The repurposing of anti-malarial as anti-cryptococcal drugs

Lynda Uju Madu

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Department of Microbial, Biochemical and Food Biotechnology

Faculty of Natural and Agricultural Sciences

University of the Free State

Bloemfontein

South Africa

Promoter:

Prof. O.M. Sebolai

Co-Promoters:

Prof. C.H. Pohl and Prof. J. Albertyn

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Author:	Lynda Uju Madu
Laboratory:	Pathogenic Yeast Research Group
	Dept. Microbial, Biochemical and Food Biotechnology
	Faculty of Natural and Agricultural Sciences
	University of the Free State
	Bloemfontein, 9301
	South Africa
	+27 51 401 2004 (telephone)
	+27 51 401 9376 (fax)
	lynda.madu@yahoo.com (e-mail)

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DEDICATION

This study is dedicated to my lovely husband - Dr Chika Egenasi and my awesome children - Chizaram and Chikamso Egenasi. Thank you for being my pillar of strength.

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patient with me. Your love, prayers and endurance on this ride, will be forever cherished.

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- **Above all, I am deeply indebted to God Almighty for the precious gift of life.**

DECLARATION

I hereby declare the work presented in the thesis is as a result of my own independent investigations. In addition, I declare this thesis has not been submitted, in full or part, to another institution for the granting of a PhD degree. There are no competing financial interests.

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Madu, Lynda Uju

Candidate for PhD degree

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Environmental & Biosafety Research Ethics Committee

02-Nov-2019

Dear **Ms. Lynda Uju Madu**

Project Title: **The repurposing of anti-malarial as anti-cryptococcal drugs**

Department: **Microbial Biochemical and Food Biotechnology Department (Bloemfontein Campus)**

APPLICATION APPROVED

This letter confirms that this research proposal was given ethical clearance by the Biosafety & Environmental Research Ethics Committee of the University of the Free State.

Your ethical clearance number, to be used in all correspondence is: **UFS-ESD2019/0133**

Please note the following:

1. **This ethical clearance is valid for one year from the issuance of this letter.**
2. **If the research takes longer than one year to complete, please submit a Continuation Report to the Ethics Committee before ethical clearance expires.**
3. **If any changes are made during the research process (including a change in investigators), please inform the Ethics Committee by submitting an Amendment.**
4. **When the research is concluded, please submit a Final Report to the Ethics Committee.**

Thank you for your application and we wish you well in all of your research endeavors.

Yours Sincerely

Prof. RR (Robert) Bragg
Chairperson: Biosafety & Environmental Research Ethics Committee University of the Free State

Directorate: Research Development
T: +27 (0)51 401 9398 | +27 (0)51 401 2075 | E: smitham@ufs.ac.za Johannes Brill Building, Room 106D, First Floor
205 Nelson Mandela Drive | Park West, Bloemfontein 9301 | South Africa
P.O. Box 339 | Bloemfontein 9300 | South Africa | www.ufs.ac.za

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THESIS SUMMARY

The safety and effectiveness of anti-fungal medicines are paramount to controlling the growth of pathogenic fungi. Following the isolation of Cryptococcus neoformans and documenting evidence that it as an aetiological agent of an often-deadly inflammatory condition of the brain, more so in people with immunosuppressive conditions, the quest to find alternative (including complementary) medicines has continued until now. The major shortcoming that is associated with the currently used anti-fungal medicines, i.e. fluconazole and amphotericin B, in South Africa, is clinical failure. This, in turn, has led to increased mortality. With the thesis, it was aimed to understand the response of cryptococcal cells towards antimalarial drugs, CQ and PQ.

In chapter 2 it was illustrated that cryptococcal cells are sensitive to light inactivation following exposure to a germicidal UV light (UVC) in the presence of CQ and PQ (both used as photosensitisers) as well as ambient air. The yielded photolytic products targeted the membranes that, in turn, led to cell death. Moreover, the treatment of internalised cryptococcal cells led to their increased sensitivity towards macrophage phagocytosis killing.

Chapter 3 highlighted the importance of PQ in controlling the growth of cryptococcal cells. The data revealed that PQ targeted cryptococcal mitochondria, an important organelle of this organisms. Given the dependence of the organism on this organelle to produce energy to sustain it, its impaired resulted in cells being vulnerable and subsequently dying. The drug also enhanced the macrophages' phagocytosis efficiency to kill internalised cryptococcal cells.

Chapter 4 considered using a lipophilic-based medium to deliver CQ, in an attempt to control of disseminated infection. The prepared TPGS-CQ micelle was then used in an *in vitro* blood-brain barrier (BBB) model, set up using a transwell plate, to control cryptococcal cells. While the micelle was not efficient in delivering the CQ, the minimal amounts that were delivered were sufficient to significantly control the growth of cryptococcal cells.

Based on these findings, it is clear that there is merit in considering CQ and PQ in the management of cryptococcal cells. The drugs could be used to complement the currently used antifungal drugs in combined therapy to establish synergism. The latter would imply that minimal concentrations would be required – thus, minimise chances to manifesting side effects. Moreover, *in vivo* studies ought to be conducted. These would be important in the establishment of their safety profiles and effectiveness a complex, eukaryotic animal like rats. To date, there are reports that highlight limitations concerning the clinical use of these drugs. These include patients underlying heart conditions as well as those with glucose 6-phosphate dehydrogenase deficiency, an enzyme that helps protect red blood cells from damage. To this end, rats with such defects can also be included in such studies for referencing purposes.

Key words: Blood-brain barrier, Chloroquine, *Cryptococcus*, CQ-TPGS, Drug-repurposing, hCMEC/D3, Macrophages, Mitochondria, Photodynamic therapy, Photosensitisers, Primaquine.

CHAPTER 1

LITERATURE REVIEW

A draft manuscript based on the chapter has been prepared and will be submitted for publication. Because of the above, repetition of some information in the document could not be avoided.

The candidate, Madu, performed a literature search and drafted the manuscript.

1.1 INTRODUCTION

Fungal species are ubiquitous and have widely been found on plant debris, soil, seawater, freshwater, animals, human skin and other organic substrates (Tovey and Green, 2005; Naranjo-Ortiz and Gabaldón, 2019). These organisms make up approximately 7% (611,000 species) of all eukaryotic species (Brown et al., 2012). Compared to bacteria, fungi are more often exploited to make food and beverages (Guynot et al., 2005). Due to the latter, the general public do not appreciate fungi serious infectious agents. At the same time, is it possible that some members of the public are familiar with non-life-threatening conditions viz. athlete's foot, that are caused by fungi (Crawford, 2009). However, fungi, like bacteria, can manifest life-threatening conditions (Cowen, 2008; Brown et al., 2012). To compound the above, some countries lack active surveillance programmes to monitor incidences of fungal infections and associated mortality (Brown et al., 2012, 2014; Schmiedel and Zimmerli, 2016). Or, if there is appreciation, there is lack of dedicated funds due to difficult socio-economic challenges that prevail, or a country may have a collapsed health infrastructure due to internal wars. These conditions make it impossible to monitor cases (Sebolai and Ogundeji, 2015).

Over the years, the emergence of mycotic agents in clinical settings has risen substantially, wherein terrestrial species that were considered to be non-pathogenic, seem to have "acquired" pathogenic qualities (Brown et al., 2012). This is more true in subjects that lack an intact immune system to ward off the invading pathogens (Badiee and Hashemizadeh, 2014). An example of an organism that has transformed how it is perceived to being regarded as an important fungal pathogen is *Cryptococcus* (*C*.)

neoformans (Casadevall and Perfect, 1998). At one point in its convoluted classification history, it was thought to be fermentative after it was isolated from a fermented fruit juice (Casadevall and Perfect, 1998). Hence, it was initially placed under *Saccharomyces* (Srikanta et al., 2014). However, it has been known for over 100 years that *C. neoformans* is pathogenic (Srikanta et al., 2014). Therefore, a substantial body of work has gone into understanding its pathobiology (Zaragoza, 2019). Thus, this write up aims to further contribute to the general understanding of *C. neoformans*. In addition, special attention was given to measures used to control the growth of cryptococcal cells and highlights issues associated with its treatment. Importantly, this contribution offers insight into alternative treatment options that can be used to circumvent the associated issues.

1.2 DESCRIPTION OF *CRYPTOCOCCUS NEOFORMANS*

C. neoformans is an encapsulated, terrestrial basidiomycetous fungus that is an obligate aerobe (DeLeon-Rodriguez and Casadevall, 2016; Mourad and Perfect, 2018). The cells of *C. neoformans* are globose to ovoid and range between 2.5 µm to 10 µm in cell diameter (Buchanan and Murphy, 1998). The classification of the members of *C. neoformans* species complex continues to change as additional genomic data becomes available. At present, *C. neoformans* has, at minimum, three distinctive genotypes *viz*. VNI, VNII, and VNB (Litvintseva and Mitchell, 2012) and *C. gattii* has been organised into four main genotypes *viz*. VGI, VGII, VGIII, and VGIV (Farrer et al., 2015), of which VGII contain additional subtypes such as VGIIa-c (Köhler et al., 2017). The two species, *i.e. C. neoformans* and *C. gattii*, are believed to have separated approximately 50 million

years ago (Kwon-Chung et al., 2014). Despite the differences in the genetic make-up of these two species, they cause the same disease in susceptible hosts (Kwon-Chung et al., 2014).

C. neoformans can primarily be found in the environment particularly in soil contaminated with bird droppings (Lin and Heitman, 2006). The spread of *C. neoformans* across the globe is in part attributed to the movement of birds, as they are considered to be the vector of transmission (Nielsen et al., 2007). It has also been suggested that cryptococcal cells can survive in sea and fresh water for a year, and thus ocean currents can serve as another mode of transport around the world (Kidd et al., 2007). These cells may become desiccated if they land on soil that has nutrient and water limitation (Maliehe et al. 2020). This phenotypic adaptation aids with survival during this period by reducing the metabolic activity of the cells, thus allowing the cell to survive for a longer period (Maliehe et al. 2020). The wind can also aid in the distribution of cells across the ecology. Here, the emergence of unfavourable conditions in the environment can trigger cells to undergo sexual reproduction (Kwon-Chung, 1976). The resultant spores, which are carried externally on a basidium, can be swept by the air, in order to colonise new niches (Maliehe et al. 2020). Unfortunately for mammalians, their bodies can become such a niche, in which cryptococcal cells *viz*. in a desiccated form or as spores, can survive (Nielsen et al., 2007).

1.3 THE JOURNEY OF CRYPTOCOCCAL CELLS IN A MAMMALIAN BODY

C. neoformans is known to possess a number of well-defined virulence factors that also allow it to colonise a mammalian host. Key among these is thermotolerance, which assists pathogenic cells to circumvent the important temperature barrier that is placed on microbes by their mammalian hosts (Yang et al., 2017). The importance of thermotolerance is more apparent when one considers *C. podzolicus*, which possess key virulence factors such as capsule, melanin production but cannot grow at 37^{o}C, thus it is non-pathogenic to humans (Perfect, 2006).

Exposure to cryptococcal cells is said to occur at an early stage in life, often when children begin to explore the world around them. The assertion was confirmed in a study by Goldman et al. in which they determined that 70% of the 120 children in New York that were surveyed, had antibodies specific for the cryptococcal antigen (Goldman et al., 2001). Typically, a cryptococcal infection begins with inhalation of windswept spores or desiccated yeast cells (Esher et al., 2018). These cells are small enough (<5 μm) to, despite airway turbulence and ciliary action, reach the alveolar space (Schop, 2007). There, they are met by cells of the innate immunity (Mukaremera and Nielsen, 2017; Setianingrum et al., 2019). In a healthy individual, immune cell are able to resolve the invading cryptococcal cells (Kwon-Chung et al., 2014). However, in a susceptible host (those with a defective immune system), the cells can, in contrast, proliferate leading to the development of pneumonia (Setianingrum et al., 2019). Another common occurrence is a case where patients remain clinically asymptomatic even in the presence of formation of small lung lymph complex of cryptococcal infection wherein the cells can be dormant

but remain viable (Salyer et al., 1974; Baker, 1976; Fisher et al., 2016). These cells can, for example, be reactivated when there is a loss of resident immunity as a result of human immunodeficiency virus (HIV) infection progression (Perfect et al., 2010). Reactivation of these dormant cells often contributes to the development of immune reconstitution inflammatory syndrome, although this inflammatory condition may be rare (Goldman et al., 2001; Boulware et al., 2010; Haddow et al., 2010).

If cells invading the lung space are not arrested, they can as “free” cells or “hiding” inside macrophages in a manner akin to a Trojan horse (Voelz and May, 2010), haematogenous disseminate to practically to every part of the body (Esher et al., 2018). The “free” cells have developed various mechanisms to evade host molecules that tag cells for phagocytosis (García-Rodas and Zaragoza, 2012). Here, cells can enlarge their polysaccharide capsule around the cell, obscuring signature molecules from host molecules (Zaragoza et al., 2008; Hommel et al., 2018). This, in turn, prevents the internalisation of the yeast by macrophages (Zaragoza et al., 2008; Hommel et al., 2018). On the other hand, cells that are hiding inside macrophages ought to negotiate the hostile environment that prevails in the phagosome. For example, cells can evoke the participation of antioxidant enzymes to resolve reactive oxygen species that are released by host immune cells (Maliehe et al. 2020). To the point, hydrogen peroxide can be decomposed by enzymes such as catalases, catalase-peroxidases, peroxidases, glutathione peroxidases and the glutathione system, peroxiredoxins, and the thioredoxin system (Tan et al., 2016). Once the cells arrive at their destination (organ), they can escape the macrophage environment in a process called vomocytosis (Ma et al., 2006).

In some instances, if there is a mechanical injury to the skin, *i.e.* to insert a medical device, it is possible to have cryptococcal cells inoculated directly onto the skin and cause primary cutaneous cryptococcosis (PCC) (Chakradeo et al., 2018). PCC is defined as the identification of *Cryptococcus* species on a skin lesion without a sign of simultaneous, disseminated infection (Wang et al., 2015). In their paper, Neuville et al. argued that only careful examination could ascertain a PCC diagnosis (Neuville et al., 2003). Moreover, they acknowledged that controversies persist on the reality of PCC as opposed to disseminated secondary cutaneous cryptococcosis (Neuville et al., 2003). However, there is reason to believe the existence of PCC, although rare, based on the findings of the French Cryptococcosis Study Group, which identified 28 cases of primary cutaneous cryptococcosis in the period 1985 – 2000 (Neuville et al., 2003). Srivastava et al. advised that all cutaneous cryptococcosis should be presumed as disseminated until proven otherwise and a hunt for other sites of infection must be immediately undertaken (Srivastava et al., 2015).

Disseminating cryptococcal cells have a particular liking for the brain (Chun et al. 2007). To get there, they ought to first negotiate the blood-brain barrier (BBB) (Santiago-Tirado et al., 2017). The BBB is a barrier that lines all capillaries in the central nervous system (CNS) consisting of tight junctions wrapped around the capillaries to control the flow of blood-borne substances in and out of the brain as well as preserve the homeostasis of the neural microenvironment (Liu et al., 2012). The neural microenvironment is vital for the proper functioning of the neurons, a boundary that separates the peripheral circulation and the CNS (Liu et al., 2012). This specialised system restricts microbe and large

molecules in the blood from entering the brain, whereas allowing the diffusion of small lipid-based and hydrophobic molecules such as hormones, oxygen and carbon dioxide (Kim, 2008).

The mechanism by which *Cryptococcus* cross the BBB is not fully understood. Nevertheless, they can successfully breach the BBB to reach the brain via several migration methods. These include paracellular, transcellular or by the so-called “Trojan horse” crossing (Kim, 2008; Casadevall, 2010; Santiago-Tirado et al., 2017). The paracellular crossing is the penetration of a circulating, free cryptococcal cell between the BBB cells with or without the disruption of tight junctions (Santiago-Tirado et al., 2017). The transcellular crossing is the penetration of brain microvascular endothelial cells without the disruption of the tight intercellular junctions by the free cryptococcal cells (Tuomanen, 1996; Santiago-Tirado et al., 2017). A number of secreted virulent factors with coordinated effort implicated in the direct migration of free cryptococcal cells across the BBB and CNS invasion are urease, metalloprotease, Mpr1, laccase, phospholipase B1, and a serine protease (Vu et al., 2019). Though urease is a well-studied virulence factor, Mpr1 (a distinctive fungalysin belonging to M36 class that are expressed in some fungal species) has only recently been recognised as a critical extracellular protein that enhances *Cryptococcus* invasion of the CNS (Vu et al., 2014; Pombejra et al., 2018; Vu et al., 2019). Regarding the “Trojan horse” mechanism, once the BBB is breached, a cryptococcal cell can exit the loaded macrophage via lytic or nonlytic extrusion (Ma et al., 2006; Voelz and May, 2010; García-Rodas and Zaragoza, 2012).

Once the yeast cells breach the BBB and reach the brain, their presence and rapid replication leads to inflammation of the meninges and brain (Honda and Warren, 2009). Symptoms are non-specific; however, may include headache, malaise, neck stiffness, among others (Sloan and Parris, 2014). A build-up of intracranial pressure and seizures commonly occur in advanced cryptococcal meningitis (Chen et al., 2013; Schmiedel and Zimmerli, 2016). This infection is the common cause of adult meningitis (Liu et al., 2012; Williamson et al., 2016).

1.4 EPIDEMIOLOGY

The first global epidemiological study into the prevalence of cryptococcal cases estimated that approximately 1 million cases were observed each year (Park et al., 2009). The report further estimated that the highest disease burden and mortality rates were noted in sub-Saharan Africa (Park et al., 2009; Sanguinetti et al., 2019). At the time, it was argued that death caused by cryptococcal meningitis may be more frequent than tuberculosis in sub-Saharan Africa (Park et al., 2009). The most common predisposition to cryptococcal meningitis globally is HIV infections (Sloan and Parris, 2014). Therefore, it is not surprising that the ratio of cryptococcosis reflects the spread of the acquired immune deficiency syndrome (AIDS) epidemic, which has sub-Saharan Africa as its epicentre (Park et al., 2009; Kwon-Chung et al., 2014). Later on, Rajasingham et al. reported that the availability of antiretroviral treatment and antifungal drug interventions have significantly impacted on the prevalence of cases (Rajasingham et al., 2017). To the point, the study reported an estimated global annual occurrence of more than 278,000 new

cases, with over 181,100 deaths (Rajasingham et al., 2017). Again, developing countries recorded the highest fatality. To illustrate this point, in sub-Saharan Africa alone, mortality due to cryptococcal meningitis is reported to be 135,900, accounting for 75% of the 181,100 annual global deaths from this infection (Rajasingham et al., 2017). This high rate of cryptococcal infection and mortality in resource-limited settings is said to be partly due to the lack of access to the standard and most effective treatment as a result of cost (Truong et al., 2018). Therefore, management of this disease and other life-threatening fungal diseases are greatly dependent on capital resources available in a specific region (Perfect and Bicanic, 2015).

Non-HIV populations also stand risks of acquiring cryptococcal infections, particularly transplant recipients and patients on immunosuppressive treatments (Mourad and Perfect, 2018). For example, about 2 - 3% of organ transplant recipients develop disseminated cryptococcal infection (Mourad and Perfect, 2018). From the above, it is clear that management is crucial to reduce mortality, and thus the next section considers how infections are currently managed.

1.5 MANAGEMENT OF CRYPTOCOCCAL INFECTIONS

1.5.1 Diagnosis

A number of methods are used to confirm a cryptococcal infection. These include direct microscopy, culture, serology, histopathology and molecular detection (De Pauw et al., 2008; Gazzoni et al., 2009; Saha et al., 2009). Most often, positive test results that are

obtained from serum, blood or cerebral spinal fluid (CSF) samples are indicative of disseminated cryptococcosis while sputum or biopsy may be required for a lung infection or a swap from a skin lesion for culturing purposes (Zhang et al., 2012; Setianingrum et al., 2019). The advent of fingerstick cryptococcal antigen has preliminary replaced the typical examination of the CSF with the Indian ink method, more so in sub-Saharan Africa (Govender et al., 2015; Williams et al., 2015). The test detects and quantifies cryptococcal polysaccharide antigen on the capsule (McMullan et al., 2012). Three formats of cryptococcal antigen (CrAg) detection tests are currently available: enzyme-linked immunoassay (EIA), latex agglutination test (LAT) and lateral flow immune-assay (LFA) (Pongsai et al., 2010; McMullan et al., 2012; Chen et al., 2014; Liang et al., 2016). Of all the CrAg formats, the LFA is most acceptable and satisfy the end-users because it is affordable, sensitive, rapid, specific and equipment-free (Sanguinetti et al., 2019). The LFA method of diagnosis is also advantageous because of early and ease of detection for immediate commencement of treatment by physicians. In turn, this may prevent the development of severe cryptococcosis in many patients (Sanguinetti et al., 2019).

1.5.2 Current treatment regimen and associated issues

The antifungal collection used for the treatment of cryptococcal infections is currently limited to three classes of drugs, which are used individually or in combination (Perfect and Bicanic, 2015). Amphotericin B, a polyene drug, is known as one of the most effective antifungal agents, exhibiting a wide-spectrum antifungal activity against both yeast-like and filamentous fungi (Mesa-Arango et al., 2012). The drug binds to the ergosterol and induces the formation of pores on the fungal cell membrane (Gray et al., 2012). From the

hydrophobic domains that are attached to ergosterol, the antifungal binds to the lipid bilayer of the fungus. As a result, multimeric pores are created, with the antifungal binding with membrane lipids (Kagan et al., 2012). The pore created increases fungal permeability to small cations like $Ca^{2+,}$ Mg^{2+} and K^{+}, thereby inducing fungal death by the quick depletion of intracellular ions (Mesa-Arango et al., 2012).

