using the identical set of complementation cell lines at full-length form. As opposed to NPD2023, the EC50 data for pentamidine lie within the nanomolar to low micromolar range based on the corrected table results, thus suggesting significantly higher activity of pentamidine in this assay system. Even though there is some variation between the different cell lines used, the general observation indicates that pentamidine is highly active against all lines tested. Thus, Figure 6.2 provides a good control example demonstrating the high sensitivity of the assay system towards pentamidine.

5.2.2 Pollastri compounds

This section highlights the EC50 values for the Pollastri compounds after correction for testing on the full complementation cell lines. The purpose of this experiment is to evaluate the overall

sensitivity profiles of the SmPDE cell lines when treated with the Pollastri compounds, and to draw comparisons amongst the wild type, the *Schistosoma* complementation cell lines, and the TbrPDEB1/B2 lines. Table 5-2 gives the complete dataset for the EC50 values after correction, and Figure 5-3 and 5-4 give examples using the NEU-0005763 compound and pentamidine respectively.

Table 5-2: Revised EC50 for Pollastri compounds tested in full-length *Schistosoma* complementation cell lines. EC50 values are reported in μM as mean, SE, and 95% CI from the verified replicate fitted outputs.

Compound	Cell line	n	Mean EC_{50} (μM)	SE	95% CI lower	95% CI upper
Pentamidine	WT	3	0.005	0.003	-0.008	0.018
Pentamidine	Sm7var	3	0.006	0.002	-0.005	0.017
Pentamidine	Sm1	3	0.005	0.001	-0.001	0.011
Pentamidine	Sm8full	3	0.008	0.004	-0.008	0.025
Pentamidine	Sm9C	3	0.017	0.013	-0.039	0.072
Pentamidine	Sm4A	3	0.004	0.002	-0.003	0.011
Pentamidine	TbrPDEB1/B2	3	0.008	0.002	0.00036	0.015
NEU-0005763	WT	4	0.698	0.055	0.522	0.874
NEU-0005763	Sm7var	4	0.149	0.008	0.124	0.173
NEU-0005763	Sm1	4	0.183	0.020	0.118	0.247
NEU-0005763	Sm8full	4	0.338	0.029	0.245	0.430
NEU-0005763	Sm9C	4	0.298	0.056	0.120	0.475
NEU-0005763	Sm4A	4	0.106	0.018	0.049	0.163
NEU-0005763	TbrPDEB1/B2	4	5.239	0.580	3.393	7.085
NEU-0005764	WT	3	1.348	0.162	0.650	2.046
NEU-0005764	Sm7var	4	0.440	0.032	0.337	0.544
NEU-0005764	Sm1	4	0.491	0.058	0.305	0.676
NEU-0005764	Sm8full	4	0.768	0.124	0.372	1.164

NEU-0005764	Sm9C	4	0.707	0.090	0.422	0.992
NEU-0005764	Sm4A	4	0.324	0.058	0.139	0.509
NEU-0005764	TbrPDEB1/B2	3	6.996	0.366	5.421	8.570
NEU-0005762	WT	3	8.154	0.414	6.372	9.935
NEU-0005762	Sm7var	4	2.430	0.388	1.196	3.665
NEU-0005762	Sm1	4	3.130	0.348	2.022	4.238
NEU-0005762	Sm8full	4	5.203	0.299	4.251	6.154
NEU-0005762	Sm9C	4	4.910	0.413	3.594	6.226
NEU-0005762	Sm4A	3	1.709	0.259	0.594	2.824
NEU-0005762	TbrPDEB1/B2	3	13.837	0.645	11.060	16.614
NEU-0005761	WT	4	8.444	0.336	7.375	9.514
NEU-0005761	Sm7var	4	3.808	0.182	3.229	4.386
NEU-0005761	Sm1	4	3.829	0.333	2.769	4.890
NEU-0005761	Sm8full	3	6.118	0.211	5.208	7.028
NEU-0005761	Sm9C	3	4.752	0.558	2.350	7.154
NEU-0005761	Sm4A	4	1.792	0.318	0.780	2.805
NEU-0005761	TbrPDEB1/B2	3	7.225	0.622	4.550	9.899
NEU-0002666	WT	3	16.963	1.002	12.653	21.273
NEU-0002666	Sm7var	3	7.617	0.144	6.999	8.235
NEU-0002666	Sm1	3	7.231	0.493	5.109	9.353
NEU-0002666	Sm8full	4	11.625	0.383	10.405	12.845
NEU-0002666	Sm9C	3	10.838	0.708	7.790	13.887
NEU-0002666	Sm4A	3	3.986	1.046	-0.514	8.486
NEU-0002666	TbrPDEB1/B2	4	28.042	1.613	22.909	33.176

The correct EC_{50} values for Pollastri compounds in the complementation lines of the entire lengths are shown in Table 5-2. From the data, we can note some variations in the apparent pharmacological properties in the compounds, where some compounds show lower EC_{50} values compared to other compounds. In summary, we can say that there is no uniform pharmacological property in the cell lines, as while some compounds show similar values in EC_{50} for some lines, others have varied values for EC_{50}. In this regard, one needs to note that the table does not present the results for Student's t-tests in pairs because the examiner did not recommend it.