Therapy using amphotericin B has been the mainstay treatment of cryptococcal meningitis in HIV and non-HIV infected patients as well as in transplant and non-transplant recipients for several decades (Mourad and Perfect, 2018). The drug is reported to cause a significant decline in yeast burden within the CNS (Sloan and Parris, 2014). However, therapy with amphotericin B has been associated with significant nephrotoxicity (Saag et al., 2000). In addition, there is the issue of poor bioavailability in the CNS due to a large molecular weight, its physical-chemical nature, very poor lipid and water solubility (Ho et al., 2016). To overcome this, formulations such as liposomal amphotericin B, amphotericin B lipid complex and amphotericin B colloidal dispersion among others are used to increase brain bioavailability (Hamill, 2013; Ho et al., 2016; Cuddihy et al., 2019), thus; improve treatment outcome. More to the point, one study indicated that formulated amphotericin B (amphotericin B-polybutylcyanoacrylate nanoparticles coated with polysorbate 80 and liposomal amphotericin) had better transport across the BBB and effectively treated mouse model cryptococcal meningitis compared to the classic amphotericin B deoxycholate (Xu et al., 2011).

Another class is the azoles with fluconazole as the prototypical drug. Evidence suggests that the co-catalysation of the heme protein and 14α-demethylation of lanosterol that is dependent on cytochrome P-450 is the primary target of azoles (Warrilow et al., 2013). The inhibition of 14α-demethylase leads to the reduction of ergosterol (the main fungal sterol) and accumulation of sterol precursors such as 14α-methylated sterols (lanosterol, 24-methylenedihydrolanosterol and 4,14-dimethylzymosterol) (Warrilow et al., 2013). Furthermore, this results in the creation of defective plasma membrane with altered structure and function (Hitchcock et al., 1990; Sanati et al., 1997).

The extensive use of this drug has resulted in fungi developing resistance (Ghannoum and Rice, 1999). Fungal mechanism of resistant to azoles involves modification of the target such as the alteration or overexpression of cytochrome P-450-dependent 14α-demethylase and active drug efflux (Jenkinson, 1996; Orozco et al., 1998). Unfortunately, this has, at times, led to extremely high doses being used for high yeast burden in order for effective treatment to be observed (Martínez et al., 2000; Mussini et al., 2004). Despite the above, monotherapy using high doses of fluconazole only for induction therapy, is still connected with a significant high mortality rate compared to when used in combination therapy (Longley et al., 2008; Nussbaum et al., 2010).

The last antifungal is a synthetic fluorinated pyrimidine *e.g.* flucytosine (Ghannoum and Rice, 1999). The antifungal activity of flucytosine is obtained from rapidly converting flucytosine into 5-fluorouracil in the cytosol of fungal cells (Bennett, 1977; Benson and Nahata, 1988). Flucytosine is an artificial fluorinated analogue of cytosine, which, on its

own, has no antifungal activity (Groll et al., 2003). The uptake of flucytosine into the fungal cell is facilitated by cytosine permease enzyme while flucytosine is rapidly deaminated to 5-fluorouracil by cytosine deaminase enzyme that is not possessed by human cells (Groll et al., 2003). 5-Fluorouracil is responsible for miscoding RNA and inhibiting DNA synthesis through two different mechanisms (Groll et al., 2003; Lewis, 2007). The lack of cytosine deaminase in human cells for 5-fluorouracil synthesis makes flucytosine an ideal drug for the exclusive disruption of nucleic acid function in fungal cells (Harris et al., 1986). Resistance incidence with the single use of flucytosine has been recorded in literature. This can occur through two distinguished mechanisms; one of which, pathogens can mutate to be deficient in cytosine permease and/or cytosine deaminase enzyme responsible for the cellular uptake and metabolism of drug respectively (Vermes et al., 2000). In addition to the above, the second mechanism is the enhanced synthesis of pyrimidines that alters with the fluorinated anti-metabolites of flucytosine and consequently diminish its anti-mycotic activity (Vermes et al., 2000; Loyse et al., 2013).

The widely acknowledged guidelines for the management of cryptococcal meningitis involves combining flucytosine with amphotericin B for two weeks (Perfect and Bicanic, 2015). This often leads to better outcomes compared to when amphotericin B is used in monotherapy (De Gans et al., 1992; Day et al., 2013; Sloan and Parris, 2014). However, regardless of the improved fungicidal activity, it has been reported that using higher doses of this combination has a number of serious adverse effects and consequences (Bicanic et al., 2008). This has resulted in the use of less toxic derivatives of amphotericin B formulations, but at a very high cost (Kagan et al., 2012).

On the other hand, the availability of flucytosine in resource-limited settings, where the disease burden is the highest, is limited by its high cost (Rajasingham et al., 2017). In the absence of flucytosine, poorer countries use fluconazole alone or in combined therapy with amphotericin B in the consolidation and maintenance treatment for cryptococcal meningitis (Perfect et al., 2010). The latter is in line with the world health organisation (WHO) guidelines that recommend the use of fluconazole in place of flucytosine in resource-limiting countries (WHO, 2011). The guidelines also recommend the use of fluconazole to treat asymptomatic cryptococcosis in patients who have an early subclinical infection with CD4 counts of <100 cells/μL (WHO, 2011). Due to these highlighted shortcomings, a number of alternative treatments have been considered and are discussed below.

1.6 ALTERNATIVE TREATMENT OPTIONS

1.6.1 Anti-virulence therapies

To complement the action of anti-*Cryptococcus* drugs, medicines that target virulence factors should be considered. Virulence factors are microbial components, *i.e.* biomolecules and structures, that are used by pathogens to colonise, invade and persist in a susceptible host (Martínez et al., 2019). More importantly, is that the production of these factors is under the control of regulatory mechanisms. The latter implies that interference with these regulatory mechanisms could affect the production of several virulence factors (Defoirdt, 2018; Martínez et al., 2019). In addition to knowing the biosynthetic pathway(s) that are involved in the production of a targeted virulence factor,

691 it is equally important to know if such a virulence factor may undergo chemical
692 modification(s) post-production that may modulate its activity (Martinez et al. 2019). The
693 latter could allow for chemical analogues to be used to disrupt gene expression or cause
694 post-translational modifications to the virulence factor. Moreover, through competitive
695 inhibition, an analogue can also be used to outcompete a critical enzyme in the
696 biosynthetic pathway. In the end, the approach that is followed should effect maximum
697 deleterious, fitness consequence for the pathogen (Do Vale et al., 2016).
698
699 *Cryptococcus* has a number of key virulence factors that can be targeted. In the write-up,
700 melanin is highlighted as such a factor. Melanin enhances the virulence of *C. neoformans*
701 by promoting its survival inside macrophages by protecting cells against oxidative
702 damage (Wang et al., 1995). In addition to the above, the melanisation of *C. neoformans*
703 can interfere with the treatment of cryptococcosis due to reduced drug susceptibility (van
704 Duin et al., 2002). Given the importance of this factor, its biosynthetic route is an ideal
705 target for drug development. The laccase enzyme has been reported to be a key enzyme
706 in the synthesis of melanin (Salas et al., 1996; Williamson, 1997). To illustrate this, the
707 deletion of the gene encoding for laccase led to reduced virulence of *C. neoformans* in *in*
708 *vivo* studies (Salas et al., 1996; Williamson, 1997). This glycosylated copper protein
709 enzyme synthesises melanin in several oxidation and reduction steps of several
710 diphenolic substrates that consist of *para*- and *ortho*-diphenols, L-dopa, monophenols,
711 and esculin, obtained extracellularly (Thurston, 1994; Almeida et al., 2015). The inhibition
712 of laccase may present with ancillary benefits. For example, the laccase enzyme is also
713 used by cryptococcal cells to produce microbial prostaglandins that are vital

immunomodulators that promote pathogenesis (Noverr et al. 2003). Fungal laccases can be inhibited by a number of compounds such as L-cystein, thiogycolic acid or diethyl dithiocarbamate (Lu et al., 2007; Baldrian, 2004). These compounds have been shown to chelate the copper at the catalytic centre of laccase, thus; disrupting the oxidation of a substrate and the subsequent transfer of electrons required to reduce oxygen (Baldrian, 2006). Further to this, a competitor for oxygen that is specific to the substrate of laccase can be considered (Baldrian, 2006). In addition, compounds that have a high-affinity for binding pigments can be used. In their papers, Larsson (1993) as well as Wang and Casadevall (1996) recognised the anti-psychotic drug, trifluoperazine, as a compound that can bind to melanin, and in turn damage the mitochondria of the highly aerobic cryptococcal cells (Eilam et al., 1987; Wang and Casadevall, 1996).

1.6.2 Immunotherapy

The immune status of a host (immuno-competent or -compromised) is important in determining the fate of invading pathogenic cells. Toward this end, the fate can be limited to three possible outcomes *i.e.* infection clearance, persistent infection or disseminated infection (Voelz and May, 2010). More importantly, the functioning of the immune system as an intact unit, wherein the interaction of innate cells with invading pathogens can lead to a secondary immunological development and the building of immunological memory, cannot be underestimated (Janeway et al., 2001). This is more critical in subjects who may have HIV, who, over time progressive lose their adaptive immunity (Janeway et al., 2001). Such occurrences create optimal conditions for even opportunistic pathogens to emerge and take hold (Janeway et al., 2001). Therefore, their innate immunity to

becomes even more pivotal. Therefore, finding medicines that can modulate the function of the immune system may prove important in clearing infections.

The existence of information demonstrating that the immune system can be modulated using compounds in the treatment of microbial infections, Antachopoulos and Walsh argued that there is still insufficient clinical data to make reliable recommendations (Antachopoulos and Walsh, 2012). Despite the latter assertion, a few successful examples are highlighted herein. Cytokines such as tumour necrosis factor-alpha (TNF-α) have been considered in boosting immunity against cryptococcosis in individuals with impaired immunity such as HIV patients (Collins and Bancroft, 1992; Heung, 2017). Fa et al. explored the therapeutic use of TNF-α as a promoter of host anti-cryptococcal responses in a murine model (Fa et al., 2019). In the study, cryptococcal cells were engineered to express murine TNF-α and were subsequently used to establish a murine model of pulmonary cryptococcosis. The study established that mice infected with the TNF-α-producing *C. neoformans* strain enhanced protective elements of host response compared to wild type strain-infected mice. These elements were Th1/Th2 cytokine balance, T-cell accumulation, antifungal activity of macrophages and the reduction of pulmonary eosinophilia (Fa et al., 2019). Collins and Bancroft documented that the administration of TNF-α enhanced macrophage's anti-cryptococcal activity *in vitro* (Collins and Bancroft, 1992). Taken together these results suggest the delivery TNF-α as a therapeutic option could be a means of complement the host immune defence against cryptococcal infections.

Cryptococcal cells are known to be poorly immunogenic due to the polysaccharide layer (capsule) that masks the antigens on the cell wall (Arana et al., 2009). To overcome this, Coelho and Casadevall suggested the introduction of polysaccharide conjugate vaccines that can trigger a strong antibody response (Coelho and Casadevall, 2016). To illustrate this, Datta et al. showed that the vaccination of experimental mice using the mimotope (P13) protein of capsular polysaccharide, conferred protection following their infection (Datta et al., 2008). In addition, the study also showed that vaccination prolonged the survival of infected mice (Datta et al., 2008). Antibodies in a form of adjunctive passive immunotherapy could also be considered (Carvalho et al., 2015). For example, antibodies against cryptococcal melanin can be raised during the course of an infection (Mitchell and Perfect, 1995). In their study, Rosas et al. showed that the injection murine-raised antibodies into mice that were lethally infected with cryptococcal cells, were able to survive longer when compared to control mice (Rosas et al., 2001). The same study further showed that the fungal burden in different organs was significantly decreased compared to infected control mice without antibody administration (Rosas et al., 2001).

1.6.3 Photodynamic treatment (PDT)

The history of using light to treat human ailments dates back to ancient time, with roots traced to Egypt, Greece and India (Azeemi and Raza, 2005). The photodynamic treatment (PDT) mechanism is based on the interaction of light and a photosensitising agent. Under light activation, the sensitiser attracts photons and transfer energy obtained from light to generate harmful radical species that are toxic to targeted cells (Baltazar et al., 2015; Liang et al., 2016). These radical species place cellular components under

oxidative stress, and in the process, kill susceptible cells (Dai et al., 2012). Therefore, PDT is well established and mainly used to treat cancers (Dai et al., 2012). However, its application has also been extended to control microbes. For example, in the 20th century, Niels Finsen, a Danish physicist successfully treated lupus vulgaris, a cutaneous infection caused by *Mycobacterium tuberculosis*, using photodynamic therapy (Daniell and Hill, 1991). Despite this early success, the advent of antibiotics halted the application of anti-bacterial photodynamic therapy (Maisch, 2009). However, the upsurge in cases of drug resistance in clinical settings has seen PDT being revisited (Abrahamse and Hamblin, 2016).

A well-known photosensitiser is curcumin, a polyphenolic compound that has a light absorbance range of 405 to 435 nm (Dahl et al., 1989). When sensitised, it displays pleiotropic binding towards many types of biomolecules, such as proteins, lipids and nucleic acids (Heger et al., 2014), and the generated radicals can lead to cell death (Dahl et al., 1989). The phototoxic effect of curcumin was found to be more significant in Gram-positive bacteria when compared to Gram-negative bacteria (Dahl et al., 1989). In addition, PDT mediated by curcumin in different species of *Candida* has been investigated (Andrade et al. 2013). In one study, light-sensitive curcumin caused extensive DNA damage following the formation of singlet oxygen in *Candida albicans*. The latter was proposed to be the likely antifungal action of curcumin-mediated PDT (Carmello et al., 2015).

The antifungal properties of porphyrins as a photosensitiser in *Candida* and *Trichophyton rubrum* PDT have been documented (Donnelly et al., 2008). Photofrin is well-known porphyrin that is used as photosensiter in cancer treatment and has also been shown to be effective on *Candida* species (Bliss et al., 2004). Notably, considerable selective toxicity of fungi over host cells has been demonstrated. Additionally, treatment is not associated with mutagenic effects or genotoxicity to either fungi or host cells. Also, there has been no report of treatment associated with fungal resistance (Donnelly et al., 2008). These molecules can be effective in killing fungal cells upon irradiation. The phototoxic activity is mostly due to the light activation of unbound porphyrins molecules in the aqueous medium thereby producing harmful radicals (Bertoloni et al., 1993; Bliss et al., 2004). After irradiation at 632.8 nm, alteration to the cytoplasmic membrane permits porphyrins penetrate into the cells facilitating translocation to the inner membranes. With continuous irradiation, intracellular targets were damaged (Bertoloni et al., 1987).

1.7 DRUG DEVELOPMENT THROUGH DRUG REPURPOSING

Drug discovery is a high-investment, time-consuming and high-risk process in traditional drug development (Truong et al., 2018). According to a report by the Eastern Research Group, the development of a new drug usually takes 10 - 15 years (Xue et al., 2018) with a low success rate of 2%, on average (Yeu et al., 2015). Even though the number of drugs approved by the Food and Drug Administration (FDA) has been on the decline since 1995, investment in drug development has been gradually on the rise (Xue et al., 2018) indicating that the cost of new drug development will continue to increase. Hence,

it is urgent to find a new strategy to discover drugs for lethal infectious diseases that currently have poor treatment options.

Drug repurposing, also called drug reprofiling, repositioning or re-tasking, is a strategy for identifying new uses of already approved drugs via applying them outside their initial medical indication scope (Pushpakom et al., 2018; Simsek et al., 2018; Truong et al., 2018). For example, a drug can be repurposed for use in a similar therapeutic area or a novel therapeutic area different from its original scope (Ashburn and Thor, 2004). Redirecting a drug for a disease in the same therapeutic area is quite a common phenomenon which is reasonable because taking a drug that works on one type of cancer and trying it on another type is an obvious example (Pushpakom et al., 2018). However, the redirecting of a drug to a completely different therapeutic area does not happen quite often and is quite more interesting, because the motivation is less obvious and more appreciated because it can extend the drug to a whole new market (Pushpakom et al., 2018). More importantly, Baker et al. argued that from a scientific point of view, it could offer further understanding of the disease mechanism of action and physiology (Baker et al., 2018).

Drug repurposing is more economically efficient and can be beneficial in identifying new therapies for diseases in a shorter time, particularly in cases where preclinical safety studies have been completed (Baker et al., 2018; Breckenridge and Jacob, 2018). A well-known drug that has been repurposed is thalidomide, a sedative initially marketed in 1957 in England and Germany for the treatment of morning sickness in pregnant women

(Ashburn and Thor, 2004). Even though thalidomide was later withdrawn because of its side effects, it was, however, serendipitously discovered to be effective for the treatment of erythema nodosum laprosum (leprosy) and for the treatment of multiple myeloma (Ashburn and Thor, 2004; Pushpakom et al., 2018).

The idea of repurposing has also been extended to the treatment of infectious diseases. In this regard, new targets and pathways have been revealed and thus exploited (Pushpakom et al., 2018). For example, studies have shown auranofin, a drug used for the treatment of rheumatoid arthritis to have a broad-spectrum antifungal activity that targets the Mia40-Erv1 pathway in the mitochondria of fungi (Thangamani et al., 2017; Wiederhold et al., 2017). The three possible targets for auranofin's antifungal activity are *mia40, acn9,* and *coa4*. Mia40p is of specific interest given its crucial role in the oxidation of proteins that are rich in cysteine and are imported to the mitochondria (Thangamani et al., 2017). Biochemical analysis confirmed that auranofin can inhibit Mia40p from interacting with its cytochrome *c* oxidase biogenesis factor Cmc1 substrate. Furthermore, this was done in a dose-dependent manner, similarly to the control (Thangamani et al., 2017).

Additionally, drugs such as aspirin and ibuprofen (anti-inflammatory drugs) as well as quetiapine and olanzapine (antipsychotic drugs) have shown anti-*Cryptococcus* activity in *in vitro* studies (Ogundeji et al., 2016, 2017). A common feature of these drugs was that they killed targeted cells by inducing ROS, which in turn, damaged their membranes. Moreover, these drugs were shown to enhance the ability of macrophages to resolved

internalised cryptococcal cells (Ogundeji et al., 2016, 2017). This may be important in controlling disseminated cryptococcal infections as these cells tend to manipulate macrophages in a Trojan-horse-like manner (Ma et al. 2006).

1.8 PURPOSE OF Ph.D. STUDY

In South Africa, the therapeutic options currently available are limited to fluconazole and amphotericin B. Unfortunately, fungal cells can develop resistance towards fluconazole, and amphotericin B can leave host organisms with adverse effects. Hence, there are reports of clinical failure that are associated with these drugs. Therefore, there is a need to consider alternative therapeutic options. A journey that is followed by cryptococcal cells when infecting a mammalian host was presented. With that, the proposed treatment options and the chosen drugs are directed at disrupting the growth of the cells at specific infection sites.

The thesis examined the possible application of antimalarials as anti-cryptococcal drugs. The antimalaria drugs, chloroquine and primaquine, were chosen as test drugs in studies presented herein, because of their reported anti-mitochondrial action in *Plasmodium* species (Foley and Tilley, 1998; Macedo et al., 2017). Given that cryptococcal cells are aerobic and thus highly dependent on their mitochondria, it was theorised that they would succumb to any form of disruption to their oxygen metabolism.

The undertaken studies were grouped into three different chapters, wherein the following were addressed:

- **Chapter 2** details the action of chloroquine (CQ) and primaquine (PQ) when used as photosensitisers that can inactivate cryptococcal cells. The chapter is aimed at addressing the possible manifestation of a skin cryptococcal infection with the following objectives:
  - To determine the effect of PDT with CQ and PQ on cryptococcal cells as well as on their membrane integrity.
  - To determine the accumulation of reactive oxygen species (ROS) after PDT.
  - To evaluate the effect of PDT on murine macrophages.
  - Lastly, to determine the effect of PDT on the phagocytic efficiency of macrophages

- **Chapter 3** examines the direct use of PQ to inhibit the general growth of cryptococcal cells. The chapter also explores the mode of action of this drug and its effects on macrophages with the objectives listed below:
  - To assess the direct effect of PQ on eight cryptococcal isolates. The effect of PQ on the cell wall ultrastructure and cytoplasmic membrane integrity was also assessed.
  - To determine the effect of PQ on the mitochondrial health of *Cryptococcus*. The accumulation of ROS and the quantification of mitochondria cytochrome *c* was also assessed after treatment with PQ.
  - The effect of PQ on murine macrophage function was also determined.

- **Chapter 4** focuses on the ability of CQ when chemically modified and not, to control the growth of cryptococcal cells in transwell plates in a model that mimics the blood-brain-barrier with the following objectives listed below:
  - To determine the effects of CQ and chloroquine-D-α-tocopheryl polyethylene glycol succinate (CQ-TPGS) micelles on hCMEC/D3 cells.
  - To assess the transportation of drugs across a blood brain-barrier (BBB) model.
  - Inhibition of cryptococcal cells using CQ and CQ-TPGS the across BBB model.

It was therefore anticipated that this thesis will provide new insight into the usage of these antimalarial as drugs that can also control the growth of cryptococcal cells. Furthermore, it is envisaged that these drugs may be used alone or in combined therapy as adjuvants, to complement the action of the currently used antifungal drugs.

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CHAPTER 2

CHLOROQUINE AND PRIMAQUINE MEDIATED PHOTODYNAMIC THERAPY INHIBITS THE GROWTH OF *CRYPTOCOCCUS NEOFORMANS* AND IMPROVES MACROPHAGE PHAGOCYTOSIS

A draft manuscript based on the chapter has been prepared and will be submitted for publication. Because of the above, repetition of some information in the document could not be avoided.

The candidate, Madu, co-designed the study, performed the experiments, analysed the data and drafted the manuscript.

2.1 ABSTRACT

Opportunistic fungal pathogens can cause different types of superficial infections or severe invasive infections, particularly in immunocompromised patients. For example, cryptococcal infections can present as cutaneous cryptococcosis in persons with impaired immunity. While it may be easy to diagnose a disseminated infection, treatment often fails due to the ineffectiveness of current antifungal agents. To this end, the current study explored the repurposing of the antimalaria drugs, chloroquine (CQ) and primaquine (PQ), as photosensitisers that may be used to inactivate cryptococcal cells. PDT was carried out using a germicidal ultraviolet (UV) lamp, which has a radiation output of approximately 500 μW/cm^2 at a distance of 20 cm. When compared to their respective controls, the three tested cryptococcal strains showed a time- and dose-dependent response to treatment. Moreover, treatment seemed to alter the ultrastructure as well as increase ($p \leq 0.05$) the loss of selective permeability of the treated cells when compared to non-treated cells. Importantly, treatment did not impair the metabolic activity of treated macrophages (which are often manipulated by cryptococcal cells for dissemination purposes) when compared to non-treated macrophages ($p \geq 0.05$). Rather, treatment improved the efficiency of treated macrophages to kill internalised cryptococcal cells when compared to non-treated macrophages ($p \leq 0.05$). Taken together, the results suggest CQ and PQ have the potential to control the growth of cryptococcal cells.

Keywords: Chloroquine, *Cryptococcus*, Macrophages, Mitochondria, Photodynamic therapy, Photosensitisers, Primaquine, Ultraviolet light.

2.2 INTRODUCTION

The skin is home to complex microbial communities, reflecting the biodiversity of the ecosystems in which they inhabit (Prescott et al., 2017). Importantly, the skin also provides the first line of protection against pathogens (Lehtimäki et al., 2017). This is done, for example, by the tactical arrangement of the dendritic cells ($CD103^{+}$) for cross-presentation of skin-tropic pathogens (Nestle et al., 2009).

A presentation of a portal of entry, *i.e*. a mechanical injury to the skin due to insertion of a medical device, can create an opportunity for commensal to invade a host. In this regard, the chances of direct inoculation are also increased. *Cryptococcus (C.) neoformans* is an example of an airborne environmental fungus that can cause a primary skin infection following direct inoculation (Neuville et al., 2003; Chakradeo et al., 2018). From that point, it is possible to have a haematological dissemination that involves cells "freely" circulating or hiding inside invaded macrophages, in a Trojan horse-like manner, after the recruitment of macrophage to the infection site (Casadevall, 2010; Voelz and May, 2010). The cells can, thereafter, practically disseminate to every part of the body (de Pauw, 2011).

In advanced countries, the prevalence of cryptococcal infection has been controlled to a minimal level (Rajasingham et al., 2017). On the other hand, sub-Saharan Africa has an estimated mortality rate of 135,900 (75%) out of the 181,000 global death recorded annually as a result of cryptococcal meningitis (Rajasingham et al., 2017). Mortality of infection and rising incidence of cryptococcosis is a challenge posed due to the limited

number of useful antifungals obtainable (Cowen, 2008). Current antifungal treatment available for treatment is limited to amphotericin B, fluconazole and flucytosine. However, flucytosine which is more effective against cryptococcal infections, is less frequently used in resource-limited settings due to cost implications. Amphotericin B and fluconazole are generally used in South Africa (Govender et al., 2015) and their mechanism of action target the cell membrane of the fungus (Ghannoum and Rice, 1999). Unfortunately, therapy with these two drugs has contributed to the unacceptably high mortality rates due to clinical failure (Truong et al., 2018). More to the fact, amphotericin B has been associated with considerable nephrotoxicity and poor ability to penetrate the blood-brain barrier due to its chemical and physical nature (Saag et al., 2000; Ho et al., 2016; Cuddihy et al., 2019). On the other hand, fluconazole has been reported to be associated with poorer clinical outcomes due to clinical relapse and the risk of inducing drug resistance (Bicanic et al., 2006; Jarvis et al., 2014; Smith et al., 2015). It is, therefore, crucial to explore alternative therapy to combat antifungal host toxicity and resistance as the evolution of this resistance is outpacing the current antifungal agents. Several treatment options have been reconsidered in the quest for alternative treatment.

The current study considers photodynamic treatment (PDT) as a possible solution to control primary cutaneous cryptococcosis (PCC). PDT has for a while, become a well-studied therapeutic option that involves the optimal combination of a light-sensitive compound (photosensitiser) and light of a specific absorption wavelength, in the presence of ambient air (Fuchs et al., 2007; Dai et al., 2012). The treatment induces the accumulation of harmful radicals that fix on cellular components of a targeted organism

leading to cell death (Fuchs et al., 2007; Dai et al., 2012; Prates et al., 2013; Baltazar et al., 2015; Saini et al., 2016). An ideal photosensitiser is a chemical compound that has suitable photo-physical characteristics that can induce specific photo-activity effect (Josefsen and Boyle, 2008). To this end, a number of compounds have been proven to be ideal photosensitisers when applied against a number of microbes (Hamblin and Hasan, 2004). A well-known photosensitiser is phenothiazinium salt (methylene blue), which has been shown to inactive the growth of bacteria, fungi and protozoa (Wainwright and Crossley, 2002).

PDT is often successful when used against aerobic microbes given their natural susceptibility to oxidative damage (Fuchs et al., 2007). In this study, CQ and PQ are repurposed as photosensitisers that may inactivate the growth of the aerobic cryptococcal cells. Traditionally, CQ and PQ are widely used in malaria therapy and they thought to disrupt the mitochondrial function of the aerobic *Plasmodium* (Slater, 1993; Foley and Tilley, 1998; Percário et al., 2012; Ha et al., 2015). It is therefore theorised that the same mitochondrial disfunction will be observed for *C. neoformans.*

2.3 MATERIALS AND METHODS

Cell cultivation and standardisation

Two clinical strains and a reference strain of *C. neoformans*, *i.e. C. neoformans* LMPE 046, *C. neoformans* LMPE 053 as well as *C. neoformans* H99, were used in the study. The clinical strains were obtained from Universitas Academic Hospital, Bloemfontein,

South Africa. These organisms were streaked out on fresh, sterile yeast-malt-extract (YM) agar (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L glucose, 16 g/L agar; Merck, South Africa). The plates were incubated for 48 h at 30^{o}C. After 48 h, a single colony was scooped with an inoculation loop and streaked onto a fresh, sterile YM agar plate before incubating for 24 h at 30^{o}C. After the incubation period, five colonies were suspended in 5 mL of sterile distilled water. A McFarland standard of 0.5 was prepared for each strain to obtain cell concentration between 0.5 x 10^{5} and 2.5 x 10^{5} colony forming units (CFU) per mL.

A murine macrophage cell line, RAW 264.7 (TIB-71 from ATCC), was cultivated in a tissue flask containing 10 mL of RPMI-1640 medium (Sigma-Aldrich, South Africa). The medium was supplemented with 20 mg/mL streptomycin (supplier), 2 mM L-glutamine (Sigma-Aldrich), 20 U/mL penicillin (Sigma-Aldrich) and 10% foetal bovine serum (Biochrom, Germany). The flask was incubated at 30^{o}C in a 5% CO_2 incubator until confluence was reached. The cells were harvested, and their viability was determined to be 90% after staining with trypan blue (Sigma-Aldrich). Thereafter, the cell concentration of macrophages was determined using a haemocytometer (Marienfield, Germany). The cell concentration was adjusted to a final cell concentration of 1 x 10^{6} cells/mL using a 10 mL solution of fresh, sterile RPMI-1640 medium. A 100 µL suspension of cells was then dispensed into wells of a sterile 96-well flat-bottom microtiter plate (Greiner Bio-One, Germany). The plate was incubated overnight in 5% CO_2 at 37^{o}C. Before use, the overnight spent media was aspirated and replaced with 100 µL of fresh, sterile RPMI-1640 media.

Preparation of photosensitisers

CQ and PQ were obtained from Sigma-Aldrich as standard powders. These compounds were first dissolved in distilled water and further diluted in RPMI-1640 medium (Sigma-Aldrich, South Africa) to prepare a stock solution of 1000 µM (CQ) and 1200 µM (PQ). These compounds were tested at final test concentrations of 5, 12.5, 25, 50, 500 µM for CQ and 0, 6, 15, 30, 60, 600 µM for PQ. The test concentrations of CQ and PQ were chosen arbitrarily, but importantly, high doses of these drugs have been reported to be well tolerated by humans with little to no side effects (Krudsood et al., 2008; Ursing et al., 2009, 2016, 2020). The UV/Vis absorption of CQ was determined to be 250 nm while that of PQ was 266 nm (data not shown), which were in the UVC range.

Light source

A germicidal ultraviolet C (UVC) lamp that was fitted in a Class II Biological safety cabinet (ESCO, South Africa) was used as the light source. The lamp is reported to have a nominal power of 30 Watt. In the current study, the cells were kept at a distance of approximately 20 cm from the lamp. At this distance, the lamp is estimated to have a radiation output of 500 µW/cm^2 (Harrington and Valigosky, 2007).

***In vitro* susceptibility assay**

A 100 µL standardised inoculum of cryptococcal cells was dispensed into wells of sterile 96-well flat-bottom microtiter plates (Greiner Bio-One). To the same wells, 100 µL of the prepared compounds were added. The effect of PDT using CQ and PQ on the metabolic activity of the standardised cryptococcal strains, *i.e.* H99, LMPE 046 and LMPE 053 were

investigated. Cryptococcal cells were grouped to yield the following CQ experimental conditions: 1) non-treated cryptococcal cells, *i.e.* 0 µM, no ultraviolet light (UVL) and dark incubation (DI) for 2 or 8 min, 2) cryptococcal cells with 5 µM of CQ and 2 or 8 min of DI, 3) cryptococcal cells with 25 µM of CQ and 2 or 8 min of DI, 4) cryptococcal cells with 50 µM of CQ and 2 or 8 min of DI, 5) cryptococcal cells with 0 µM of CQ and 2 or 8 min of UVL, 6) cryptococcal cells with 5 µM of CQ and 2 or 8 min of UVL, 7) cryptococcal cells with 25 µM of CQ and 2 or 8 min of UVL and 8) cryptococcal cells with 50 µM of CQ and 2 or 8 min of UVL. For PQ, the following experimental conditions were set up: 1) non-treated cryptococcal cells, *i.e.* 0 µM, no UVL and dark incubation (DI) for 2 or 8 min, 2) cryptococcal cells with 6 µM of PQ and 2 or 8 min of DI, 3) cryptococcal cells with 30 µM of PQ and 2 or 8 min of DI, 4) cryptococcal cells with 60 µM of PQ and 2 or 8 min of DI, 5) cryptococcal cells with 0 µM of PQ and 2 or 8 min of UVL, 6) cryptococcal cells with 6 µM of PQ and 2 or 8 min of UVL, 7) cryptococcal cells with 30 µM of PQ and 2 or 8 min of UVL and 8) cryptococcal cells with 60 µM of PQ and 2 or 8 min of UVL. In the above conditions, cryptococcal cells were allowed to interact with CQ and PQ drug concentrations for 30 min before either DI or UV exposures. Following the treatment, the cells were reacted with a tetrazolium salt (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, XTT; Sigma-Aldrich) and menadione (Sigma-Aldrich). The plates were incubated for 3 h at 37ºC in 5% CO_2. After 3 h of initiating the tetrazolium reaction, the absorbance of the wells was measured at 492 nm to estimate the metabolic activity of the cells.

Effect of PDT with CQ and PQ on the ultrastructure and membrane integrity of cryptococcal cells

To assess the impact of PDT on the cells, scanning electron microscope (SEM) was performed on the same cells as detailed in the *in vitro* susceptibility assay section. The SEM protocol was based on the method detailed by Swart and co-workers (Swart et al., 2010). In brief, after treatment, cells of the same experimental condition were aspirated and pooled together into 1.5 mL plastic tubes (Merck, South Africa). The plastic tubes were centrifuged for 5 min at 1000 x *g* at 30°C to pellet the cells. The pelleted cells were then fixed with 1 mL of 3% phosphate-buffered glutaraldehyde (Merck, South Africa) for 3 h and washed afterwards with the same buffer. The cells were fixed a second time with 1% sodium buffered osmium tetroxide glutaraldehyde (Merck, South Africa) for 1.5 h. Thereafter, graded acetone series of 30, 50, 70, 95 and 100% were used to dehydrate the fixed cells. Following this, cells were critical-point dried, mounted on stubs and coating with gold. The samples were viewed with a scanning electron microscopy (Shimadzu SSX-550 Superscan, Tokyo, Japan).

Two assays were also performed to investigate the effect of treatment on the integrity of the cytoplasmic membrane *viz*. a propidium iodide (PI; Sigma-Aldrich) assay and Toxilight (Lonza Rockland, Inc., United States) assay. The PI stain is excluded by a healthy cell with an intact membrane (Hussain et al., 2019) while Toxilight measures the amount of adenylate kinase (AK), an intracellular enzyme that is only secreted into the cultivation media of dead cells once they have lost selective permeability.

At the end of treatment (as indicated in the *in vitro* susceptibility assay section), the contents of the wells were aspirated and dispensed to corresponding wells in either black (PI) or white (AK) microtiter plates (Greiner Bio-One). For Pl exclusion assay, 1 µL of PI stain (10 µg/mL) was added to the wells to react with the cells. The plate was incubated in the dark for 1 h at room temperature and induced fluorescence measured at an excitation of 485 nm and emission of 538 nm with a fluorescence plate reader (Thermo-Scientific, United States). The reader converts logarithmic signals to relative fluorescence units. For AK assay, 100 µL of Toxilight reagent was added to the wells to react with the supernatant and the plate incubated at room temperature for 10 min in the dark. The induced luminescence was measured after the incubation period with a luminometer (Thermo-Scientific, United States). The reader converts logarithmic signals to relative luminescence units.

Reactive oxygen species assay

The levels of accumulated ROS were measured using 2',7-dichlorofluorescein diacetate (DCFHDA; Sigma-Aldrich, South Africa). In brief, following the treatment of cells as indicated in the *in vitro* susceptibility assay section, the micro-well contents were aspirated and dispensed to corresponding wells in black microtiter plate. Thereafter, 10 µL of DCFHDA (1 mg/mL) was reacted with 90 µL of the aspirated cells suspension. The plates were incubated at room temperature for 30 min in the dark. Induced fluorescence was measured at excitation and emission of 485 / 535 nm using a fluorescence plate reader (Thermo-Scientific, United States).

Determination of the effect of PDT on murine macrophages

The effect of PDT using CQ and PQ on the metabolic activity of murine macrophages was also investigated. The macrophages that were grouped to yield the following CQ experimental conditions: 1) non-treated macrophages, *i.e*. 0 µM, no UVL and dark incubation (DI) for 8 min, 2) macrophages with 5 µM of CQ and 8 min of DI, 3) macrophages with 50 µM of CQ and 8 min of DI, 4) macrophages with 500 µM of CQ and 8 min of DI, 5) macrophages with 0 µM of CQ and 8 min of UVL, 6) macrophages with 5 µM of CQ and 8 min of UVL, 7) macrophages with 50 µM of CQ and 8 min of UVL and 8) macrophages with 500 µM of CQ and 8 min of UVL. For PQ, the following experimental conditions were set up: 1) non-treated macrophages, *i.e*. 0 µM, no UVL and dark incubation (DI) for 8 min, 2) macrophages with 6 µM of PQ and 8 min of DI, 3) macrophages with 60 µM of PQ and 8 min of DI, 4) macrophages with 600 µM of PQ and 8 min of DI, 5) macrophages with 0 µM of PQ and 8 min of UVL, 6) macrophages with 6 µM of PQ and 8 min of UVL, 7) macrophages with 60 µM of PQ and 8 min of UVL and 8) macrophages with 600 µM of PQ and 8 min of UVL. In the above conditions, macrophages were allowed to interact with CQ and PQ drug concentrations for 30 min before either DI or UV exposures. Following treatment, macrophages were reacted with XTT (Sigma-Aldrich) and menadione (Sigma-Aldrich) before incubating for 3 h at 37^{o}C in 5% CO_2. After 3 h, the absorbance of the wells was measured at 492 nm to estimate the metabolic activity of macrophages.

Effect of PDT on the phagocytic efficiency of macrophages

We explored the influence of CQ and PQ PDT on the phagocytic capability of macrophages against cryptococcal cells. In this experiment, a number of co-culture (1 (macrophage): 1 (cryptococcal cell)) experimental conditions were set up for CQ: 1) non-treated co-cultured cells, *i.e*. 0 µM, no UVL and dark incubation (DI) for 2 min, 2) co-cultured cells with 5 µM of CQ and 2 min of DI, 3) co-cultured cells with 12.5 µM of CQ and 2 min of DI, 4) co-cultured cells with 25 µM of CQ and 2 min of DI, 5) co-cultured cells with 0 µM of CQ and 2 min of UVL, 6) co-cultured cells with 5 µM of CQ and 2 min of UVL, 7) co-cultured cells with 12.5 µM of CQ and 2 min of UVL and 8) co-cultured cells with 25 µM of CQ and 2 min of UVL. For PQ, the following experimental conditions were set up: 1) non-treated co-cultured cells, *i.e*. 0 µM, no UVL and dark incubation (DI) for 2 min, 2) co-cultured cells with 6 µM of PQ and 2 min of DI, 3) co-cultured cells with 15 µM of PQ and 2 min of DI, 4) co-cultured cells with 30 µM of PQ and 2 min of DI, 5) co-cultured cells with 0 µM of PQ and 2 min of UVL, 6) co-cultured cells with 6 µM of PQ and 2 min of UVL, 7) co-cultured cells with 15 µM of PQ and 2 min of UVL and 8) co-cultured cells with 30 µM of PQ and 2 min of UVL.

Following the treatment, the plate was incubated at 37^{o}C in 5% CO_2 for 18 h. After the incubation period, the supernatant was aspirated and replaced with 200 µL of phosphate-buffered solution (PBS; Sigma, Aldrich) to wash out the non-internalised cryptococcal cells. The macrophages with internalised cryptococcal cells were harvested by transferring to 1.5 mL plastic tubes (Merck, South Africa). Macrophages were then lysed using 300 µL of 0.1% Triton X-100 (Sigma-Aldrich, South Africa). At this concentration,

Triton X-100 has been reported not to have any effect on the viability of fungi but can lyse macrophage cells (Levitz et al., 1997; Shen et al., 2018). A 10 times serial dilution using distilled water was carried out on cryptococcal cells and 50 µL of the dilution was plated out on YM agar plates. The plates were incubated for 48 h at 30^{o}C and colony-forming units (CFUs) were counted.

Statistical analyses

For each study, three independent experiments were performed. GraphPad Prism 8.3.1 was used to calculate mean values and the standard deviation of the means. The same programme was used to perform the multiple comparison test using Tukey as an option. A *p* value of less than or equal to 0.05 was considered significant. To this end, a bar that has a different alphabet to the other implies there is a significance difference while those with the same alphabet are not significantly different. The data were also tested for normality of distribution using the Shapiro-Wilk test with alpha value of 0.05. Thus, a *p* value less than 0.05 indicated abnormal distribution of the data points.

2.4 RESULTS

Cryptococcal cells were susceptible to the photodynamic action of CQ and PQ

The response of cryptococcal strains, i.e. H99, LMPE 046 and LMPE 053, to photodynamic inactivation using CQ and PQ are summarised in Figure 1A (2 min data) and Figure1B (8 min data).