Another critical aspect about Table 5-2 is the inclusion of standard errors and 95% confidence intervals, which help to get a better representation of the uncertainty associated with each of the fitted EC_{50} values. This aspect is particularly significant in analyzing the entire length complementation lines' response, as it helps in determining the precision of each estimated value without having to refer to any form of significance value.

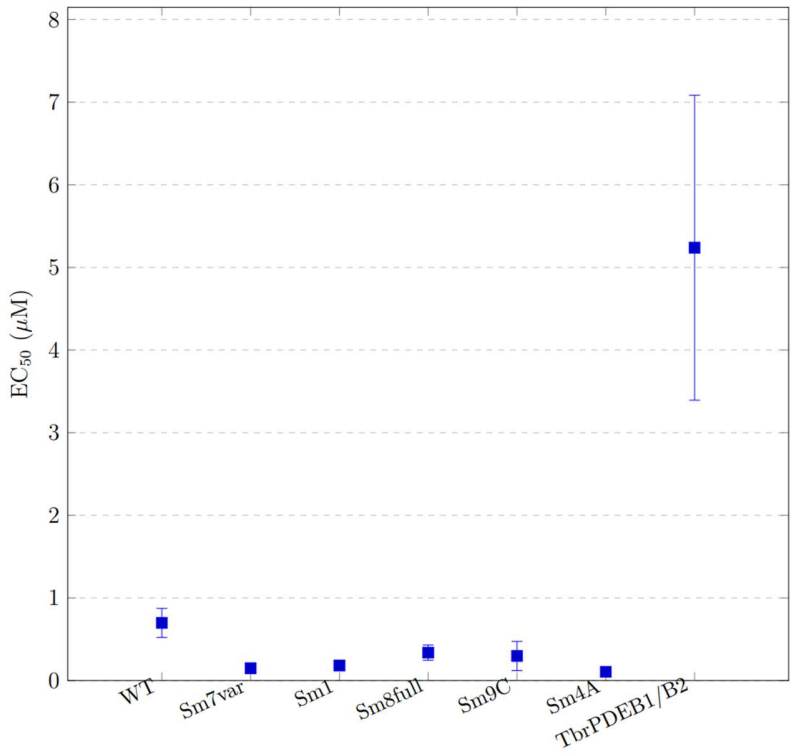


Figure 5-3: Representative EC50 summary for Pollastri compound NEU-0005763.

The figure 5-3 shows the typical representation of the EC50 plot of NEU-0005763 for all of the complementation cell lines in their entirety. The figure provides a way of comparing the EC50 fits obtained for the wild-type control, the different SmPDE cell lines, and the TbrPDEB1/B2

cell lines. Overall, the response obtained from NEU-0005763 differs from one cell line to another. However, the magnitude of this difference is more of a descriptive value than a statistically significant one. Therefore, figure 6.3 complements table 6.2 with regards to Pollastri compounds.

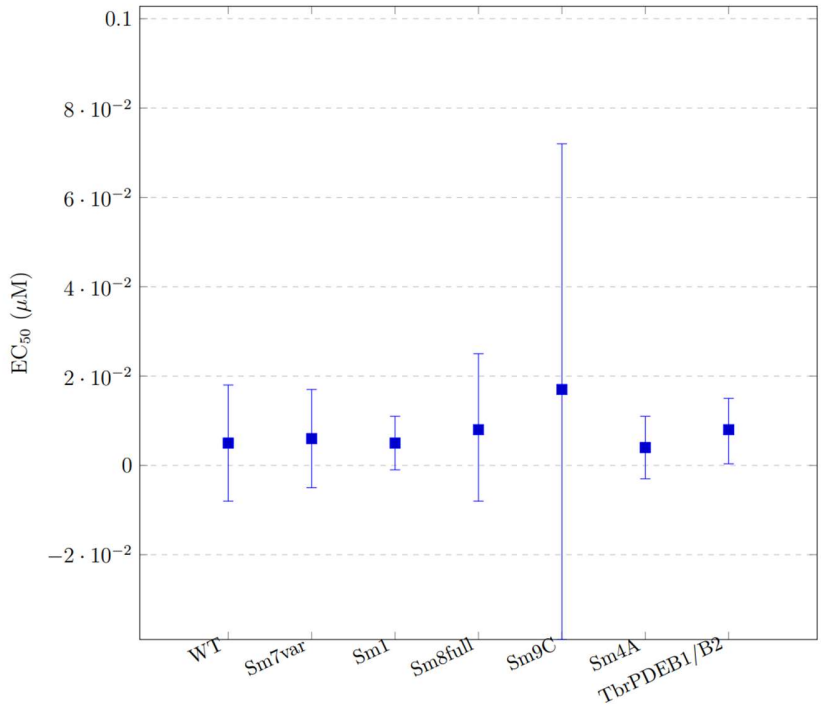


Figure 5-4: Representative EC50 summary for Pollastri pentamidine control.

Figure 5-4 shows the EC50 values for pentamidine as a function of the representative full-length cell line data set. Not surprisingly, pentamidine displays an EC50 that is significantly lower than that of the experimental compounds, suggesting a much higher level of effectiveness than the latter. This figure therefore serves as a benchmark against which the Pollastri dataset can be measured, since the sensitivity of the assay system as expected is high, and responses from the Pollastri compounds can be compared to those of an active compound. Like the other figures in this chapter, the differences shown should not be taken as statistically significant.

5.3 Drug sensitivity of catalytic-domain complementation cell lines

In this part, the corrected EC_{50} values of catalytic-domain complementation cell lines will be presented. This experiment is done to determine whether introducing the catalytic domain on the scaffold TbrPDEB1 produces a unique pharmacological profile compared with the wild-type

construct and other catalytic-domain constructs. The results are presented in a descriptive manner using the mean value, standard error, and 95% confidence interval without conducting a pairwise Student's t-test, according to the examiner's recommendation. The data of the entire corrected EC_{50} values is presented in Table 5-3, and the sample presentation for compound NPD226 and NPD1014 is illustrated in Figure 5-5 and 5-6, respectively.

Table 5-3: Revised EC50 summary table for catalytic-domain complementation cell lines. EC50 values are reported in µM as mean, SE, and 95% CI from the verified replicate fitted outputs.

Compound	**Cell line**	**n**	**Mean EC_{50} (µM)**	**SE**	**95% CI lower**	**95% CI upper**
Pentamidine	WT	3	0.006	0.001	0.000265	0.011
Pentamidine	Sm7var catalytic domain	3	0.005	0.002	-0.004	0.015
Pentamidine	Sm4A catalytic domain	3	0.013	0.003	-0.002	0.027
Pentamidine	TbrPDEB2 catalytic domain	3	0.007	0.004	-0.010	0.024
NPD226	WT	3	0.629	0.143	0.013	1.245
NPD226	Sm7var catalytic domain	3	0.282	0.030	0.154	0.411
NPD226	Sm4A catalytic domain	3	0.247	0.101	-0.186	0.681
NPD226	TbrPDEB2 catalytic domain	3	0.341	0.031	0.209	0.474
NPD008	WT	3	1.778	0.375	0.165	3.390
NPD008	Sm7var catalytic domain	3	1.047	0.257	-0.058	2.153
NPD008	Sm4A catalytic domain	3	0.486	0.097	0.068	0.905
NPD008	TbrPDEB2 catalytic domain	3	0.748	0.187	-0.054	1.550

NPD029	WT	3	9.132	0.890	5.302	12.962
NPD029	Sm7var catalytic domain	3	6.310	0.777	2.966	9.654
NPD029	Sm4A catalytic domain	3	3.561	0.408	1.806	5.315
NPD029	TbrPDEB2 catalytic domain	3	4.947	0.031	4.816	5.078
NPD1014	WT	3	7.952	0.720	4.855	11.050
NPD1014	Sm7var catalytic domain	3	2.168	0.008	2.132	2.205
NPD1014	Sm4A catalytic domain	3	1.859	0.011	1.811	1.907
NPD1014	TbrPDEB2 catalytic domain	3	2.377	0.108	1.912	2.841
NPD274	WT	3	14.577	0.780	11.219	17.934
NPD274	Sm7var catalytic domain	3	10.283	0.026	10.171	10.395
NPD274	Sm4A catalytic domain	3	10.983	0.101	10.549	11.417
NPD274	TbrPDEB2 catalytic domain	3	10.860	0.360	9.310	12.410

The corrected half-maximal effective concentrations (EC_{50}) for the catalytic-domain complementation cell lines are shown in Table 5-3 and include the wild-type control, the Sm7var catalytic-domain, Sm4A catalytic-domain, and TbrPDEB2 catalytic-domain lines. From the results obtained, pharmacological response differs with chemical compound and cell lines. In general, pentamidine shows the smallest EC_{50} values, and hence it appears as the most potent agent in the current dataset. Other chemical compounds such as NPD029, NPD1014, and NPD274 show significantly higher EC_{50} values. NPD226 and NPD008 lie within the low-to-medium categories, while NPD029, NPD1014, and NPD274 have increasingly higher EC_{50} values.

Secondly, the catalytic-domain cell lines do not act similarly. More specifically, Sm7var and Sm4A catalytic-domain lines often give lower EC_{50} values than the wild-type controls for some chemicals like NPD226, NPD008, NPD029, and NPD1014. Also, the catalytic-domain cell line shows a characteristic different response from that of wild-type. Notably, the TbrPDEB2 catalytic domain has a different response, although not identical with Sm7var or Sm4A catalytic domain. Catalytic-domain complementation might modify the pharmacological response of the system. However, these results should be viewed comparatively and not as statistical significance since the pairwise Student's t-test was not performed.

Table 5-3 is especially crucial in the present context as it gives not only the estimated mean EC_{50} values but also the standard errors and 95% confidence intervals. Hence, the current corrected table meets the examiner's criteria of presenting EC_{50} estimates alongside model uncertainties. In other words, Table 6.3 provides a firmer basis for comparison among the various catalytic-domain lines than before.