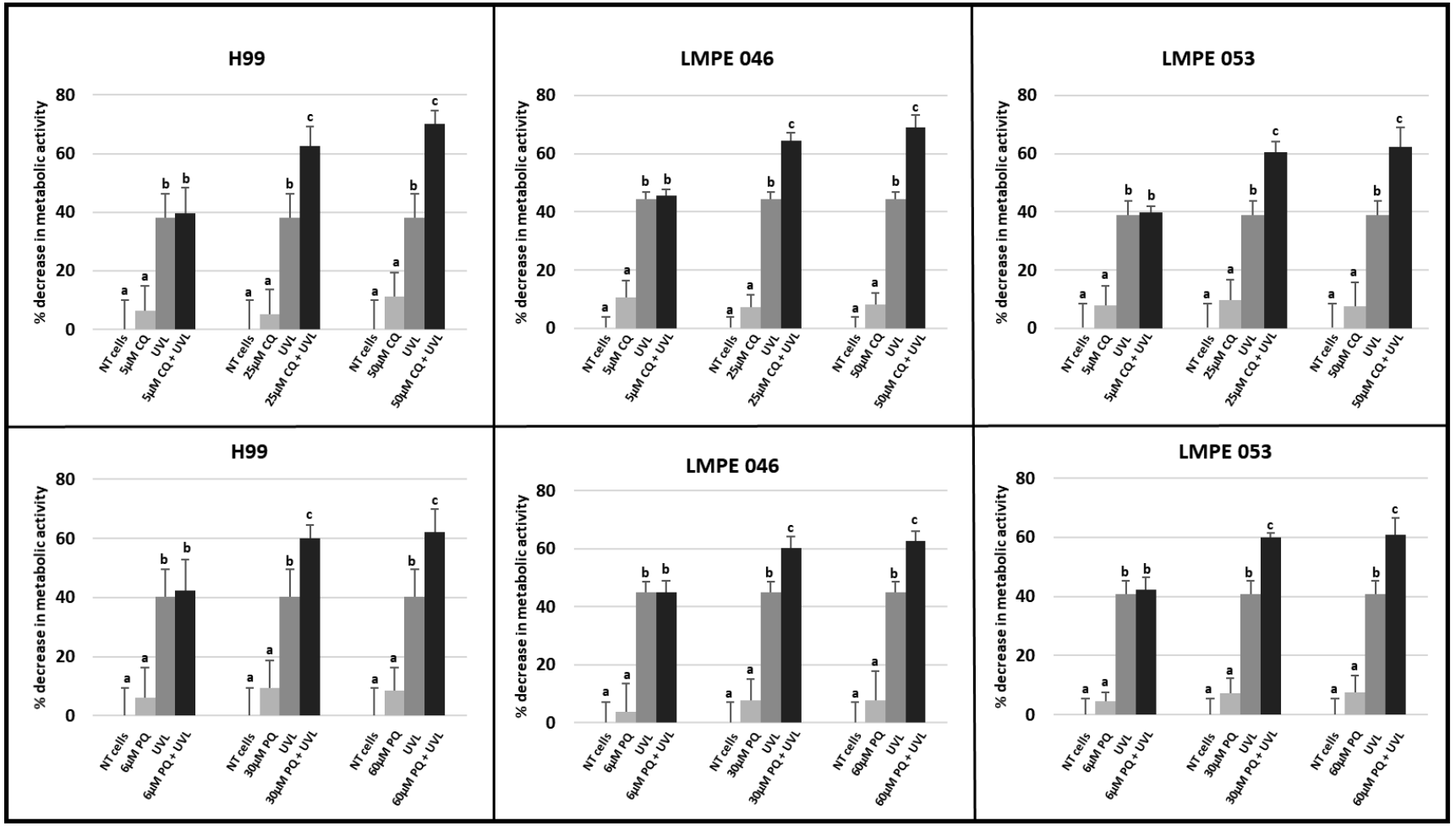


Figure 1A: *The metabolic activity of strains of C. neoformans H99, C. neoformans LMPE 046 and C. neoformans LMPE 053 following PDT with CQ and PQ at 2 min UVL exposure. CQ- or PQ-treated cryptococcal cells showed no significant reduction in their metabolic activity compared to their non-treated cells. However, there was a significant decrease in the metabolism of 2 min UVL-treated cells as well as PDT-treated cells when compared to the NT cells. CQ = chloroquine; NT = non treated; PDT = photodynamic therapy; PQ = primaquine; UVL = ultraviolet light.*

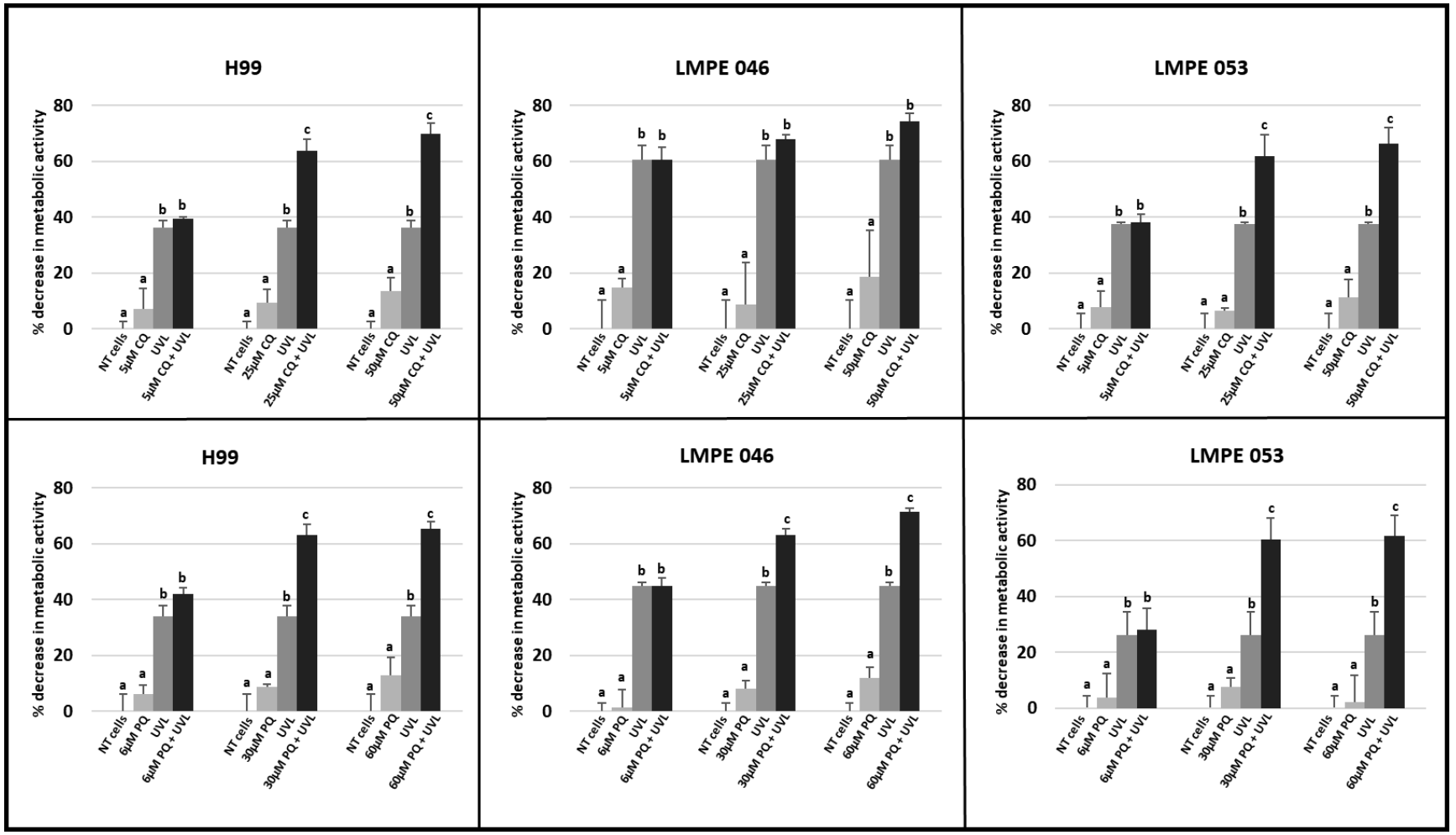


Figure 1B: *The metabolic activity of strains of C. neoformans H99, C. neoformans LMPE 046 and C. neoformans LMPE 053 following PDT with CQ and PQ at 8 min UVL exposure. CQ- or PQ-treated cryptococcal cells showed no significant reduction in their metabolic activity compared to their non-treated cells. Nevertheless, there was a significant decrease in the metabolism of 8 min UVL-treated cells as well as PDT-treated cells when compared to the NT cells. CQ = chloroquine; NT = non-treated; PDT = photodynamic therapy; PQ = primaquine; UVL = ultraviolet light.*

In Figure 1A, there was no significant difference (H99; $p \geq 0.5$, LMPE 046; $p \geq 0.2$ and LMPE 053; $p \geq 0.4$) in the absorbance readings obtained for the non-treated cells (0 μM and no UVL) when compared to cells treated with either CQ (5, 25 or 50 μM) or PQ (6, 30 or 60 μM) in the absence of UVL – suggesting there is no effect when exposed to drug-alone. However, there is a clear UVL effect (0 μM) for all the strains as their metabolic activity was significantly decreased ($p \leq 0.04$) when compared to their respective non-treated cells. Therefore, when PDT was implemented for all the strains at either 5 μM of CQ or 6 μM of PQ, there was no significant difference ($p \geq 0.5$) with their respective UVL-alone data were considered. Despite the noted UVL effect, an increase in either drug's concentration led to significant ($p \leq 0.01$) and compounded combinatorial effect compared to their respective non-treated cells as well as UVL-only treated cells ($p \leq 0.05$).

At 8 min UVL exposure (Figure 2B), a similar response pattern was observed, with some noticeable differences between the two-time points. For example, strain 046 showed no significant difference ($p \geq 0.2$) between the UVL exposed cells and cells exposed to a combinatorial effect of UVL and 25 μM or 50 μM of CQ. Moreover, an increase in the UVL exposure time (from 2 min to 8 min) did not yield a greater compounded, combinatorial effect. Based on this determination, it was decided to use a maximum UVL exposure time of 2 min. In addition, to use either CQ at 25 μM or PQ at 30 μM, unless otherwise stated.

CQ and PQ photosensitisation is detrimental to cryptococcal ultrastructure and cell membranes

The SEM image of PDT treatment with CQ and PQ for strains H99, LMPE 046 and LMPE 053 are presented in Figure 2A and 2B, respectively.

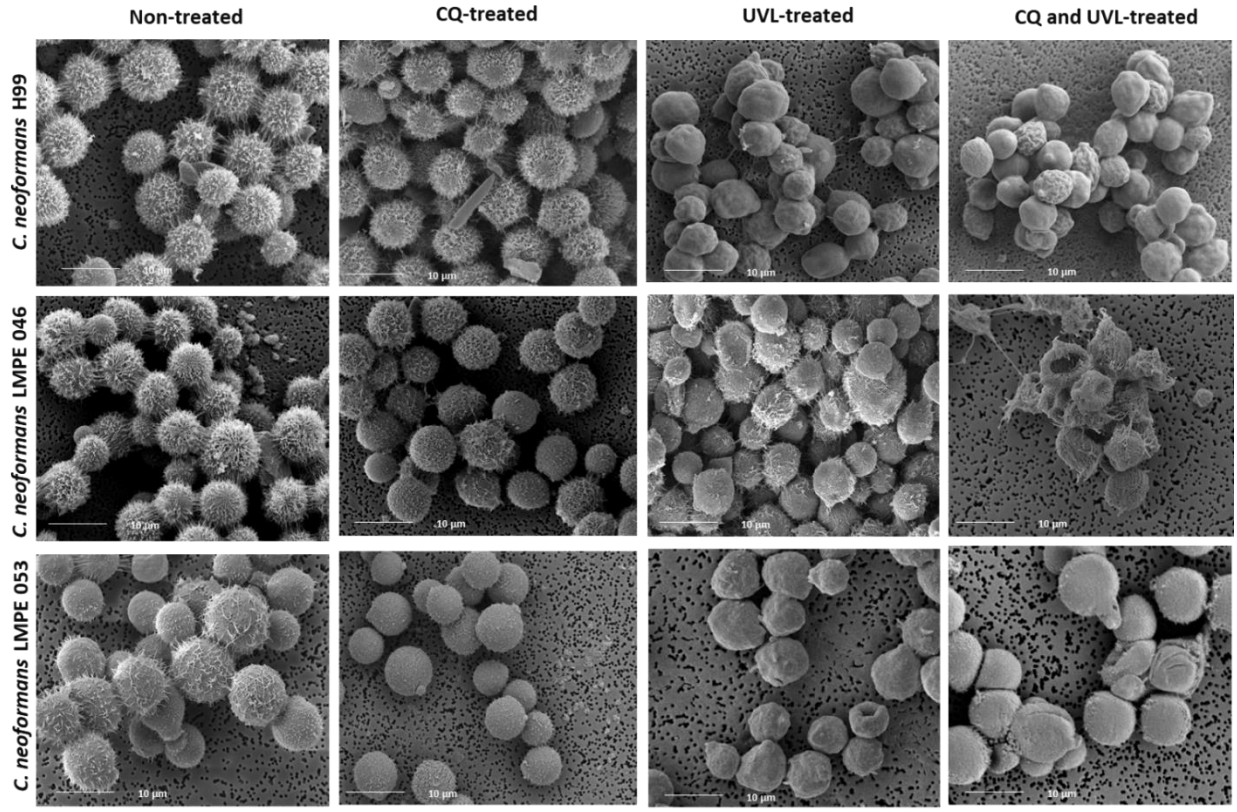


Figure 2A. *SEM images of morphological variation on the ultrastructure of cryptococcal isolates after PDT using CQ. The non-treated cells appear healthier with bigger cell diameter and are covered with extracellular matrix. Similarly, CQ-treated cells are comparable in their cell diameter; however, there was a reduction in the extracellular matrix of CQ-treated cells. In contrast, cells exposed to UVL-only treatment as well as PDT led to a ruptured cell wall with significantly smaller cell diameter for all three isolates. CQ = chloroquine; NT = non-treated; PDT = photodynamic therapy; PQ = primaquine; UVL = ultraviolet light.*

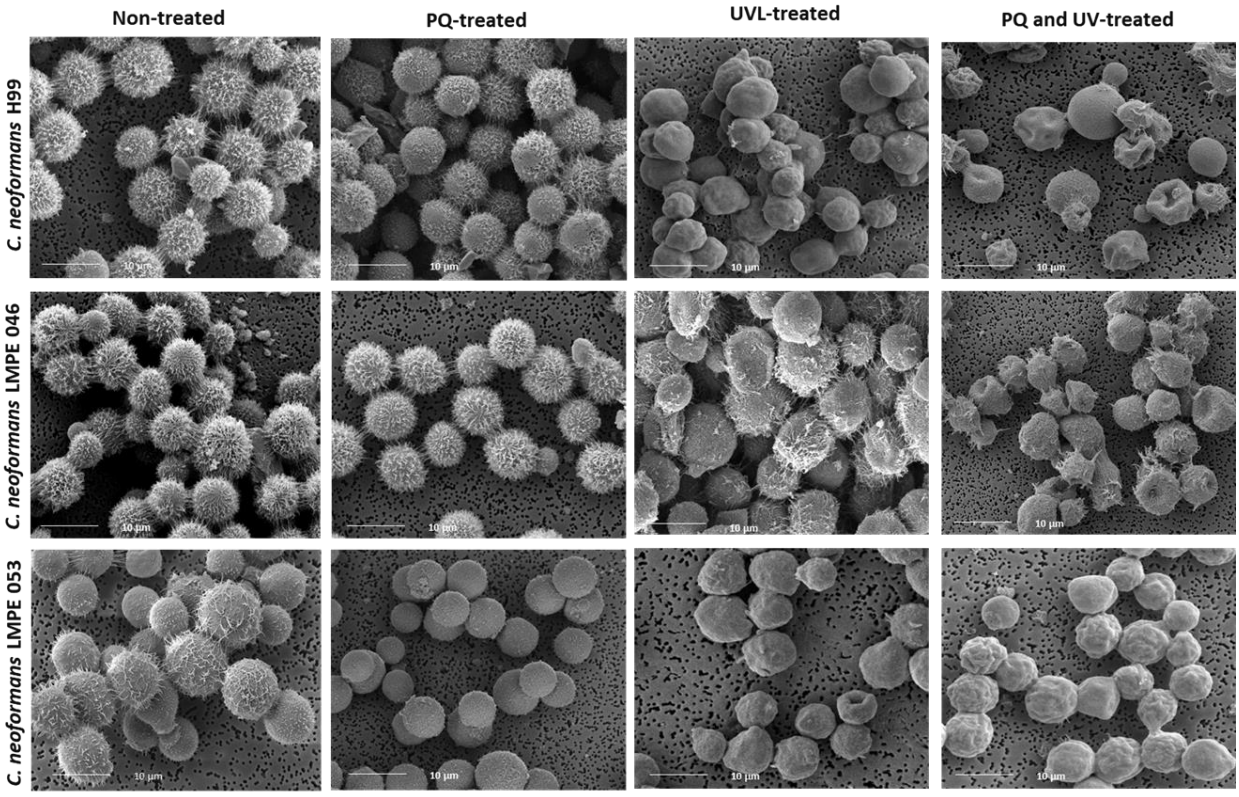


Figure 2B. *SEM images of morphological variation on the ultrastructure of cryptococcal isolates after PDT using PQ. PQ-treated cells were comparable to the non-treated cells with no notable difference in the cell diameters. Nevertheless, UVL-treated cells and PDT led to ruptured cells and significantly smaller cell diameter for all three isolates due to the effect of the treatment. CQ = chloroquine; NT = non-treated; PDT = photodynamic therapy; PQ = primaquine; UVL = ultraviolet light.*

In Figure 2A, the outer structure of the non-treated cells (with neither CQ nor UVL) of all three strains appears intact, healthier and covered with an extracellular matrix with an average cell diameter of 3.6 µm (+/- 0.1) for H99, 3.7 µm (+/- 0.1) for LMPE 046 and 3.8 µm (+/- 0.3) for LMPE 053. Cells treated with 25 µM CQ-alone seem to have little difference as seen by a reduction in the extracellular matrix in their ultrastructure without significant reduction ($p \geq 0.05$) in the cell diameter for H99 (3.5 µm (+/- 0.3)), LMPE 046 (3.6 µm (+/- 0.3)) and LMPE 053 (3.6 µm (+/- 0.3)) compare to the non-treated cells. 2 min UVL-alone treated cells without a drug, however, appear to have pronounced difference on the cells' appearance. For example, cells appear to have rough surfaces

and have also lost more of the extra-cellular matrix as well as significantly ($p \leq 0.05$) smaller cell diameter for H99 (2.9 µm (+/- 0.3)), LMPE 046 (2.6 µm (+/- 0.3)) and LMPE 053 (3.0 µm (+/- 0.3)). There was also a notable rupture of cell ultrastructure compared to the non-treated cells. The combinatorial effect of UVL and CQ had far more visible detrimental effect ($p \leq 0.05$) on the cells' ultrastructure with a significant reduction in cell diameter for H99 (2.5 µm (+/- 0.1)), LMPE 046 (2.3 µm (+/- 0.3)) and LMPE 053 (2.6 µm (+/- 0.3)) compared to the non-treated cells. A similar pattern was observed for Figure 2B images of PQ PDT. Treatment with PQ-only was not so effective with no significant reduction ($p \geq 0.05$) in the cell diameter compared to the non-treated cells of H99 (3.6 µm (+/- 0.1)), LMPE 046 (3.7 µm (+/- 0.1)) and LMPE 053 (3.8 µm (+/- 0.3)). However, there were visible negative effect of 2 min UVL-alone on H99 (2.9 µm (+/- 0.3)), LMPE 046 (2.6 µm (+/- 0.3)) and LMPE 053 (3.0 µm (+/- 0.3)) as well as UVL and 30 µM PQ for H99 (2.5 µm (+/- 0.2)), LMPE 046 (2.5 µm (+/- 0.4)) and LMPE 053 (2.7 µm (+/- 0.1)) compared to non-treated cells ($p \leq 0.05$).

To further assess the integrity of the cell wall following treatment, the accumulation of PI inside the cells (Figure 3), as well as the release of AK enzyme into the cultivation media (Figure 4), were considered.

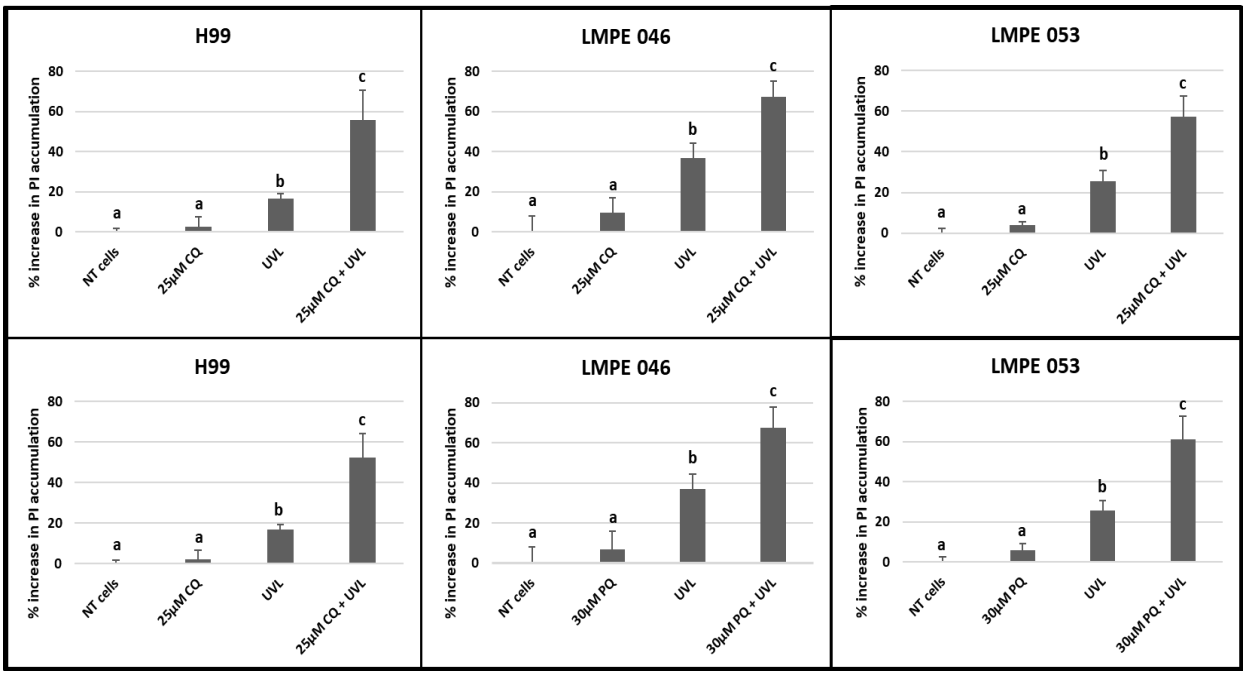


Figure 3. *The accumulation of PI stain in cells with damaged cell membrane. The effect of PDT with either CQ or PQ was reflected in the accumulation of PI inside affected cells. Results obtained for non-treated cells and either CQ or PQ were comparable as there was no significant accumulation of PI in drug-alone treated cells. CQ = chloroquine; NT = non-treated; PDT = photodynamic therapy; PI = propidium iodide; PQ = primaquine; UVL = ultraviolet light.*

For PI, the data for the non-treated cell was comparable, *i.e*. no significant difference ($p \geq 0.1$), to that of the drug-alone test, *i.e.* 25 µM of CQ (2% for H99; 10% for LMPE 046 and 4% for LMPE 053) or 30 µM of PQ (2% for LMPE 046; 7% for H99 and 6% for LMPE 053). However, the exposure of cells to 2 min UVL led to a UVL-effect ($p \leq 0.05$). Despite this UVL effect, when combined (25 µM of CQ and 2 min UVL as well as 30 µM of PQ and 2 min UVL), there was a significant accumulation ($p \leq 0.03$) of PI inside the cells when the data was compared to that of non-treated cells and also cells exposed to UVL-alone ($p \leq 0.05$).