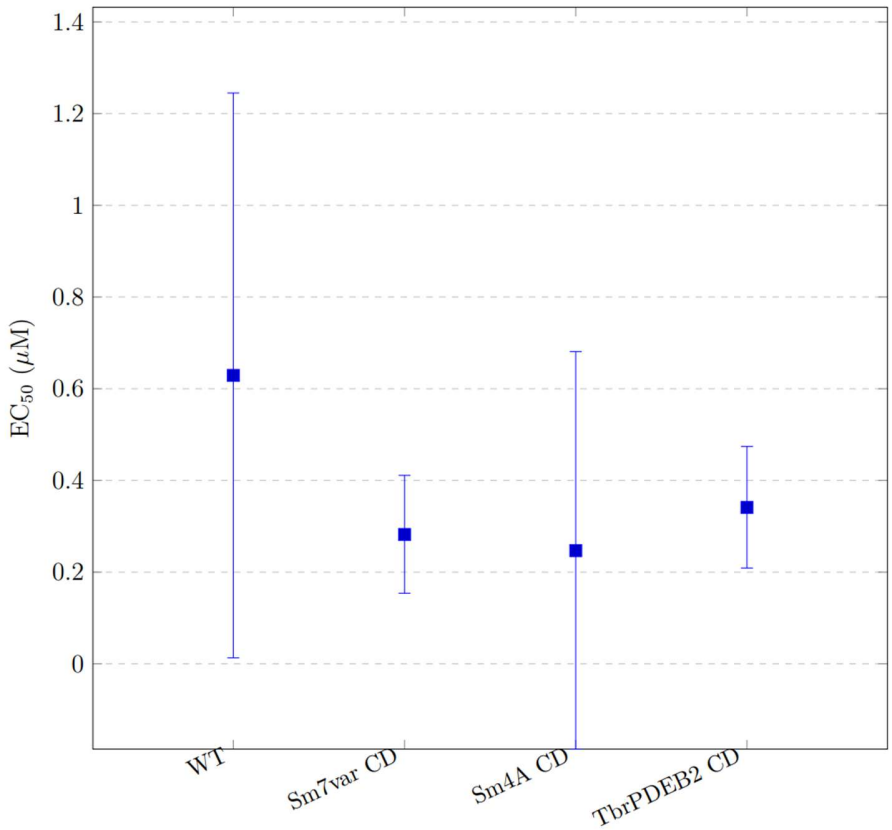


Figure 5-5: Representative EC50 summary for catalytic-domain construct NPD226

The EC50 profile for NPD226 in the catalytic-domain complementation cell lines is depicted by Figure 5-5. From this figure, the wild-type control shows the highest mean EC50 value while

the Sm7var catalytic-domain and Sm4A catalytic-domain cell lines show low EC50 values with the latter giving the lowest mean EC50 value in this case. The TbrPDEB2 catalytic-domain cell line lies between these two ranges. This could be because the catalytic-domain substitution background affects the sensitivity towards NPD226 with the catalytic-domain substitution cell lines being more sensitive than the wild-type control.

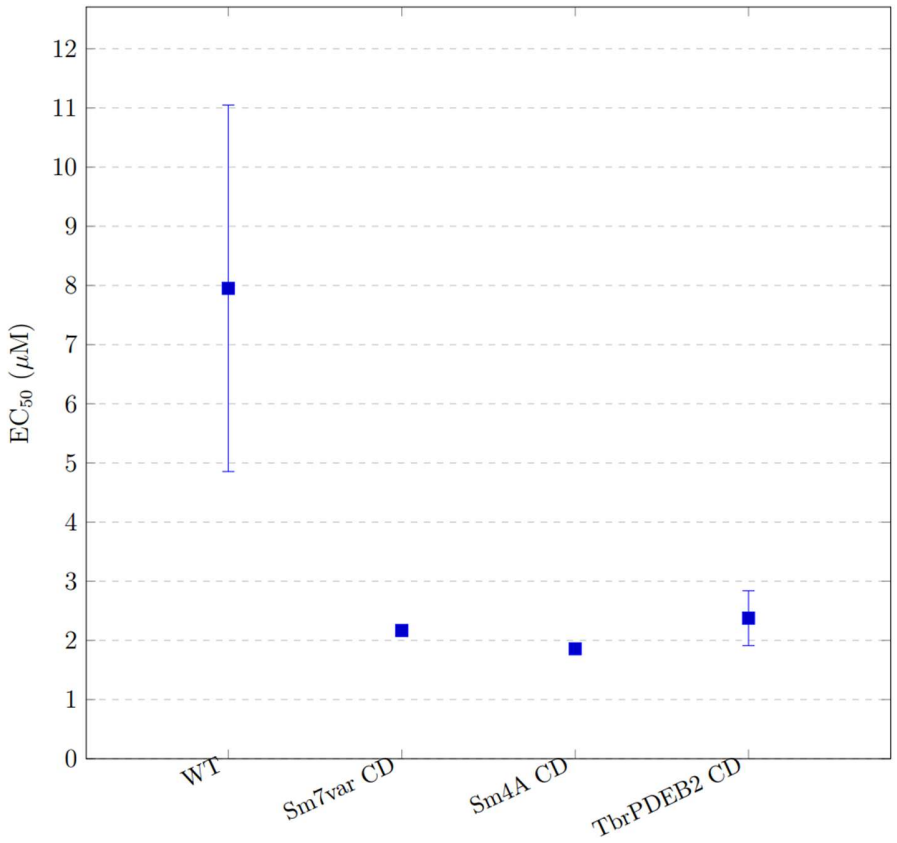


Figure 5-6: Representative EC50 summary for catalytic-domain construct NPD1014.

The EC50 profile of NPD1014 is shown in Figure 5-6 using catalytic domain complementation cell lines like Figure 5-5. As would be expected, the mean EC50 was highest in the wild-type controls compared to the catalytic domain lines. This trend of reduced EC50s was seen in the Sm4A and Sm7var catalytic domain lines, which showed more dramatic EC50 changes than the other catalytic domain cell lines examined. Similarly, the TbrPDEB2 catalytic domain line had an EC50 lower than the wild type but still slightly higher than Sm4A and Sm7var catalytic domains. Overall, this graph confirms the trend reported in Table 5-3.

5.4 Summary of pharmacological findings

This chapter offers a corrected pharmacological profiling of the full-length and catalytic domain complementation cell lines. Contrasting the earlier version of this report, all presented EC50 values are fully formatted with associated SE and 95% CI. This will make the interpretation of the fitted EC50 estimates possible as opposed to presenting just a point estimate in isolation. With this presentation, the results will offer a clearer and methodologically more sound basis for comparing drug responses across compounds and cell lines.

In the case of the Augustyn full-length dataset, the results show considerable variance in apparent potency between the different compounds. Pentamidine showed the lowest EC50 values in all full-length lines, supporting its status as the control compound with high apparent potency. The test compounds showed varying potencies as well with NPD2023 having some of the lowest EC50 values in the full-length complementation lines while NPD3024, NPD306, and NPD311 exhibiting average to intermediate EC50 values. NPD305, NPD3265, NPD264, NPD1246, and NPD274 on the other hand showed high EC50 values, thus demonstrating low potency. It is also apparent from the Augustyn results that full-length cell lines do not respond equally to each drug; some drugs exhibit only small differences while others display larger spreads of fitted EC50 estimates.

The Pollastri dataset showed a somewhat different but similar pharmacological profile. Again, pentamidine showed the lowest EC50 values in the full-length complementation lines, which shows its high apparent potency within this construct. Out of the Pollastri compounds, NEU-0005763 and NEU-0005764 showed relatively low EC50 values compared to the rest, indicating their relatively high apparent potency in this system. NEU-0005762, NEU-0005761, and NEU-0002666 in comparison to other compounds showed high EC50 values, hence relatively low apparent potency. Another interesting pattern in this dataset was that the TbrPDEB1/B2 background was shown to have among the highest EC50 values in response to various compounds while the full-length SmPDE lines, specifically Sm7var and Sm4A, had the lowest fitted values to certain compounds.

Similarly, the catalytic domain dataset supports the conclusion that the pharmacological profile of a particular complementation construct might be influenced by whether a full-length protein or only catalytic domain was used. In the catalytic-domain constructs, just like in the two previously mentioned datasets, pentamidine showed the lowest EC50 values. NPD226 and

NPD008 showed EC50 in the low-to-intermediate range, while NPD029, NPD1014, and NPD274 had higher values. From this dataset, it appears that Sm7var catalytic domain and Sm4A catalytic domain often show lower EC50 values than the wild-type background for certain compounds, such as NPD226, NPD008, NPD029, and NPD1014. TbrPDEB2 catalytic domain also showed unique values, which, however, did not coincide perfectly with those of schistosome constructs.

Thus, from this chapter, the major conclusion is the description of the pharmacological behavior of the tested lines as opposed to claiming any kind of statistical differences between them. As per the suggestions made by examiners, no Student's t-test comparisons for EC50 values were performed. The results have been provided in the form of a descriptive pharmacological profiling using EC50, SE, and 95% CI.

Chapter 6: General Discussion and Conclusions

6.1 Introduction to the general discussion

In this chapter, the findings of the thesis have been consolidated in totality, and their importance has been analyzed with regard to the objectives of the study. In particular, the contribution made by the study has been analyzed, its findings have been interpreted in the context of previous literature, and the strengths and weaknesses of the approach taken have been evaluated. The impact that these findings have on future investigations into the phosphodiesterases of *Schistosoma mansoni* has been discussed.

6.2 Summary of the main findings

6.2.1 Identification and cloning of selected SmPDEs

One of the main discoveries made during the course of the thesis is that of the cloning and preparation of a selection of *Schistosoma mansoni* phosphodiesterases for functional studies in heterologous expression systems. Indeed, the preparation of expression constructs corresponding to selected full-length SmPDEs as well as a following set of catalytic domain constructs aimed for domain swap complementation in *Trypanosoma brucei* were made throughout the duration of the thesis project and served as a molecular foundation for the rest of the complementation and pharmacology experiments carried out in the thesis.

While being based on past studies conducted in the De Koning laboratory, the current thesis went further by breaking out of the functional paradigm created in the former. Indeed, while earlier studies focused on complementing certain SmPDEs, the current thesis managed to expand the scope of experiments by producing an increased number of full-length as well as catalytic domain SmPDE constructs and optimizing their expression method. Therefore, the current thesis was not limited to cloning experiments carried out before but took them one step further.

6.2.2 Functional complementation of full-length SmPDEs in *T. brucei*

Another important finding from this thesis is that the full-length versions of SmPDE7var, SmPDE1, SmPDE8, and SmPDE9C were able to be used for complementing the conditional *T.*

brucei PDEB1/B2 knockout system, producing viable and complemented double knockouts in an inducible fashion via tetracycline addition. Through growth analysis, it has been established that such complemented lines remain alive after the induction of the heterologous SmPDE gene and become nonviable upon removal of the inducer, which confirms that viability in such condition depends on continuous expression of the introduced SmPDE. In conclusion, the presented data shows that each of the four full-length SmPDEs provides enough phosphodiesterase activity for maintaining the vital role of cAMP regulation in trypanosomes that is performed by TbrPDEB1/B2.