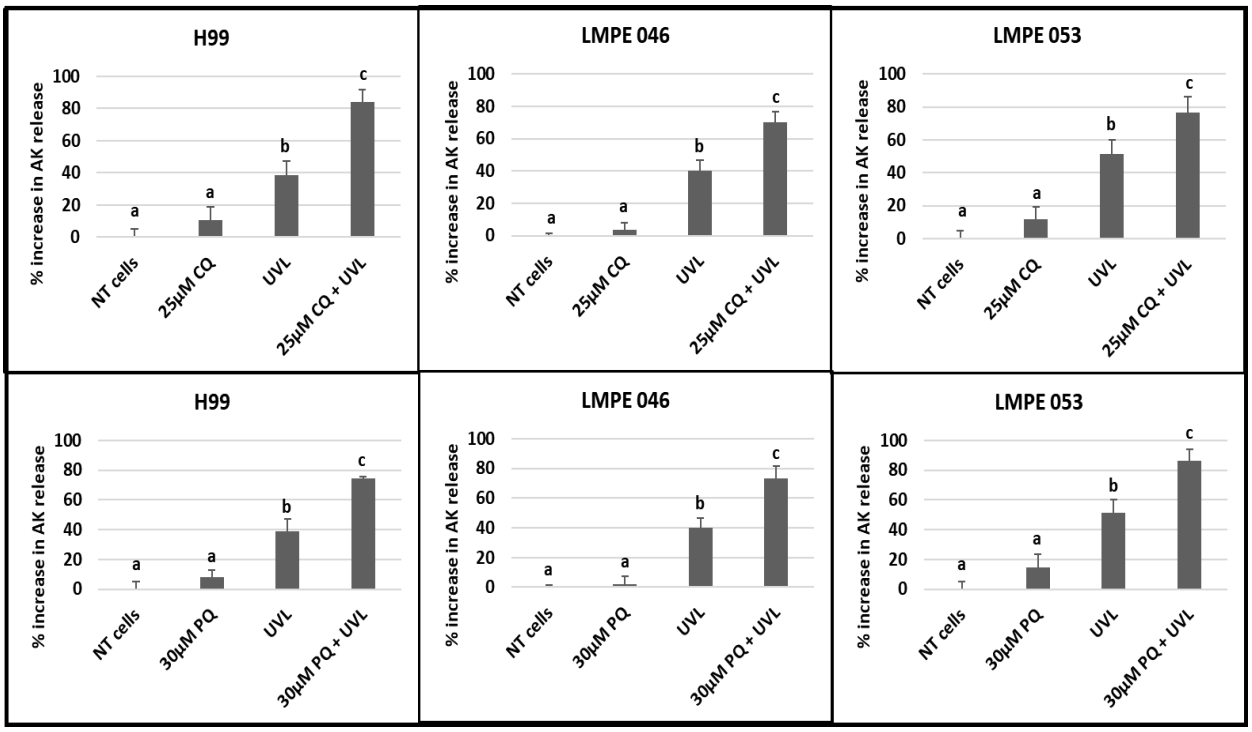


Figure 4. *The released AK from disrupted cells membrane. Cells treated with UVL-alone and UVL and either drug (CQ or PQ) were distinguished by the inability to preserve the integrity of the cell wall as evident by the significant release of cytoplasmic AK compared to the non-treated cells. However, CQ- or PQ-treated cells did not release any significant amount of the AK enzyme when compared to non-treated cells. CQ = chloroquine; NT = non-treated; PDT = photodynamic therapy; PQ = primaquine; UVL = ultraviolet light.*

For Toxilight, the data for non-treated cell was comparable, *i.e*. no significant difference ($p \geq 0.2$), to that of drug-alone test, *i.e.* 25 µM of CQ (11% for H99; 4% for LMPE 046 and 12% for LMPE 053) or 30 µM of PQ (8% for H99; 2% for LMPE 046 and 14% for LMPE 053). However, the exposure of cells to UVL led to a UVL effect ($p \leq 0.05$). Despite this UVL effect, when combined (25 µM of CQ and 2 min UVL as well as 30 µM of PQ and 2 min UVL), there was a significant accumulation ($p \leq 0.01$) of AK in the cultivation media when the data was compared to that of non-treated cells as well as cells exposed to UVL-alone ($p \leq 0.05$).

CQ and PQ PDT induces accumulation of ROS

The ROS data is summarised in Figure 5.

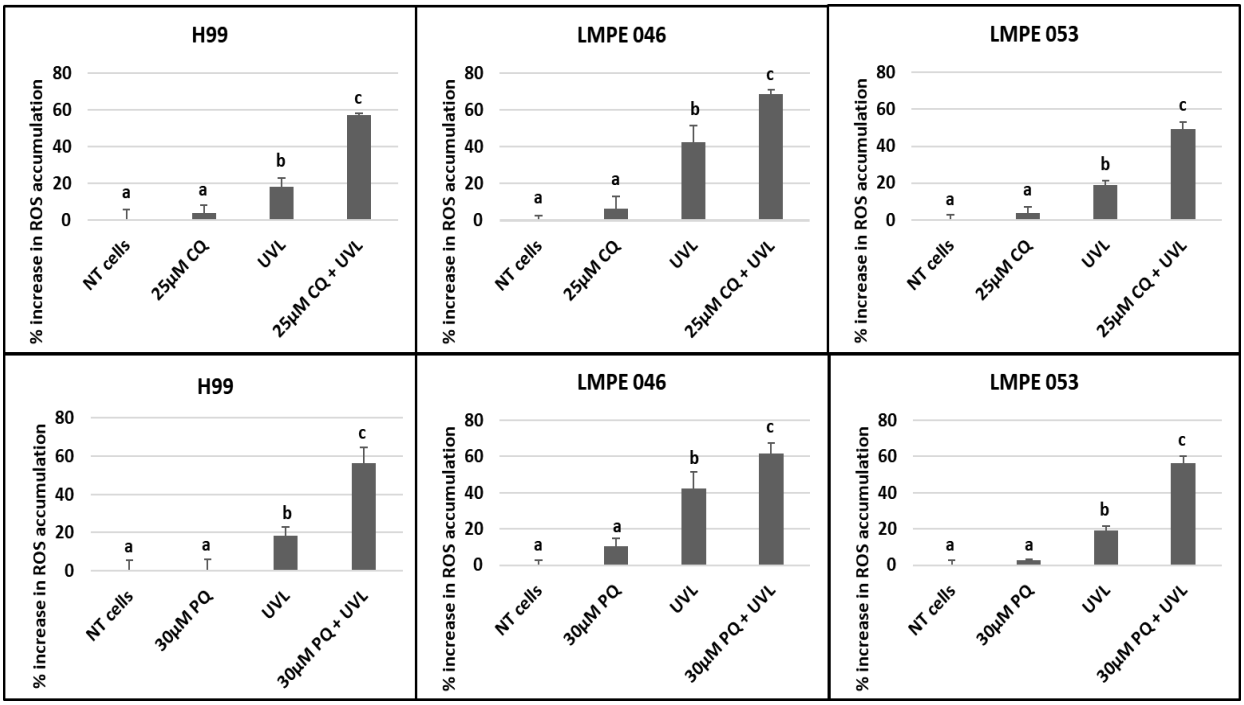


Figure 5. *Accumulation of ROS. The effect UVL and PDT led to a significant accumulation of excessive ROS compared to the non-treated cells. Results obtained CQ- or PQ-alone treated cells were comparable to that the non-treated cells as expected. CQ = chloroquine; NT = non-treated; PDT = photodynamic therapy; PQ = primaquine; UVL = ultraviolet light.*

The data for non-treated cells (H99 and LMPE 053) was comparable, *i.e*. no significant difference ($p \geq 0.4$), to that of drug-alone test, *i.e*. 25 µM of CQ (3% for H99; 7% for LMPE and 4% for LMPE 053) or 30 µM of PQ (0.5% for H99; 11% for LMPE 046 and 3% for LMPE 053). Moreover, all the strains experienced a UVL effect when their UVL data was compared to the non-treated cells' data ($p \leq 0.05$). When PDT was implemented for all the strains (25 µM of CQ and 2 min UVL as well as 30 µM of PQ and 2 min UVL), there was a significant accumulation ($p \leq 0.01$) of ROS when the PDT data was compared to that of non-treated cells as well as cells exposed to UVL-alone ($p \leq 0.04$).

PDT improves the activity of macrophages

Figure 6 summarises the response of macrophages to the effect of PDT.

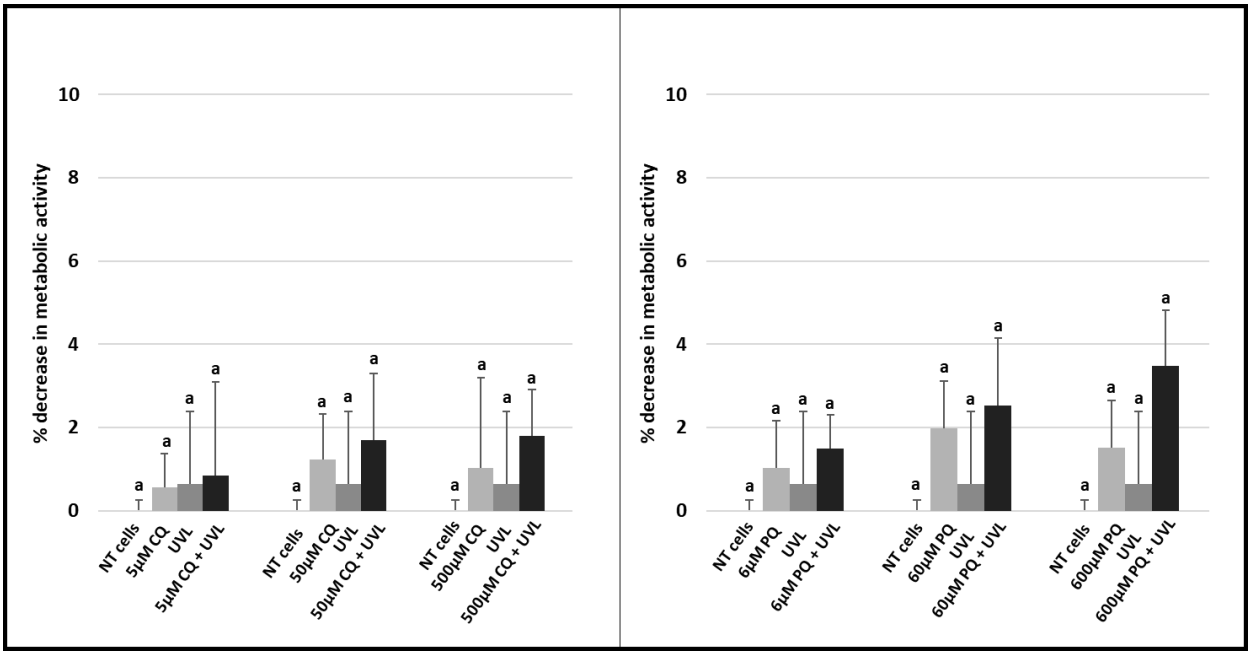


Figure 6. *Impact of photodynamic treatment on the health of macrophages. Macrophage cells can withstand exposure to PDT using either CQ or PQ even at high concentrations and longer exposure to UVL as there was no significant reduction in the metabolism of macrophages compared to the non-treated macrophages. CQ = chloroquine; NT = non-treated; PDT = photodynamic therapy; PQ = primaquine; UVL = ultraviolet light.*

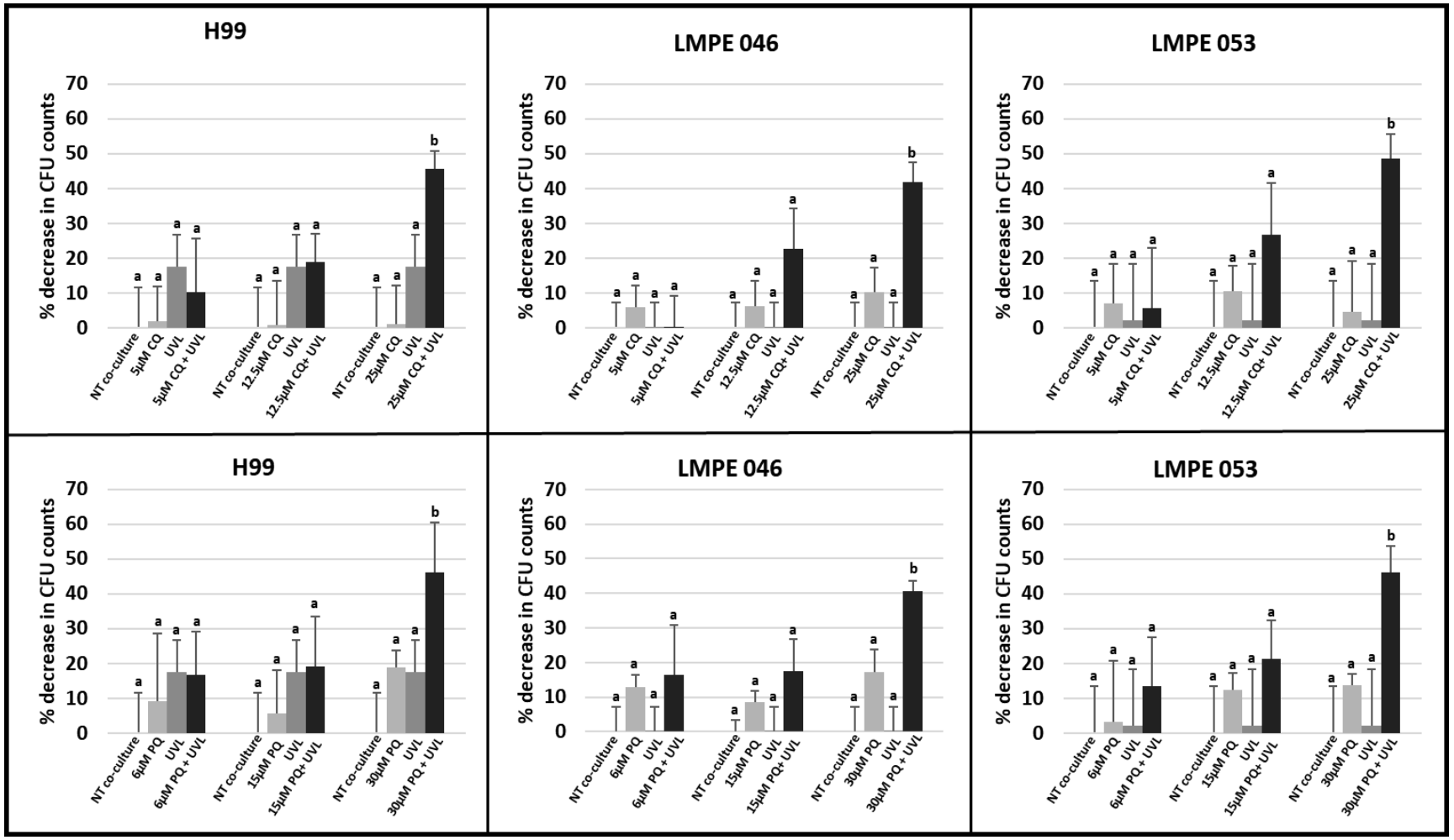
There was no notable difference ($p \geq 0.6$) in the absorbance readings obtained for the macrophages that were non-treated (0 µM, no UVL and in 8 min of DI) when compared to macrophages that were exposed to either CQ alone (5, 50 or 500 µM), UVL-alone (8 min) or PDT (5 µM and UVL for 8 min, 50 µM and UVL for 8 min or 500 µM and UVL for 8 min). The same observation was noted for PQ's experimental conditions.

This suggests that there is no effect when exposed to either drug alone, even at high concentrations, there was also no significant ($p \geq 0.08$). However, the highest decrease in metabolic activity of macrophages was observed when they were subjected to the

effect of UVL and either of CQ or PQ. Though, the later was not significant compared to the non-treated macrophages. Taken together, the above result reveals that less than 10% of macrophages were affected.

PDT improves the phagocytic competence of macrophages

Figure 7 represents the CFU counts of H99, LMPE 046, LMPE 053 recovered after the co-culture with macrophages exposed to different experimental conditions. Co-culture treated with either CQ (5, 12.5 or 25 μM) or PQ (6, 15 or 30 μM) did not show any significant decrease ($p \geq 0.3$) in CFU counts compared to the non-treated co-cultures. There was also a decrease in the CFU counts of co-cultures exposed to the effect of only UVL; however, it was not significant ($p \geq 0.4$) compared to the co-culture cells without any treatment. Exposure of co-culture to PDT with either CQ (5 or 12.5 μM) or PQ (6 or 15 μM PQ) did not result in any significant ($p \geq 0.09$) CFU counts compared to untreated co-culture. Nevertheless, a significant increase ($p \leq 0.05$) in CFU counts was obtained with the co-cultures were subjected to the effect of UVL and 25 μM CQ or 30 μM PQ.



2074

2075 **Figure 7.** *PDT with CQ and PQ enhances the phagocytic competence of macrophage cells. The internalisation of*
2076 *cryptococcal cells by macrophages appeared to be significantly enhanced with the combined effect of UVL and either CQ*
2077 *or PQ as reflected by the reduction in CFU counts. CQ = chloroquine; NT = non-treated; PDT = photodynamic therapy; PQ*
2078 *= primaquine; UVL = ultraviolet light.*

2.5 DISCUSSION

Fungal infections may manifest life-threatening diseases (Cowen, 2008). To compound this, deficiencies in the current antifungal regimen has often led to treatment failure in clinical settings. This has necessitated the need to consider repurposing CQ and PQ as photosensitisers that can be used to inactivate cryptococcal cells. The current study provides evidence that exposing these antimalaria drugs to UVL was sufficient to sensitise them to display an anti-cryptococcal quality. More to this point, the photo-excitation of CQ may have led to the production of CQ-induced hydroxyl radicals (OH·), superoxide anion and hydrogen peroxide while that of PQ may lead to the formation of monoprotonated (PQH(2+)*) and non-protonated (PQ(+)*) cation radical (Viola et al., 2007; Kristensen et al., 2009). More importantly, it is theorised that these resultant radicals from the photolysis of these compounds, may have led to cells losing their cell wall integrity and selective permeability and in turn, led to the observed cell death. These results support the findings in literature wherein the susceptibility of *C. neoformans* and *Candida albicans* to PDT has a direct correlation with cell membrane permeability function (Fuchs et al., 2007; Giroldo et al., 2009). To illustrate this point, Fuchs et al. demonstrated the use of polycationic conjugate of polyethyleneimine and photosensitiser chlorin(e6) compromised the integrity of cell walls of *C. neoformans* following PDT (Fuchs et al., 2007).

Numerous limitations faced with this type of therapy still need to be overcome for it to be fully appreciated as a therapy for infectious diseases. The crucial issues to address will be modes of delivery of both the light and the photosensitiser to sites of infection. The

current knowledge on PDT delivery for infection is limited to the parts of the body where light can reach quite easily such as skin and body cavities (Dai et al., 2012). More to the point, antimicrobial PDT is better applied exclusively to localised diseases in contrast to systemic infections such as cryptococcal pneumonia, cryptococcal meningitis and sepsis among others (Fuchs et al., 2007; Kharkwal et al., 2011). Importantly, it is necessary to determine the appropriate illumination devices with well-defined parameters and accurate dosimetry (Kharkwal et al., 2011).

In conclusion, the current study has given insight into the potential of CQ and PQ as photosensitisers when used in *in vitro* studies. However, further *in vivo* studies should be carried out to validate the obtained results wherein several aspects of photosensitisers need to be well defined in a clinical setting. These include the dose to be delivered and patients acceptability (Kim et al., 2016). Moreover, care should be taken to prevent continuous photo-reaction by keeping patients away from sources of UVC, as this radiation is damaging. Fortunately, UVC radiation from the sun is filtered by the atmosphere, and, therefore, does not reach the earth's surface.

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CHAPTER 3

PRIMAQUINE, AN ANTIMALARIAL DRUG THAT CAN CONTROL THE GROWTH OF CRYPTOCOCCAL CELLS

A draft manuscript based on the chapter has been prepared and will be submitted for publication. Because of the above, repetition of some information in the document could not be avoided.

The candidate, Madu, co-designed the study, performed the experiments, analysed the data and drafted the manuscript.

3.1 ABSTRACT

The study investigated the effect of primaquine on the growth of some *Cryptococcus* isolates, whose infection can be deadly, especially in persons with HIV/AIDS. We show that primaquine had a significant inhibitory effect on the metabolic activity of all tested cryptococcal isolates when compared to non-treated cells. When tested at 60 µM, a more than 50% reduction in the metabolic activities of all tested cryptococcal cells was achieved. Moreover, at this concentration, the drug was able to affect mitochondrial function adversely, as treated cells displayed loss of mitochondrial membrane potential, dislodging of cytochrome *c* (cyt *c*) and overproduction of reactive oxygen species (ROS). It is our reasoned summation that the produced ROS targeted the cell membranes, causing an increase in membrane permeability compared to non-treated cells. Concerning the effect of this drug on macrophages, we show that when tested at 1200 µM (20x the 60 µM), these immune cells did not experience a 50% reduction in their metabolic activity after a 24 h period. Thus, suggesting it may be safe to administer to macrophages over this period. Remarkably, the addition of primaquine to macrophages significantly enhanced their phagocytic efficiency against cryptococcal cells by a maximum of 32% when tested at 30 µM (0.5x the 60 µM). The latter may be critical in controlling the dissemination of cryptococcal cells inside macrophages, which they often manipulate in a Trojan horse-like manner.

Keywords: *Cryptococcus*, Drug-repurposing; Macrophages; Mitochondria; Primaquine.