The data obtained is quite different from the results previously obtained in the laboratory of De Koning and represents a valuable extension to the previous research. Namely, the earlier work has shown that SmPDE4A, but not the other tested SmPDEs in the initial framework with a C-terminal GFP tag, complements the *T. brucei* PDEB1/B2 knockout system. At the same time, SmPDE11 could not complement this system because of its preference for hydrolyzing cGMP rather than cAMP. Contrasting to this data, this thesis demonstrates that the lack of complementation for SmPDE11 was not caused by intrinsic inability to complement the *T. brucei* system but rather by expression strategy chosen, and the same applies to at least four other SmPDE enzymes.

Functionally, this means that the absence of complementation previously reported cannot be regarded as indication of inability to complement this *T. brucei* system, because of the dependence of complementation on expression strategy chosen for heterologous protein. In parallel, growth analysis revealed that despite the fact that such SmPDEs are able to complement this *T. brucei* system, they may not recapitulate all properties of the native enzyme in trypanosome since some complemented strains grow slower than wild type. Overall, such data clearly indicates that several full-length SmPDEs may serve as functional cAMP hydrolyzing enzymes in trypanosomes.

6.2.3 Catalytic-domain complementation as a refined strategy

The next major result that emerged from the thesis work was that the use of catalytic-domain replacement represented an advanced form of complementation test which was sometimes more useful than using full-length heterologous protein. This is because, in the event of failure in full-length complementation, this may not only be due to problems in enzymatic activity but may also be due to factors such as localization, stability, or even compatibility of the foreign protein

in the trypanosome cell environment. To overcome this problem, the catalytic domains of select SmPDEs were used and cloned in the same frame with TbrPDEB1, thus retaining the native trypanosome protein structure for the sake of localization and stability, while only testing the capability of the schistosome catalytic domain for enzymatic activity.

Using this methodology, the catalytic domains of SmPDE4A and SmPDE7var succeeded in complementing the PDEB1/B2 double-knockout, whereas the catalytic domain of SmPDE11 failed to do so. These two former domains satisfied the molecular criteria required for successful complementation and allowed growth in the absence of tetracycline. Hence, these two catalytic domains were capable of providing sufficient phosphodiesterase activity when placed in the TbrPDEB1 backbone structure. On the other hand, SmPDE11 was incapable of creating a double knockout complementation line due to its inability to complement the endogenous *T. brucei* enzyme system.

Molecularly, the success of the catalytic domains of SmPDE4A and SmPDE7var, compared with their failure in the full-length form, indicates that full-length heterologous proteins are not necessarily needed for the successful complementation reaction. This means that the problem may lie elsewhere, such as localization and regulation. It appears that the catalytic domains can actually hydrolyze enough cAMP for the parasite to survive; thus, it is likely that the full-length proteins' inability to do so is partly related to localization and regulation issues. Meanwhile, the inability of the SmPDE11 catalytic domain to complement shows that the preservation of the TbrPDEB1 backbone alone cannot ensure complementation; in other words, catalytic compatibility is still crucial.

So, the catalytic-domain complementation test is therefore a valuable method in understanding complementation experiments since it enables one to discriminate between enzymatic capability alone and the ability to localize and regulate appropriately. Because in this method, the TbrPDEB1 backbone structure was maintained, the host specificity was retained for both localization and regulation via GAF domains. Thus, the catalytic-domain complementation proved itself to be a more informative approach to both complementation studies and pharmacological analysis carried out in this thesis.

6.2.4 Pharmacological profiling of the complementation lines

The pharmacological data presented in this thesis show that the complementation lines represent an important tool for identifying general trends in compound activity against the full-

length and catalytic-domain constructs. In the case of the reference drugs, pentamidine always showed the lowest EC50 value and thus proved to be the most active compound in the assay system. The experimental drugs, on the other hand, showed considerable variation in their EC50 value, with some compounds being more potent (having lower micromolar activity) than others, demonstrating a pronounced difference in apparent potency.

In the full-length background, it became clear that pharmacological response was affected not only by the chemical composition of the drug but also by the PDE construct in which the compounds were tested. While some compounds showed relatively equal EC50 values in all three complementation lines, others demonstrated considerable variability in the pharmacological response profile. As a general trend, the Augustyn set consisted of compounds with lower, middle, and higher EC50 values, while the Pollastri set included compounds that showed distinctly different pharmacological responses in the same panel of complementation lines.

Pharmacological analysis of the catalytic domain lines revealed that the response to the compounds may also be modified if the full-length PDE construct is replaced with only the catalytic domain of the enzyme. Specifically, in several cases, the Sm7var and Sm4A catalytic-domain lines showed reduced EC50 values compared to the control line. Overall, these findings suggest that pharmacological profiling using the complementation approach is sensitive to the differences in the target PDE construct. Thus, this chapter clearly shows that the complementation lines are capable of providing meaningful data about the general response to a broad range of compounds and can be used for pharmacological analysis.

6.3 Critical interpretation of the findings in the context of the broader field

6.3.1 What this thesis adds beyond previous functional complementation work

Firstly, this thesis contributes an important improvement in the functional complementation experiments of *Schistosoma mansoni* phosphodiesterases. While Munday et al. (2020) demonstrated proof-of-principle functional complementation with SmPDE4A and illustrated its success at rescuing the crucial *Trypanosoma brucei* PDEB1/PDEB2 system in a heterologous system, there remained a lingering question about whether or not the negative outcomes of

attempts to complement using other full-length SmPDEs indicated their inability to substitute for the TbrPDEB1/PDEB2 pair. The current study addresses this issue head-on.