3.2 INTRODUCTION

The recent increase in the number of people with immunosuppressive conditions has led to a parallel rise in the number of opportunistic fungal infection cases in clinical settings (Jerez-Puebla, 2012; Sebolai and Ogundeji, 2015; Esher et al., 2018). Cryptococcal meningitis is an example of such an infection, caused by the opportunistic basidiomycetous yeast, *Cryptococcus neoformans* (Esher et al., 2018). Cryptococcal meningitis is regarded as AIDS-defining (Esher et al., 2018), and without the prospect of treatment, a person may die within three months (Perfect et al., 2010). In developed countries, there has been a significant reduction in AIDS-related fungal disease burden (Rajasingham et al., 2017). However, the opposite is true in developing countries (Warkentien and Crum-Cianflone, 2010; Warnock, 2007). In sub-Saharan Africa, mortality due to cryptococcal meningitis is reported to be 135,900, accounting for 75% of the 181,100 annual global deaths caused by this infection (Rajasingham et al., 2017). This high rate is said to be partly due to the lack of access to effective antifungal drugs (Truong et al., 2018). The current collection of antifungals is limited to three classes of drugs, namely polyenes, azoles and pyrimidine that may be used independently or in combination with other antifungals (Perfect and Bicanic, 2015). Amphotericin B and fluconazole are mostly used in Southern Africa for the treatment of cryptococcal infections (Govender et al., 2013). Both these drugs exert their mode of action by targeting the fungal membranes (Ghannoum and Rice, 1999). Treatment of this infection with amphotericin B and fluconazole is unfortunately associated with clinical failure and unacceptably high mortality rates (Truong et al., 2018). More to the point, fluconazole has been reported to be associated with clinical relapse, poorer clinical outcomes, and the

risk of inducing drug resistance (Bicanic et al., 2014; Smith et al., 2015). On the other hand, amphotericin B has been associated with significant nephrotoxicity and poor bioavailability in the central nervous system due to its physicochemical nature (Saag et al., 2000; Ho et al., 2016; Cuddihy et al., 2019). Therefore, there is a need to find alternative anti-*Cryptococcus* drugs that are more effective. In the quest for alternative treatment, several compounds have been reconsidered in recent times. Drug repurposing has become of increasing interest as it bypasses the process of establishing a new drug (Deftereos et al., 2011), but it rather seeks to find a new therapeutic use of an already approved drug outside its original scope (Deftereos et al., 2011; Pushpakom et al., 2018). Our group has successfully shown the effectiveness of several non-traditional anti-*Cryptococcus* drugs (Ogundeji et al., 2016, 2017, 2018). In the current study, we examine the effect of the anti-malarial, primaquine (PQ), on cryptococcal cells. Furthermore, the current study also assessed the effect of this drug on macrophages as a way to control the possible growth of cryptococcal cells inside macrophages.

PQ is known to interfere with the transport of electrons in the mitochondria of parasites (Foley and Tilley, 1998; Percário et al., 2012). The above makes this drug ideal for targeting the mitochondria of cryptococcal cells as they are non-fermentative – thus, are inherently susceptible to oxidative stress as a result of any impairment to their oxygen metabolism. Importantly, the idea of re-purposing PQ is not foreign, as it has successfully been shown to control the growth of *Pneumocystis jirovecii* (Toma, 1991; Noskin et al., 1992). This fungus, similar to an extent to *Cryptococcus*, is an obligate fungus that lives in the exclusively aerobic environment of the lungs. In the study above, PQ was shown

to effectively control mild-to-moderate cases of *Pneumocystis jirovecii* pneumonia in patients with AIDS (Noskin et al., 1992). Importantly, in the above study, the most common adverse effect was the development of an erythematous rash (Noskin et al., 1992). The latter speaks to the suitability of using PQ to control fungal infections with minimum side effects.

3.3 MATERIALS AND METHODS

Strains, cultivation and standardisation

The clinical cryptococcal strains that were used in the study were *C. neoformans* LMPE 014, *C. neoformans* LMPE 046, *C. neoformans* LMPE 053 and *C. neoformans* H99 as well as *C. gattii* LMPE 045, *C. gattii* LMPE 048, *C. gattii* LMPE 052 and *C. gattii* R265. These strains were streaked out on yeast-malt-extract (YM) agar (3 g/l yeast extract, 3 g/l malt extract, 5 g/l peptone, 10 g/l glucose, 16 g/l agar; Merck, South Africa) plates for 48 h at 30^{o}C. From the 48 h culture, a colony was streaked out onto a YM agar plate and incubated for 24 h at 30^{o}C. After 48 h, five colonies were suspended in 5 mL of distilled water. The turbidity of each strain suspension was adjusted using a spectrophotometer to a final inoculum cell concentration of between 0.5 x 10^5 and 2.5 x 10^5 colony forming units (CFU) per millilitre as per EUCAST guidelines (Arendrup et al., 2015).

To cultivate the murine macrophage cell line RAW 264.7 (ATCC TIB-71), RPMI-1640 medium (Sigma-Aldrich, South Africa) supplemented with 20 mg/mL streptomycin (Sigma-Aldrich) and 2 mM L-glutamine (Sigma-Aldrich), 20 U/mL penicillin (Sigma-

Aldrich) as well as 10% fetal bovine serum (Biochrom, Germany) was used. For each experiment, the cells were grown until they reached a confluency of 80-90% in 5% CO_2 at 37^{o}C. Before use, the viability of the macrophages was determined to be above 90% for each biological repeat using trypan blue (Sigma-Aldrich). These cells were then standardised to 1 x 10^6 cells/mL and seeded in a transparent 96-well microtiter plate (Greiner Bio-One, Germany). The plate was then placed overnight in a 5% CO_2 incubator at 37^{o}C.

Preparation of drug

Primaquine diphosphate (PQ) was obtained as a standard powder from Sigma-Aldrich. This drug (4.55 mg) was first dissolved in 1 mL of distilled water and further diluted in 9 mL of RPMI 1640 medium. The amount of water in RPMI 1640 did not exceed 1%. PQ was used at final test concentrations of 12 µM, 60 µM and 120 µM. These concentrations were arbitrarily chosen, and importantly high doses of PQ are often well tolerated with no evidence of side effects in humans (Krudsood et al., 2008; Fernando et al., 2011; Berman et al., 2018).

Susceptibility assay

The broth microdilution protocol was modified from the EUCAST method by (Arendrup et al., 2015). In brief, into wells of a transparent 96-microtiter plate (Greiner Bio-One), a 100 µL suspension of standardised cryptococcal cells were dispensed. To the same wells, a 100 µL working solution of PQ was added, and thus the cells were allowed to react with PQ at final concentrations of 12 µM, 60 µM and 120 µM. A control of non-treated cells

(exposed to 0 µM) was also included. The prepared plates were incubated at 35^{o}C (+/- 2^{o}C) for 48h. At the end of incubation, the metabolic activity was measured by reacting cells for a further 3h with a tetrazolium salt (XTT; Sigma-Aldrich) in the presence of menadione (Sigma-Aldrich) as per the XTT protocol by Kuhn et al. (2003). After a 3h period, the absorbance of the plates was read at 492 nm using a spectrophotometer (Biochrom EZ Read 800 Research, United Kingdom). The percentage change in the metabolic activity of treated cells, when compared to non-treated (control) cells, was calculated to determine the effectiveness of the test concentrations. The 60 µM concentration was shown to reduce the metabolic activity of all tested cells by more than 50% and was thereafter used in subsequent experiment. Moreover, strains that were proven to be the most susceptible (*C. neoformans* LMPE 046), most resistance (*C. neoformans* LMPE 053) as well as the reference strain (*C. neoformans* H99), were also used further in the subsequent studies.

Effect of PQ on cryptococcal cell wall ultrastructure, capsule shedding and cytoplasmic membrane integrity

To visualise the damage induced by PQ on the outer structure of cryptococcal cells, scanning electron microscopy (SEM) was performed. The protocol was modified from the Swart et al. method (Swart et al., 2010). Cells of *C. neoformans* H99, *C. neoformans* LMPE 046 and *C. neoformans* LMPE 053 were prepared as mentioned above. At the end of the treatment period, the contents of the wells (cells suspended in media) were aspirated and transferred to 1.5 mL plastic tubes. Thereafter, the tubes were centrifuged to dispose of the media. The cells were fixed with phosphate-buffered 3% glutaraldehyde

(Merck), rinsed with the same buffer and fixed again with 1% buffered osmium tetroxide glutaraldehyde (Merck). The fixed cells were dehydrated using a graded ethanol series of 30, 50, 70, 95 and 100%. Afterwards, the cells were critical point dried and mounted on stubs. The stubs (with the specimen) were coated with gold and viewed with SEM (Shimadzu SSX-550 Superscan, Tokyo, Japan). Untreated control cells were included.

To determine the effect of PQ on the integrity of the cytoplasmic membrane, a propidium iodide (PI; Sigma-Aldrich) stain exclusion assay was performed. The protocol was modified from the Hussain et al. method (Hussain et al., 2019). The stain cannot cross intact membranes of viable cells and thus tends to cross and accumulate inside dead cells. The standardised cryptococcal cells were allowed to react with PQ for 6 h or 24 h in a black 96 well plate (Greiner Bio-One). At the end of each time interval, the cells were reacted with 1 µL of the PI stain (10 µg/mL). The plate was then incubated in the dark for 1 h at room temperature. After the incubation period, the induced fluorescence was measured using a fluorescence plate reader (Thermo-Scientific) at excitation of 485 nm and emission of 538 nm. To complement the above experiment, the effect of PQ on the leakage of adenylate kinase to the cultivation media was measured. The extracellular leakage of adenylate kinase is used as a reporter for the loss of cytoplasmic membrane integrity or cell lysis (Jacobs et al., 2013). The Toxilight assay was performed according to the manufacture's protocol (Lonza Rockland, Inc., United States). Cryptococcal cells were treated likewise with PQ for 6 h or 24 h in a transparent 96 well plate (Greiner Bio-One). At the end of each time interval, 20 µl of the supernatant was aspirated and dispensed into wells of a white 96-well plate. Into this, 100 µl of the Toxilight reagent was

added. The plate was then incubated for 5 min in the dark at 37^0C. The induced luminescence was then measured with a Fluoroskan Ascent FL (Thermo-Scientific).

The effect of PQ on cryptococcal mitochondrial health

Changes in mitochondrial membrane potential (ΔΨm) was measured using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), which is a membrane-permeant dye that forms an aggregate in the matrix of healthy mitochondria but is excluded in a dysfunctional mitochondrion (Yao et al., 2006). The assay was performed according to the manufacture's protocol (Life Technologies, United States). The assay was performed using cells that were reacted with PQ-treated for 6 h or 24 h inside a black 96 well plate. The cells were immediately reacted with 10 µL of JC-1 and incubated for a further 20 min in the dark at room temperature. The induced fluorescence was measured using the Fluoroskan Ascent FL. Mitochondrial membrane potential (ΔΨm) was calculated as the ratio of aggregated JC-1 (unhealthy cells; at excitation 540 nm / emission = 570 nm) and monomer (unhealthy cells; at excitation = 485 nm / emission = 535 nm) in order to deduce changes in depolarization.

To examine if PQ may lead to accumulation of ROS as a result of impaired mitochondrial membrane function, the amount of ROS produced was measured using 2',7-dichlorofluorescein diacetate (DCFHDA, Sigma-Aldrich). The protocol was modified from the Rastogi et al., method (Rastogi et al., 2010). In brief, the cells were likewise treated for 6 or 24 h. Thereafter, they were reacted with 10 µL of DCFHDA. The plate was

incubated in the dark for 30 min at room temperature. The induced fluorescence was measured using a fluorescence plate reader (excitation = 485 nm / emission = 535 nm).

To further characterise the impairment of the mitochondrial membrane, the dislodging of cytochrome *c* (cyt *c*) from the mitochondria was assessed following PQ treatment. The protocol was modified from the Choi and Lee method (Choi and Lee, 2015). Following the treatment of cells for 3 h, they were separated from the media by centrifugation and transferred a 1.5 mL plastic tube that contained 1 mL of a buffer solution (50 mM Tris (Sigma-Aldrich), 2 mM EDTA (Sigma-Aldrich), 1 mM phenylmethylsulfonyl fluoride (pH 7.5; Sigma-Aldrich). The contents were prepared for homogenised with 0.5 mm diameter zirconium glass beads (BeadBug, Benchmark Scientific Inc.). The plastic tube was placed on a microtube homogeniser (BeadBug, Benchmark Scientific Inc.) to achieve mechanical rupture. After homogenisation, 1 mL of 2% glucose (Merck) was added to the plastic tube. The tube was then centrifuged for 10 min at 2000 x *g*, and the supernatant (representing cytoplasm with the dislodged cyt *c*) was aspirated. The tube was momentarily kept of ice. The pellet (containing mitochondria) was resuspended in 1 mL of a second buffer solution containing 50 mM Tris, 2 mM EDTA (pH 5.0) and incubated for 5 min. The tube was again centrifuged for 30 sec at 5000 x *g*. The supernatant was disposed, and the pellet was suspended in 1 mL of a third buffer solution containing 2 mg/mL of Tris-EDTA (Sigma-Aldrich). A 100 µL suspension of this reaction buffer (containing mitochondria) was then dispensed into a 96-well microtiter plate (Greiner Bio-One). Similarly, a 100 µL suspension of the cytoplasm supernatant (which was momentarily kept on ice) was also dispensed into other wells of the same microtiter plate. All the well contents were then

reacted with 100 µL of 1000 mg/mL ascorbic acid (Sigma-Aldrich) for 5 min at room temperature. The absorbance of each plate was read at 550 nm using a spectrophotometer (Biochrom EZ Read 800 Research).

The effect of PQ on murine macrophages

The effect of PQ on macrophage health was assessed by testing a range of doses that would be effective without inducing intolerable adverse effects, i.e. killing 50% or more of the macrophage population. From the overnight incubated macrophages, the media was aspirated and 100 µL of fresh, sterile media was added to macrophages inside the wells. To this, 100 µL of PQ (at twice the desired strength) was added. At the end, macrophages were exposed to the following final concentrations of PQ: 0 µM (0x the 60 µM), 30 µM (0.5x the 60 µM), 60 µM (yielded more than 50% reduction in cryptococcal metabolic activity), 300 µM (5x the 60 µM), 600 µM (10x the 60 µM) or 1200 µM in the wells (20x the 60 µM). The plates were then incubated at 37^0C in a 5% CO_2 incubator for 6 h or 24 h. After each incubation period, the metabolic activity was determined by allowing macrophages to react with the XTT salt in the presence of menadione for a further 2 h period inside the same 5% CO_2 incubator as per the XTT protocol by Kuhn et al. (2003). The absorbance of the plates was measured at 492 nm with a plate reader (Biochrom EZ Read 800 Research). Non-treated macrophages were included as a control.

Effect of PQ on murine macrophage phagocytic efficiency

We further investigated if PQ treatment may improve the phagocytic efficiency of macrophages by enumerating colonies of recovered cryptococcal cells after they were

co-cultured with macrophages. The protocol was modified from the Mazzolla et al., (Mazzolla et al., 1997) and Harrison et al., (Harrison et al., 2000) methods. For this experiment, PQ was used at a final concentration of 0 µM (0x the 60 µM), 6 µM (0.1x the 60 µM) and 30 µM (0.5x the 60 µM) in the wells. The overnight macrophage media was aspirated and replaced with 100 µL of fresh media that contained twice the PQ concentration gradient. To the same wells, cryptococcal cells were added yield a 10 (macrophages): 1 (fungus) effector to target ratio. For control, cryptococcal cells were added to wells with the same concentration gradients but in the absence of macrophages. The microtiter plates were incubated for 18 h at 37^0C in a 5% CO_2 incubator. After which, the supernatant was aspirated, and 200 µl of phosphate-buffered solution (PBS; Sigma, Aldrich) was added to the wells to wash-off non-internalised cryptococcal cells. To harvest macrophages with internalised cryptococcal cells, cold PBS was added to the wells. The contents of the wells (containing mobilised macrophages) were then aspirated and transferred to 1.5 mL plastic tubes. To this, 200 µl of 0.1% Triton X-100 (Sigma-Aldrich) was added to lyse macrophages. The recovered cryptococcal cells were then diluted 1:10. A 50 µl dilution solution, with recovered cryptococcal cells, was spotted onto a YM agar plate. A spread-plate method was carried out to create a lawn of cryptococcal cells. The YM plate was then incubated at 30^oC for 48 h before colonies were counted.

Statistical analyses

For each study, three independent experiments were performed. GraphPad Prism 8.3.1 was used to calculate mean values and the standard deviation of the means. The same programme was used to perform the multiple comparison test using Tukey as an option.

A *p* value of less than or equal to 0.05 was considered significant. To this end, a bar that has a different alphabet to the other implies there is a significance difference while those with the same alphabet are not significantly different. The data were also tested for normality of distribution using the Shapiro-Wilk test with alpha value of 0.05. Thus, a *p* value less than 0.05 indicated abnormal distribution of the data points.

3.4 RESULTS

Cryptococcal cells are susceptible to the antifungal action of PQ

The response of cryptococcal cells to the different concentrations of PQ are shown in Table 1. Each strain was affected in a dose-dependent manner when subjected to increasing concentrations of PQ. When comparing the response of all the eight tested strains, it was noted that *C. neoformans* LMPE 046 was the most susceptible strain, while *C. neoformans* LMPE 053 was the most resistant. In the study, 60 µM and 120 µM led to more than 50% reduction in the metabolic activity of all tested strains when compared to the non-treated cells. However, it is important to point out that given the wide concentration range that was used in the current study, it is possible that another concentration(s) between 12 µM and 60 µM, may also yield a more than 50% reduction in the metabolic activity of the cells.

Table 1. The effect of primaquine on some species of *Cryptococcus* genus.

Details of species		Drug response			
		XTT (492 nm) reading	% Reduction in M.A.		
Species name	**Strain number**	**0 µM treatment**	**12 µM treatment**	**60 µM treatment**	**120 µM treatment**
C. neoformans	LMPE 014	0.37 (0.01)	8.2 (8.2)	56 (0.6)	63 (1.0)
C. neoformans	LMPE 046	0.89 (0.07)	23 (7.9)	74 (1.2)	82 (1.9)
C. neoformans	LMPE 053	0.22 (0.01)	1.7 (0.9)	51 (1.5)	51 (1.2)
C. neoformans	LMPE 150 (H99)	0.72 (0.08)	2.8 (8.8)	63 (5.6)	64 (6.3)
C. gattii	LMPE 045	0.29 (0.03)	5 (11.0)	52 (4.0)	55 (3.0)
C. gattii	LMPE 048	0.30 (0.03)	16 (9.0)	53 (3.4)	60 (1.9)
C. gattii	LMPE 052	0.93 (0.01)	17 (2.8)	52 (0.8)	55 (0.7)
C. gattii	LMPE 109 (R265)	0.65 (0.03)	22 (4.0)	57 (2.0)	81 (0.6)

% Reduction in M.A. = percentage reduction in metabolic activity. Values represent the mean of biological triplicate and in brackets are the standard error values.

PQ negatively affects cryptococcal cell walls and cell membranes

The SEM data revealed that the ultrastructure of cells treated with PQ differed from non-treated cells (Figure 1).

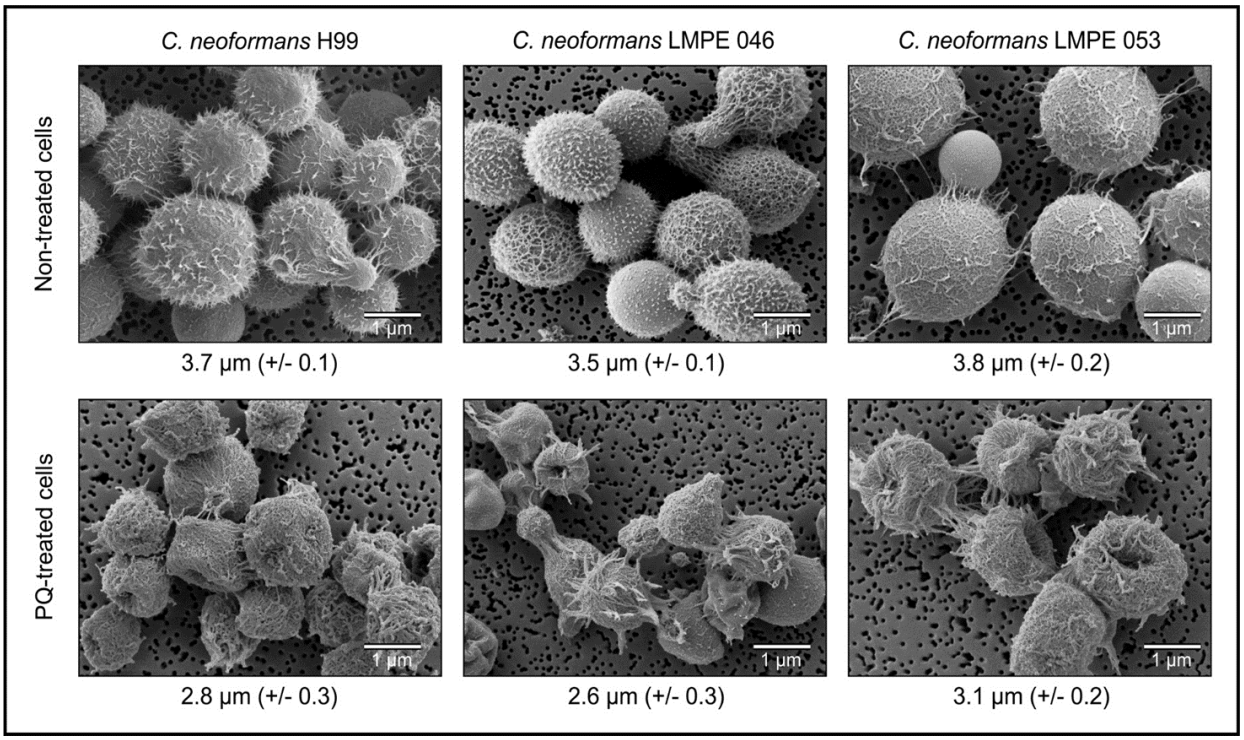

Figure 1. *Scanning electron micrographs showing the effect of PQ on treated cells when compared to non-treated cells. Treatment seems to have induced an ultrastructural change, which is evidenced by collapsed walls (PQ-treated cells) when compared to non-treated cells*.

All cells (non-treated and treated cells) were observed to be covered by an extracellular matrix, which could be the capsule. Although there were no observable differences concerning the matrix, PQ-treated cells seemed to have collapsed cell walls. Furthermore, PQ-treated cells were smaller in size when compared to the non-treated cells. PQ-treatment also led to the impairment of the cytoplasmic membrane's integrity; a feature commonly seen in dead cells. To the point, the treated cells showed a significant

intracellular accumulation of the PI stain, *i.e.* 6 h ($p \leq 0.03$) and 24 h ($p \leq 0.01$), when compared to non-treated cells (Figure 2).

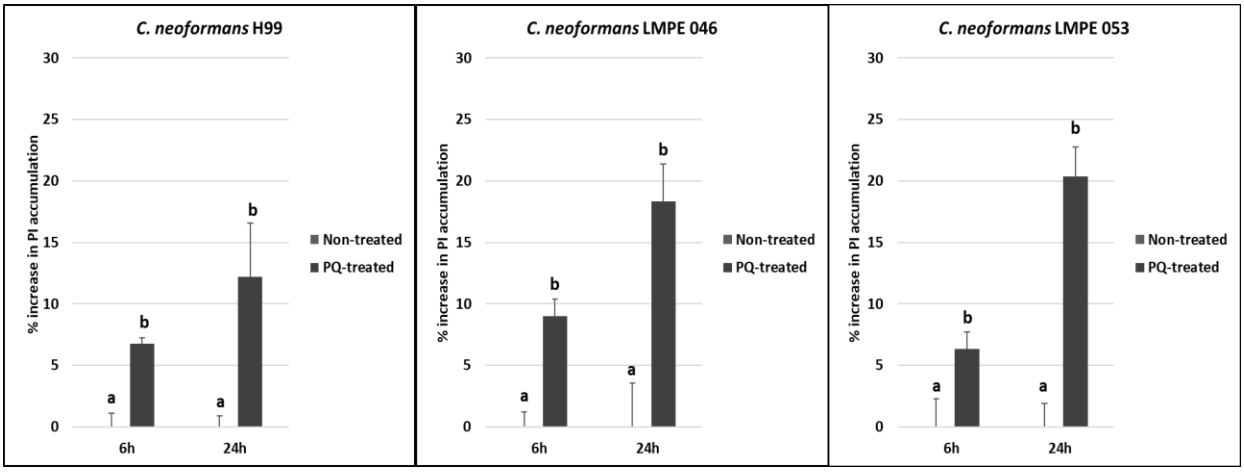

Figure 2. *Assessment of membrane integrity. An exclusion assay using propidium iodide was used and it revealed that all PQ-treated cells significantly accumulated the stain while non-treated cells (assumed to be healthy) excluded the stain.*

The PI data was complemented by Toxilight data wherein the treated cells were revealed to significantly leak the adenylate kinase, *i.e.* 6 h ($p \leq 0.04$) and 24 h ($p \leq 0.01$), into the media when compared to non-treated cells (Figure 3). Thus, further confirming the assumption of membrane integrity loss.

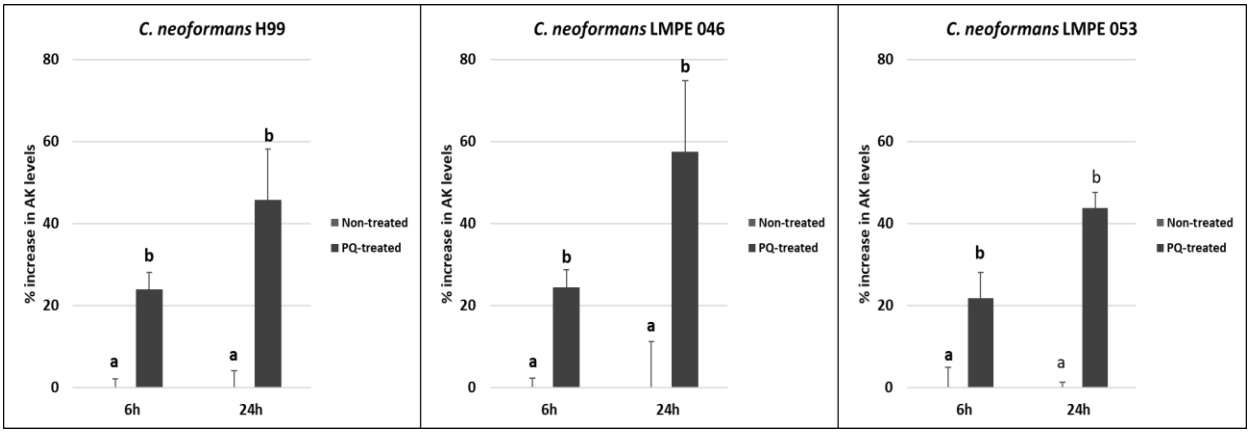

Figure 3. *Assessment of membrane integrity. All PQ-treated cells were characterised by the inability to retain adenylate kinase, which is indicative of loss of integrity. All treated cells had significantly secreted this cytosolic enzyme into the culture media when compared to non-treated cells.*

PQ negatively affects cryptococcal mitochondria

The oxygen metabolism in cryptococcal cells is central to their survival, as it provides a means to acquire energy to support cellular processes. Thus, impairment of this metabolism would be lethal as cells cannot switch over to a fermentative pathway. In the study, we show that PQ-treatment led to a significant decrease in membrane potential (ΔΨm) for all treated cells, *i.e.* 6 h ($p \leq 0.02$) and 24 h ($p \leq 0.01$), compared to non-treated cells (Figure 4).

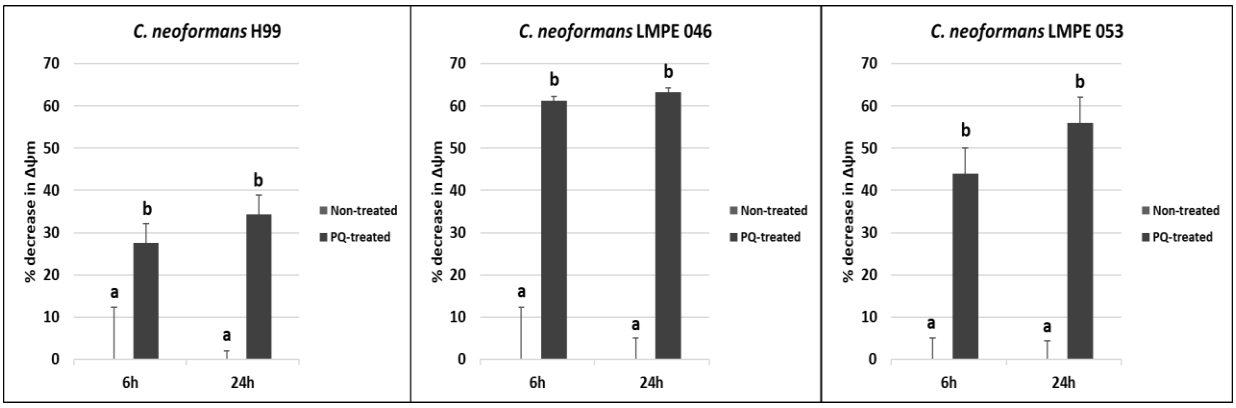

Figure 4. *Assessment of mitochondria membrane potential. PQ treatment led to all cells showing a significant loss of membrane potential when compared to non-treated cells. The finding pointed towards an impaired mitochondrion.*

Coupled to this, we also noted a significant release of the cyt *c* from the mitochondria ($p \leq 0.02$) to the cytoplasm ($p \leq 0.04$) in all cells following exposure to PQ, compared to their respective non-treated cells after 3 h (Figure 5).

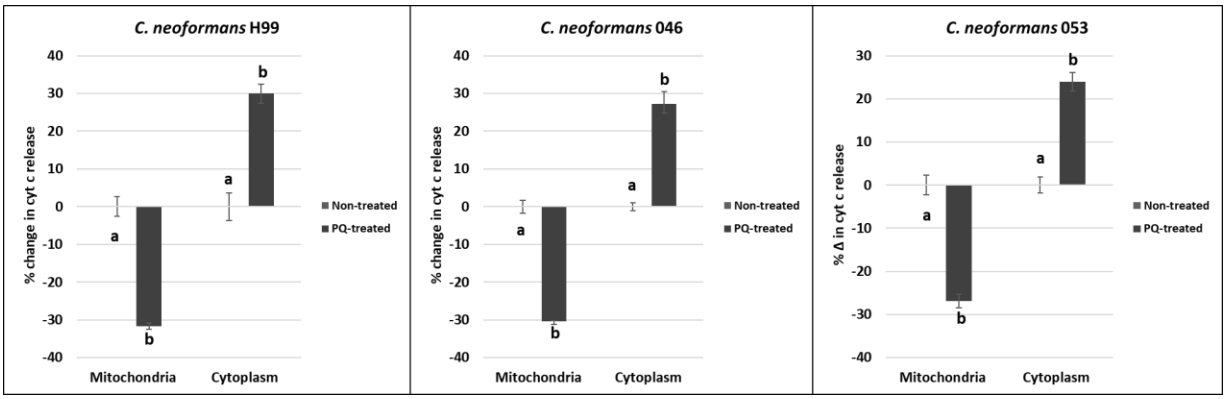

Figure 5. *Using cyt c as an indicator for an impaired mitochondrion. PQ treatment led to a dysfunctional mitochondrion characterised by the dislodging of this electron carrier (cyt c) from the mitochondria to the cytoplasm when compared to non-treated cells ($p \leq 0.05$). Healthy cells (symbolised by non-treated cells) are often characterised by mitochondria with intact cyt c*

These results suggest that the mitochondrial membranes are not energised or charged correctly to produce ATP. Moreover, the dislodging of the cyt *c* also suggests that oxygen will not receive a full complement of electrons to be reduced. Because of the latter, harmful forms of oxygen may be produced. Therefore, it was not surprising to note that PQ-treatment led to an increase in ROS production at 6 h ($p \leq 0.04$) and a more significant increase at 24 h ($p \leq 0.02$), compared to their respective non-treated cells (Figure 6).

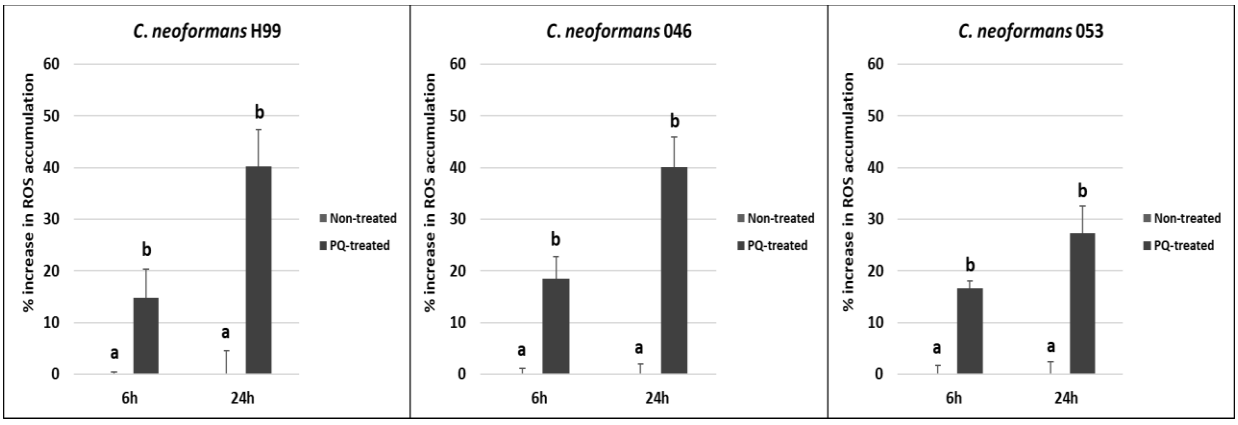

Figure 6. *Using excessive ROS production as an indicator of impaired mitochondria. All PQ-treated cells showed a distinctive overproduction of ROS when compared to the non-treated cells. This may be due to the inability to relay electrons to oxygen considering the absence of cyt c in the mitochondria as shown in Fig. 7.*

Based on these results, it is reasonable to conclude that exposure to PQ led to cell death via oxidative damage. The observed ROS may have targeted cell membranes – causing this important cellular constituent to lose its ability to control the trafficking of molecules in and out of the cells (Figures 2 and 3).

PQ improves the phagocytic efficiency of macrophages

Given that cryptococcal cells are eukaryotic, it becomes equally important to show the effect that this drug may have on host cells. In the study, macrophages were assessed – as cryptococcal cells have been shown to survive and reproduce inside them (Casadevall, 2010). Furthermore, cryptococcal cells are known to manipulate these cells in a Trojan horse-like manner for dissemination purposes (Casadevall, 2010). Therefore, for this drug to be considered as a candidate drug for controlling cryptococcal cells, their effect on macrophages was thus assessed.

Circulating monocytes have been reported to have a half-life of approximately 24 h in the blood (Monie, 2017). Thus, we assessed the effect of PQ on the health of macrophages over a 6 and 24 h period. It was observed that when macrophages were exposed to 60 µM, a 4% ($p = 0.1$) and 18% ($p \leq 0.02$) reduction in metabolic activity was achieved at 6 h and 24 h when compared to the non-treated cells. At the highest concentration of 1200 µM (20x the 60 µM); a reduction of 12% ($p \leq 0.01$) and 37% ($p \leq 0.0001$) was achieved at 6 h and 24 h (Figure 7). Both these percentages suggest that a concentration greater than 1200 µM is required to negatively affect more than 50% of the macrophage

population. Therefore, this drug at the said concentrations would be safe to administer to macrophages over a 24 h period.

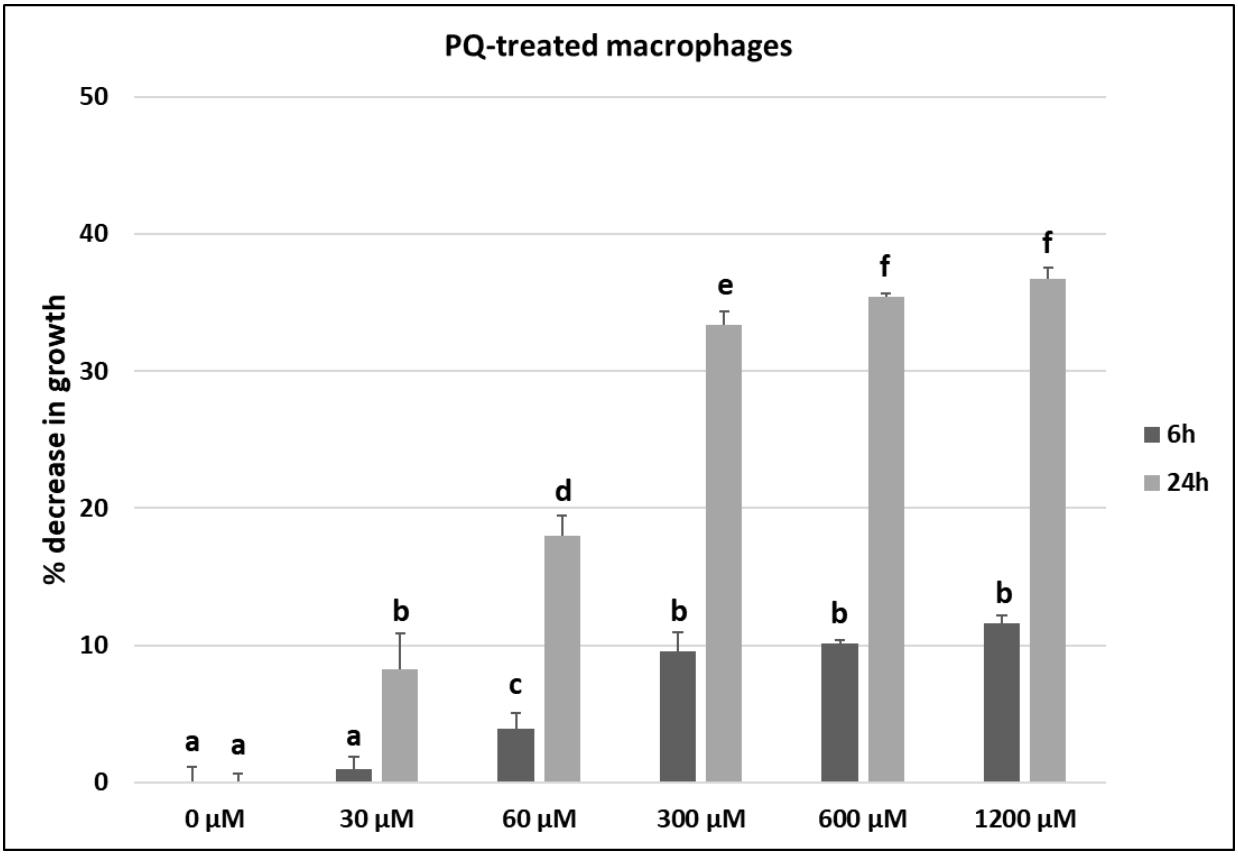

Figure 7. *Assessment of PQ-treatment on the health of macrophages. Results show that macrophages can withstand exposure to a wide concentration range of PQ over a 24 h period. To the point, at 20x the 60 µM, i.e. 1200 µM, up to 50% of the macrophage population was not negatively affected. This suggested it would be safe to administer this drug at 60 µM (as shown in the current study) to sensitise them.*

Nonetheless, for our chemo-sensitising studies, we chose to use lower concentrations 0 µM (0x the 60 µM), 6 µM (0.1x the 60 µM) and 30 µM (0.5x the 60 µM) to expose macrophages to minimal amounts of PQ. On their own, macrophages were able to phagocytose cryptococcal cells but not significantly enough at 0 µM ($p \geq 0.1$) and 6 µM ($p \geq 0.1$). However, their efficiency seems to be enhanced in the presence of PQ (Figure 8) as fewer colonies were recovered on agar plates after cryptococcal cells were co-

cultured with macrophages in the presence of 30 µM PQ, compared to untreated controls. More to the point, in the presence of 30 µM PQ, the phagocytosis of *C. neoformans* H99, *C. neoformans* LMPE 046 and *C. neoformans* LMPE 053 was increased by 31% ($p \leq 0.01$), 22% ($p \leq 0.02$) and 32% ($p \leq 0.01$), based on the CFU counts.

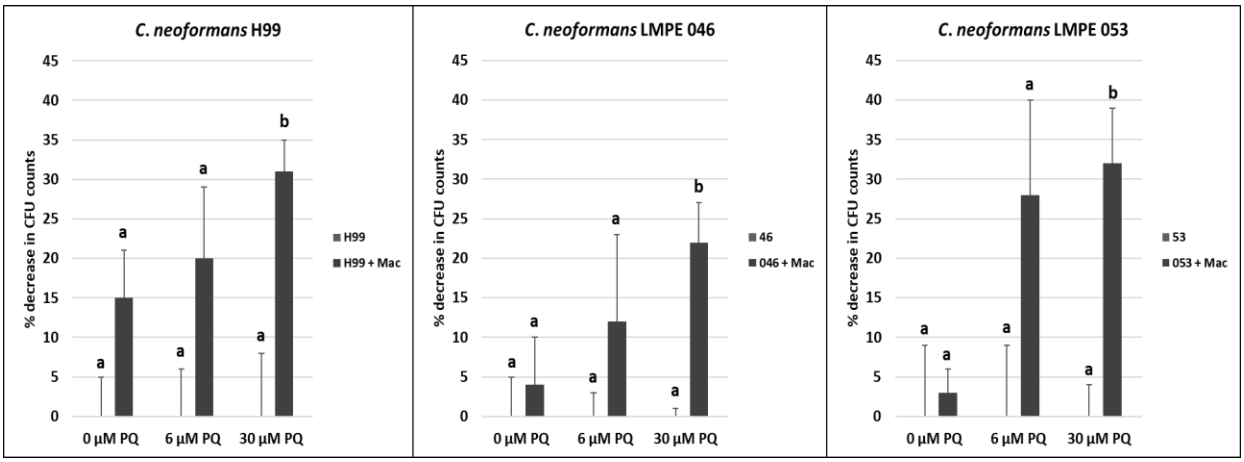

Figure 8. *PQ enhances the phagocytic efficiency of macrophages. Exposure of macrophages to PQ seemed to sensitise them to significantly phagocytose more cryptococcal cells than when not exposed to the drug.*

3.5 DISCUSSION

Cryptococcosis is one of the major fungal infections in persons infected with HIV, and its disease burden is reported to be highest in sub-Saharan Africa (Rajasingham et al., 2017). To complicate this, the current cryptococcal treatment regimen is often characterised by clinical failure, and medicines like flucytosine are not readily available in Africa due to cost. Moreover, Truong et al. further added that drug development is hampered by the unwillingness of pharmaceutical companies to invest in drugs that cannot assure a large financial return (Truong et al., 2018). Therefore, there is a need to consider other treatment options, such as drug repurposing.

In the current study, we sought to repurpose PQ. This drug belongs to the class of aminoquinolines, and it is widely used for the treatment of malaria (Bawa et al., 2010; Chu and White, 2016). However, the drug has successfully been used outside its prescribed scope in the treatment of *Pneumocystis* pneumonia (Toma, 1991; Noskin et al., 1992). The above allows us to place our current antifungal results in context with previous work. We also sought to explain the mechanism through which PQ causes fungal growth inhibition. When the obtained results were taken together, it led us to theorise that the treated cells were killed by oxidative damage due to the excessive production of ROS in cells with impaired mitochondrial function.