As evidenced by the data in this thesis, complementation capacity does extend beyond SmPDE4A. By proving that certain other full-length, untagged SmPDE proteins can serve as complements in place of TbrPDEB1/PDEB2, the present research indicates that prior failures were not sufficient to rule out the possibility of successful replacement. This constitutes a significant step forward because it allows us to reassess the previous system as more than an experiment in which enzyme identity determines success; here we take into account construct type, host-cell compatibility, and much more. In other words, not only do we provide additional positive examples of complementation, we interpret them in context – biologically.

The third major advance made by the research conducted for this thesis lies in methodology. The use of catalytic domain complementation in this case is preferable to the prior method of employing whole, full-length proteins. This is due to the decoupling of catalytic activity from structure and context in this approach. In turn, this leads to better-informed interpretation of negative results. When the catalytic domain of a protein complements successfully when integrated into the scaffold of TbrPDEB1, a failure of the full-length protein to play that role suggests problems with localization or stabilization.

6.3.2 What the successful and unsuccessful complementation results mean biologically

The biological relevance of the obtained results demonstrates that there exists a range of SmPDEs that might have enough phosphodiesterase activity for cyclic nucleotide breakdown in a cAMP-dependent trypanosome to provide complementation. This allows assuming that these enzymes are not just similar in structure to any particular known group of phosphodiesterases but are indeed biologically active in living cells. Nevertheless, this cannot be interpreted as full equivalence to trypanosome enzymes since not all complemented strains demonstrated similar growth dynamics to wild-type strains, meaning that functional complementation might suffice for viability but does not guarantee that heterologous PDE would provide the same regulation or efficiency as endogenous TbrPDEB1/B2.

As for the unsuccessful cases, they give us important information. The failure of SmPDE11 in this case is further confirmed since both previous tests and the catalytic-domain approach showed it as unsatisfactory. The point is that SmPDE11 prefers to hydrolyze cGMP instead of

cAMP, which explains why it could not complement this system. It is crucial to state that the use of heterologous complementation as a means of checking enzymatic activity proved to be more specific here since the inability of an enzyme to complement this system confirms its biological specificity.

Comparing the full-length method and catalytic-domain method shows how much impact regulation has on functionality. Namely, the success of SmPDE4A and SmPDE7var catalytic domains in the TbrPDEB1 system proves that, in some situations, catalytic ability is enough to function properly in a host system if its own regulation and localization are preserved. This means that, in many cases, failed attempts to complement systems via full-length heterologous expression might appear since PDE function is dependent on something else beside catalysis itself. In other words, PDEs function within a certain structural and regulatory environment provided by domain architecture, localization signal, and other features peculiar to host species. Therefore, our biological analysis concludes that successful complementation is provided due to both catalytic activity and compatibility with regulation.

Summing up all the above facts, we conclude that the *Trypanosoma brucei* heterologous platform has proven itself well-suited for studies. Its main advantage is in providing a viable cell-based system in which a certain defined enzymatic activity is required for survival. In other words, *Trypanosoma brucei* heterologous system can be seen as extremely useful for studying biological function, but the presence of its host-specific characteristics makes it still just a substitute.

6.3.3 What the pharmacological findings mean for SmPDE target validation

As suggested above, the pharmacological data corroborate the utility of complementation lines as important platforms for target-oriented profiling, although only conditionally. First of all, it cannot be stated directly from the obtained EC50 values that any particular SmPDE is proven to be a valid therapeutic target since it was shown that complementation leads to the formation of reproducible and construct-dependent pharmacological profiles. At the same time, the presented results demonstrate that the complementation approach can be successfully applied as an intermediate step toward validating target orientation of a compound.

At the same time, one should bear in mind the limitations associated with using such an approach. Differences between EC50 values indicate that the biological background can significantly affect the perceived effect of the tested compounds, although these data do not

provide evidence for biochemical specificity toward a given SmPDE. One should remember that cellular assay allows assessing compound efficacy due to several different parameters (compound penetration, metabolism, etc.) that go far beyond actual interaction with the protein target. Based on this assumption, one can conclude that the presented results can serve as supportive information only.

It is necessary to emphasize that SmPDE target validation poses several unique challenges due to the specific nature of *Schistosoma* parasite. The current study indicates that complementation-based approaches can play the role of an intermediate stage between purely biochemical screenings and direct testing in schistosomes themselves. This strategy has several key advantages over other possible methods: on one hand, it preserves cellular context; on the other hand, it provides much better experimental opportunities compared with whole-parasite testing. Thus, the most important role of complementation lies in its capability of narrowing down the gaps between biochemical and pharmacological validation stages.

The data presented above allow proposing the following three-stage target validation scheme involving SmPDEs: at first, cloning and complementation should prove that an SmPDE or its catalytic domain is functional; second, compound profiling within complementation systems can show that pharmacological response depends on background PDE type; third, parasite-native studies will have to prove that this response produces antischistosomal effects.

6.4 Strengths of the study

One notable strength of this thesis project was the direct application of functional methods used previously in the De Koning laboratory with significant improvements over several shortcomings associated with those previous studies. For example, the current work moves beyond the restricted proof-of-principle phase and includes functional complementation of other phosphodiesterase enzymes of *S. mansoni* and extends analysis of constructs from both full-length and catalytic domains. This combination is beneficial for interpretational purposes since it provides multiple levels at which differences may be observed between constructs and at least in part discriminates between catalytic activity and protein context, localization, and regulation.

The second strength of the thesis was the design and use of manageable models of complementation in *T. brucei* providing biological relevance to this study on a cellular level. An additional benefit is provided by combining molecular cloning, complementation analysis,

growth phenotyping, and drug sensitivity testing in one single methodology. Lastly, an improvement in the way pharmacological data are reported in this study is seen through the inclusion of EC_{50} values with their standard error and 95% confidence interval.

6.5 Limitations of the study

It is important to identify and list several limitations of the current research. The first one is that not all SmPDEs of *S. mansoni* could be complemented in the *T. brucei* cell line. This means that despite receiving important data concerning the behavior of some SmPDEs within this particular platform, we failed to build a comprehensive functional map of all members of this phosphodiesterase family. Another limitation related to heterologous complementation of proteins in *T. brucei* cell line implies that our results might be affected by the context in which SmPDEs and their catalytic domains were active. Just because SmPDEs showed activity within the context of *T. brucei* cells does not mean that they performed their native functions and had a similar distribution and regulation pattern as in *Schistosoma mansoni* parasites.

Another limitation is related to the pharmacological aspect of our experiments. Although the corrected EC50 values allowed us to create a more accurate description of responses of compounds tested against SmPDEs, such findings cannot be considered mechanistically conclusive per se. Differences between fit of the obtained EC50 values are caused by many reasons other than simple interaction with targets, including their uptake, accumulation within cells, and biological characteristics of the host itself. Furthermore, since there is no availability of representative raw data for fitting curves for all EC50 values, we could not visualize them all. Most importantly, our research did not eliminate the necessity for experiments on whole parasites themselves.

6.6 Future directions

6.6.1 Further functional analysis of non-complementing SmPDEs

One significant next step includes the additional functional characterization of the SmPDEs which failed to complement in the present study. Inconclusive results do not necessarily mean that the proteins were biologically inactive, as they could result from problems associated with the method used for expression, the host cell used, the localization of the protein, or the regulation of its activity, rather than from a lack of enzymatic activity. Further studies must

include tests for these SmPDEs using different constructs and/or expression systems, as well as additional assays designed to differentiate between an inability to properly integrate into the cell and an inability to perform their enzymatic activity.

6.6.2 Expansion of catalytic-domain swap approaches

The catalytic-domain strategy is an especially illuminating development of the hypothesis and should be continued in future experiments. In particular, the testing of other SmPDE catalytic domains on the TbrPDEB1 scaffold would further elucidate whether these domains can be used in the trypanosome cAMP regulation network or not. This would provide an even clearer basis for comparing the results obtained for full-length proteins and catalytic domains. A further analysis of catalytic domains could also highlight certain commonalities among all members of the phosphodiesterase family that are not currently evident based on the results gathered so far.

6.6.3 Broader pharmacological screening and orthogonal validation

Another key aspect is the need for pharmacological screening using the new complementation strains produced in this study. In terms of the results obtained in the current study, the ability of the technology to identify compound-dependent and background-dependent responses has been successfully demonstrated; nonetheless, future studies should focus on employing larger numbers of compounds and comparisons between several PDE proteins. In parallel, pharmacological data acquired with complementation strains should be supported by orthogonal approaches that include enzymatic activity assays, different cell-based systems, or additional approaches for assessing the engagement of the target.

6.6.4 Validation in schistosomes and not only in *T. brucei*

Although the *T. brucei* complementation system represents an important tool for experimentation, it must not be considered the ultimate solution; further steps need to be taken for validating results obtained by using this approach. One such step involves performing validation experiments on *S. mansoni* itself, to test if molecules showing promise through the complementation system affect viability, mobility, or reproductive capabilities of the parasite. Such an experiment is necessary since the activity of a potential drug target on the parasite may behave differently compared to its behavior inside a heterologous organism, owing to differences in protein expression conditions, transport mechanism, access to certain organs, and other factors.

6.6.5 Structural and mechanistic follow-up studies

The findings of this thesis also call for further structural and mechanistic studies to better understand their significance. Since it is clear from the above results that complementation success depends on catalytic complementarity and overall protein context, the next step will be to look into structural reasons why some proteins or catalytic domains are able to successfully complement the enzyme while others fail at that. Some examples could be sequence comparison, mapping of protein domains, structure-based modeling, and studying substrate specificity or regulation mechanisms. This would help explain why some SmPDE enzymes or their domains are capable of functioning in the system while others are unable to do so.

6.7 Overall conclusion

In this regard, this thesis significantly contributes to the functional characterization of SmPDEs by expanding heterologous complementation beyond the narrow boundaries of previous studies and improving the boundaries themselves with the help of a catalytic domain strategy. First, the research proves that several full-length SmPDE genes can complement the lack of essential PDE function in *Trypanosoma brucei* cells. Second, it shows that a catalytic domain substitution is a better way to evaluate functional compatibility compared to full-length proteins. Third, it establishes that both types of complementation lines are appropriate candidates for pharmacological testing.

Overall, the results presented in this research provide valuable insight into the functional characteristics of SmPDEs and prove that functional compatibility depends not only on the catalytic activity of the protein itself but on the overall structure. Therefore, these results have practical application value for future research related to antischistosomal drug development.

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