Macrophages were used in the study as a model because they are often recruited in response to the presence of invading pathogenic cells. Unfortunately for host organisms, cryptococcal cells can manipulate macrophages and use them as Trojan horses to disseminate without triggering an immune response (Casadevall, 2010; Voelz and May, 2010). Therefore, it becomes important to evaluate the effect of this drug on macrophages and its ability to kill more internalised cryptococcal cells. Herein, we show that PQ sensitised macrophages to significantly kill cryptococcal cells that were internalised. Based on the chemistry of PQ, *i.e.* that it is a lipophilic, weak base, it is possible that it may accumulate inside macrophages by ion trapping to, in turn, adjust the internal pH of macrophages from 4.5 to around 7 as previously documented chloroquine by Levitz et al. (1997). The raised pH may impair the proteolytic activity of enzymes and availability of nutrients crucial to the growth and survival of internalised microorganism (Seglen, 1983; Geary et al., 1986; Newman et al., 1994). Moreover, cryptococcal cells are said to

replicate optimally at a low pH, such as in the acidic phagosomes, but not at a higher pH (DeLeon-Rodriguez and Casadevall, 2016). While PQ is a weak base, its accumulation inside some organelles has been observed. To the point, van Weert et al. documented the accumulation of PQ inside endosomes led to pH neutralisation (van Weert et al., 2000). However, given that PQ it is a weak base is also conceivable that a high concentration may be required to achieve accumulation. It is also conceivable that another killing method (independent of pH neutralisation), may also participate in the killing of the internalised cells and reduction of non-lytic exocytosis as previously suggested by (Ma et al., 2006; Nicola et al., 2011) or cells may be dying from direct contact with PQ and not enhanced macrophage function.

In conclusion, the current study presented a case for PQ to be used alone or as adjuvants to complement the current anti-*Cryptococcus* drugs that are used routinely in clinical settings. *In vivo* studies should now be considered to determine if success can be achieved against a systemic cryptococcosis infection. While the idea of drug repurposing holds promises, it is possible that it may not always yield desired outcomes. To illustrate this point, the repurposing of sertraline, an anti-depressant drug, as an anti-*Cryptococcus* drug showed encouraging results with *in vitro* and *in vivo* studies but failed in phase III clinical trial (Zhai et al., 2012; Smith et al., 2015; Trevinõ-Rangel et al., 2016; Rhein et al., 2019;). One of the important considerations for repurposing PQ may concern prescribing it to persons with the glucose 6-phosphate dehydrogenase deficiency, which may see such subjects experiencing a dose-dependent haemolysis (Avalos et al., 2018; Recht et

al., 2018). More so, prescribing PQ to such subjects in sub-Saharan Africa where malaria is problematic.

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CHAPTER 4

CHLOROQUINE AND D-α-TOCOPHERYL POLYETHYLENE GLYCOL SUCCINATE MIXED MICELLES FOR TARGETING DRUG DELIVERY ACROSS *IN VITRO* BLOOD BRAIN BARRIER TO INHIBIT GROWTH OF CRYPTOCOCCAL CELLS

A draft manuscript based on the chapter has been prepared and will be submitted for publication. Because of the above, repetition of some information in the document could not be avoided.

The candidate, Madu, co-designed the study, performed the experiments, analysed the data and drafted the manuscript.

4.1 ABSTRACT

Cryptococcus (*C.*) *neoformans* is a fungal pathogen that can cause life-threatening cryptococcal meningitis mostly in immunocompromised patients. This fungus has a tropism for the brain and is one of the few pathogens that possess invasion strategies to breach the blood-brain barrier (BBB). Current treatment for the management of this infection has proved difficult partly due to the inability of some drugs to cross the BBB. In this study, an attempt was made to disperse chloroquine (CQ) in D-α-tocopheryl polyethylene glycol succinate (TPGS) to assist CQ to cross an *in vitro* BBB model made up by a monolayer of hCMEC/D3 cells. Next, the effect of CQ-TPGS micelles on the health of hCMEC/D3 was assessed. Lastly, the ability of the reformulated drug to cross the hCMEC/D3 monolayer and kill cryptococcal cells (seeded in the basolateral chamber of a transwell plate) was also assessed. For comparison, an unformulated, standard powder of CQ was included for reference. Here, it was shown that the CQ-TPGS micelles, similarly to CQ, was not toxic to hCMEC/D3 cells, as it did not negatively affect the metabolic activity of hCMEC/D3 cells at used test concentrations. While the CQ-TPGS micelles were able to cross the monolayer of hCMEC/D3 cells, it was not as efficient as the standard CQ, which readily dissolves in water. Despite the inefficiency of CQ-TPGS micelles to cross the monolayer when compared to CQ, it was still able to reach cryptococcal cells in the basolateral chamber and significantly reduce their CFU counts ($p \leq 0.05$) after treating them for 24 h when compared to non-treated cells. Concerning the CQ, it was also able to significantly reduce cryptococcal CFU counts ($p \leq 0.03$) when compared to non-treated cells. Taken together, the results highlight the potential of CQ

and CQ-TPGS micelles as drugs that can cross the brain endothelial cells, without negatively affecting it, to possibly control the growth of cryptococcal cells in the brain.

Keywords: Blood-brain barrier, Chloroquine, *Cryptococcus*, CQ-TPGS micelles, hCMEC/D3 cells.

4.2 INTRODUCTION

Infections of the central nervous system (CNS) are often fatal, and in some cases, can lead to 100% mortality (Rushing and Burns, 2001). It is reported that globally, each year, over 1.2 million people are affected by meningitis (Cain et al., 2019). Meningitis is an inflammatory response in the cerebrospinal fluid (CSF) compartment that can be caused by bacteria, fungi and parasites (Thigpen et al., 2011). Importantly, meningitis requires immediate medical treatment to prevent complications such as altered mental condition ranging from delirium to coma and focal neurologic signs or seizures, which can further lead to death (Salimi et al., 2016). Invasion of the CNS by pathogens can either be through transneuronal or haematogenous routes to gain access to the CNS parenchyma (Cain et al., 2019).

In 1912, the phrase ‘‘blood-brain barrier” (BBB) was used to explain the observation that the developing mammalian brain could exclude dyes that were administered (Battelli and Stern, 1912). It was later shown that the BBB was composed of distinct brain capillary endothelial cells in contrast to other endothelial cells in the body (Daneman and Prat, 2015). These capillary endothelial cells have tight, and adherent junctions that limit paracellular passage that allows for only low permeability of small hydrophilic and lipophilic molecules (Daneman and Prat, 2015; Poller et al., 2008). Because of the latter, some studies have shown that the BBB is responsible for maintaining the neuroparenchyma microenvironment by restricting the entry of pathogens, toxins, buffers variations in blood and brain, immune cells, and molecules into the CNS (Barker and Widner, 2004; Wilson et al., 2010; Wolburg and Lippoldt, 2002).

Fungal invasion of the CNS occurs principally in persons with a defective T-cell response (Engelhardt et al., 2017). The most common cause of fungal meningitis is *C. neoformans*, an encapsulated basidiomycetous yeast (Buchanan and Murphy, 1998). *C. neoformans* can traverse the BBB either as a free yeast or hidden inside an infected macrophage (Casadevall, 2010; Kaufman-Francis et al., 2018; Santiago-Tirado et al., 2017; Sorrell et al., 2016; Voelz and May, 2010). As a yeast, the CNS invasion is possible by the participation of the host's Annexin A_2 and hyaluronic receptor (CD44), as well as pathogen's secreted metalloproteinase (Mpr1) (Jong et al., 2012; Pombejra et al., 2017; Vu et al., 2014).

Unfortunately, with cryptococcal meningitis, the rate of mortality and morbidity is unacceptably high in spite of several strategies for fungal management (Mourad and Perfect, 2018; Truong et al., 2018). These strategies include the administration of amphotericin B and fluconazole (Ghannoum and Rice, 1999). For example, the clinical application of amphotericin B against cryptococcal meningitis is restricted due to its inability to cross the BBB (Saravolatz et al., 2003). In addition, complications of nephrotoxicity limit the use of this drug (Laniado-Laborín and Cabrales-Vargas, 2009). In contrast, fluconazole achieves very high penetration into the CSF. However, it is not an ideal treatment option either due to its fungistatic nature (Felton et al., 2014).

In this study, it was sought to examine the ability of CQ to cross a monolayer of hCMEC/D3 cells that was set up in a transwell plate. While CQ can easily be reconstituted in water (hydrophilic), it was also reformulated by dispersing it in a lipid carrier, D-α-

tocopheryl polyethylene glycol succinate (TPGS; derivative of vitamin E), to prepare a CQ- TPGS micelles. Therefore, the two preparations were directly compared. In addition, it was sought to determine if these preparations could cross an hCMEC/D3 monolayer that was seeded on the insert *i.e*. apical chamber (which represent the blood side) of a transwell plate, and kill cryptococcal cells that were seeded at the bottom of a transwell plate, i.e. basolateral chamber (which represented the brain side).

4.3 MATERIALS AND METHODS

Preparation of drugs

A standard powder of CQ was obtained from Sigma-Aldrich, South Africa. A stock solution of CQ was prepared by dissolving 1 mg in 1 mL of deionised water. For CQ reformulation, a colloidal dispersion method was used. This protocol was modified from Meng et al. (2017). In brief, a 50 mg micellar carrier composed of 10 mg of CQ and 40 mg of TPGS (a kind donation from Prof. P. Kumar, University of Witwatersrand, South Africa) was dissolved in 10 mL ethanol. These were vortex mixed on a magnetic stirrer at 300 rpm for 30 min at room temperature. The prepared solution was transferred to a glass petri-dish, which was left in a fume hood overnight to evaporate the ethanol. The resultant film of polymer re-hydrated in 10 mL of deionised water. The latter was vortex mixed on a magnetic stirrer at 300 rpm for 30 min at room temperature to form a micellar suspension. A standard powder of aspirin was obtained from Sigma-Aldrich. 1 mg of aspirin was dissolved in 1 mL of absolute ethanol to prepare a stock solution.

Cell cultivation and standardisation

C. neoformans LMPE 053 strain that is held as a stock culture at the University of the Free State, South Africa, was used in the study. This fungus was streaked out on a yeast-malt-extract (YM) agar (10 g/L glucose, 3 g/L malt extract, 3 g/L yeast extract, 16 g/L agar, 5 g/L peptone; Merck, South Africa) plate. The YM plate was incubated for 48 h at 30^{o}C to obtain single colonies. A colony, from the 48 h culture, was then streaked out on a fresh YM agar plate and incubated for 24 h at 30^{o}C. Thereafter, five colonies were scooped and suspended in 5 mL of distilled water. The cell numbers were counted using a haemocytometer and the cell concentration was adjusted to reach a concentration of 1 x 10^4 colony forming units (CFUs) per millilitre in 10 mL of RPMI-1640 medium (Sigma-Aldrich, South Africa). The cell suspension was then seeded in the basolateral chamber of a transwell plate with a 12 mm membrane insert and 0.4 µm pore size (BD Falcon, Corning).

An immortalised human cerebral microvascular endothelial cell line (hCMEC/D3; Millipore Sigma, USA) was cultivated in EBM-2 Endothelial Cell Growth Basal Medium (Lonza Rockland, Inc., United States) that was supplemented with FBS (5%), hydrocortisone (1.4 µM), penicillin-streptomycin (1%), HEPES (10 mM), ascorbic acid (5 µg/mL), basic fibroblast growth factor (bFGF) (1 ng/mL), human vascular endothelial growth factor (hVEGF) (2 ng/mL) and chemically defined lipid concentrate (1%) as recommended by Lonza. Prior to seeding, a T75 tissue flask (Nunc, Denmark) was coated with rat tail collagen type 1 solution (Millipore, USA) for 1 h at 37^{o}C to enhance cell adhesion and proliferation. For each experiment, a flask was incubated at 37^{o}C in 5% CO_2 until 90%

confluence was reached. Viability of hCMEC/D3 cells was determined to be 90% using trypan blue (Sigma-Aldrich). Using a haemocytometer, cells were standardised to 1 x 10^5 cells/mL in a 50 mL centrifuge tubes (Becton-Dickinson Labware, United States) containing 10 mL of EBM-2 media The cells were then seeded either in a transparent 96-well microtiter plate (Greiner Bio-One, Germany) or a transwell plate with a 12 mm membrane insert and 0.4 µm pore size (BD Falcon, Corning).

For cultivation in a 96-well plate, a 100 µL cell suspension in wells, containing the standardised hCMEC/D3 (1 x 10^5 cells/mL in EBM-2 media) cells, were incubated overnight at 37^{o}C and 5% CO_2. The next day, the overnight media was first aspirated and replaced with 100 µL of fresh, sterile EMB-2 media.

For cultivation in a transwell plate, a 350 µL cell suspension that contained the standardised hCMEC/D3 cells (1 x 10^5 cells/mL) in EBM-2 media, was seeded on the apical chamber of the transwell. On the basolateral chamber, 700 µL of the EBM-2 media was dispensed. To monitor the development of a confluent monolayer, an inverted microscope (Olympus, Japan) was used. In addition, the transepithelial electrical resistance (TEER; ERS-2 (EMD Millipore, USA)) values were recorded every second day. The culture media in both chambers were aseptically changed every second day. A peak TEER reading of 97 Ω.cm^2 was reached on day 10 and there was no significant increase between day 10 and day 12 (96 Ω.cm^2). The latter suggested the formation of a tight, confluent monolayer (Figure 4).

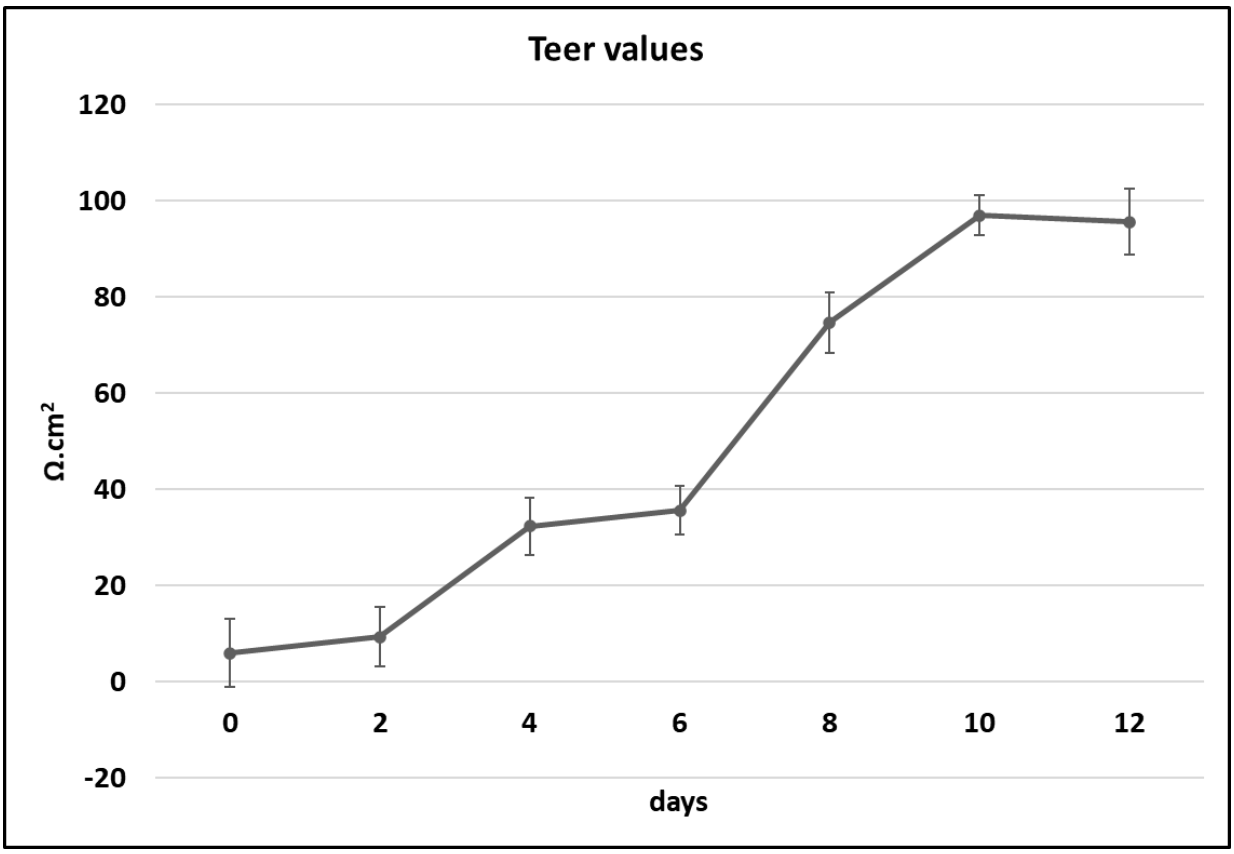

Figure 4. *A plot of TEER reading of hCMEC/D3 monolayer over a period of 12 days. Ω.cm² = ohm per centimetre square*

Cytotoxicity assay: the effects of CQ and CQ-TPGS micelles on hCMEC/D3 cells

This protocol was modified from (Ogundeji et al. (2017) and Meng et al. (2017). To the wells containing seeded hCMEC/D3 cells, a 100 µL solution of either CQ, CQ-TPGS micelles or TPGS was added. CQ was tested at final concentrations of 0 µg/mL (non-treated cells), 26 µg/mL, 52 µg/mL, 129 µg/mL or 258 µg/mL, CQ-TPGS at 156 µg/mL (1 part CQ to 4 parts TPGS), 258 µg/mL (1 part CQ to 4 parts TPGS), 645 µg/mL (1 part CQ to 4 parts TPGS), or 1290 µg/mL (1 part CQ to 4 parts TPGS). TPGS was tested at 1032 µg/mL (4 parts TPGS). The plate was incubated in a 5% CO_2 incubator at 37^{o}C for 24 h. After 24 h, the metabolic activity of the cells was measured using a tretrazolium salt, *i.e.* 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium

hydroxide) (XTT; Sigma-Aldrich), in the presence of menadione (Sigma-Aldrich, South Africa). After initiating the tetrazolium reaction for 3 h in the same humidified incubator, absorbance reading was measured at 492 nm with a plate reader (Biochrom EZ Read 800 Research, United Kingdom). A vehicle (water) control was included and used to normalise the data by subtracting the background.

Drug transport assay across BBB model

The assay was modified from the protocols of Cucullo et al. (2008) and Yang et al. (2019). Before commencement, the 12-day-old monolayer (in the apical chamber) was washed with phosphate-buffered saline (PBS; Sigma-Aldrich, South Africa). Thereafter, to the same apical chamber, 52 µg/mL of CQ in 350 µL of MOPS buffer (3-(N-morpholino) propane sulfonic acid; Sigma-Aldrich) or CQ-TPGS micelles (258 µg/mL in 350 µL of MOPS buffer) were dispensed. A control drug, aspirin (52 µg/mL in 350 µL of MOPS buffer), was also dispensed to the apical chamber. Aspirin has been reported not to efficiently cross the BBB (Kalathil et al., 2016). Moreover, a MOPS buffer control, *i.e.* devoid of CQ, CQ-TPGS or aspirin, was also dispensed to the apical chamber. In a separate experiment, a transwell plate was set up as detailed above; however, it was not overlaid with hCMEC/D3 cells on the apical chamber. All the transwell plates were incubated for 3h at 37^{o}C. After incubation, a 100 µL solution was aspirated from the basolateral chamber and was transferred to corresponding transparent, 96-well microtiter plates. The absorbance of the microtiter plates was read with a spectrophotometer at 340 nm, as a measure to determine the paracellular permeability of the compounds. After the

assay, TEER readings of the monolayer in the transwell plate were taken to ensure the integrity of the monolayer was still intact.

Inhibition of cryptococcal cells using CQ and CQ-TPGS

The protocol was modified from the protocols of Santiago-Tirado et al. (2017) and Vu et al. (2014). The transwell plates with cryptococcal cells were prepared as detailed in 4.3.2 and 4.3.4. To initiate the challenge study, to the apical chamber that was seeded with hCMEC/D3 monolayer, 350 uL of CQ (52 µg/mL) or CQ-TPGS micelles (258 µg/mL) was added. A control experiment was also included wherein the cryptococcal cells were not exposed to CQ or CQ-TPGS. The transwell plate was incubated for 24 h at 37^{o}C in 5% CO_2. After 24 h, the transwell plate was disassembled and all the contents in the basolateral chamber were aspirated and transferred to 1.5 mL plastic tube. A 1 in 10 dilutions was made using distilled water. After this, 100 µL of the dilution dispensed to the centre of a corresponding YM agar plate before being spread to create a confluent lawn. The YM plates were incubated at 30^{o}C for 48 h. After incubation, recovered colonies were counted.

Statistical analyses

For each study, three independent experiments were performed. GraphPad Prism 8.3.1 was used to calculate mean values and the standard deviation of the means. The same programme was used to perform the multiple comparison test using Tukey as an option. A *p* value of less than or equal to 0.05 was considered significant. To this end, a bar that has a different alphabet to the other implies there is a significance difference while those

with the same alphabet are not significantly different. The data were also tested for normality of distribution using the Shapiro-Wilk test with alpha value of 0.05. Thus, a *p* value less than 0.05 indicated abnormal distribution of the data points.

4.4 RESULTS

The effect of CQ and CQ-TPGS on hCMEC/D3 metabolic activity

Figure 1 summarises the response of hCMEC/D3 cells to CQ, CQ-TPGS micelles and TPGS-alone. When CQ was tested at 26 or 52 µg/mL and CQ-TPGS micelles at 156 or 258 µg/mL, no reduction in the metabolic activity was observed, when compared to the non-treated cells (0 µg/mL). Rather at these concentrations, the metabolic activity seemed to have been significantly enhanced. To the point, there was a 10% increase at 26 µg/mL of CQ ($p \leq 0.03$) and 25% at 52 µg/mL of CQ ($p \leq 0.02$) in the metabolic activity. And with CQ-TPGS micelles, there was a 42% increase at 156 µg/mL ($p \leq 0.02$) and 57% at 258 µg/mL ($p \leq 0.02$) in the metabolic activity. However, at higher concentrations of CQ (129 or 528 µg/mL) and CQ-TPGS micelles (645 or 1290 µg/mL), there was a significant reduction in the metabolic activity of the cells ($p \leq 0.02$). The observed reduction in the metabolic activity is due to the action of CQ and not TPGS in the micelles – as the usage of TPGS-alone at a high concentration, *i.e.* 1032 µg/mL, promoted the metabolic activity of tested cells by 98% when compared to the control cells ($p \leq 0.001$).

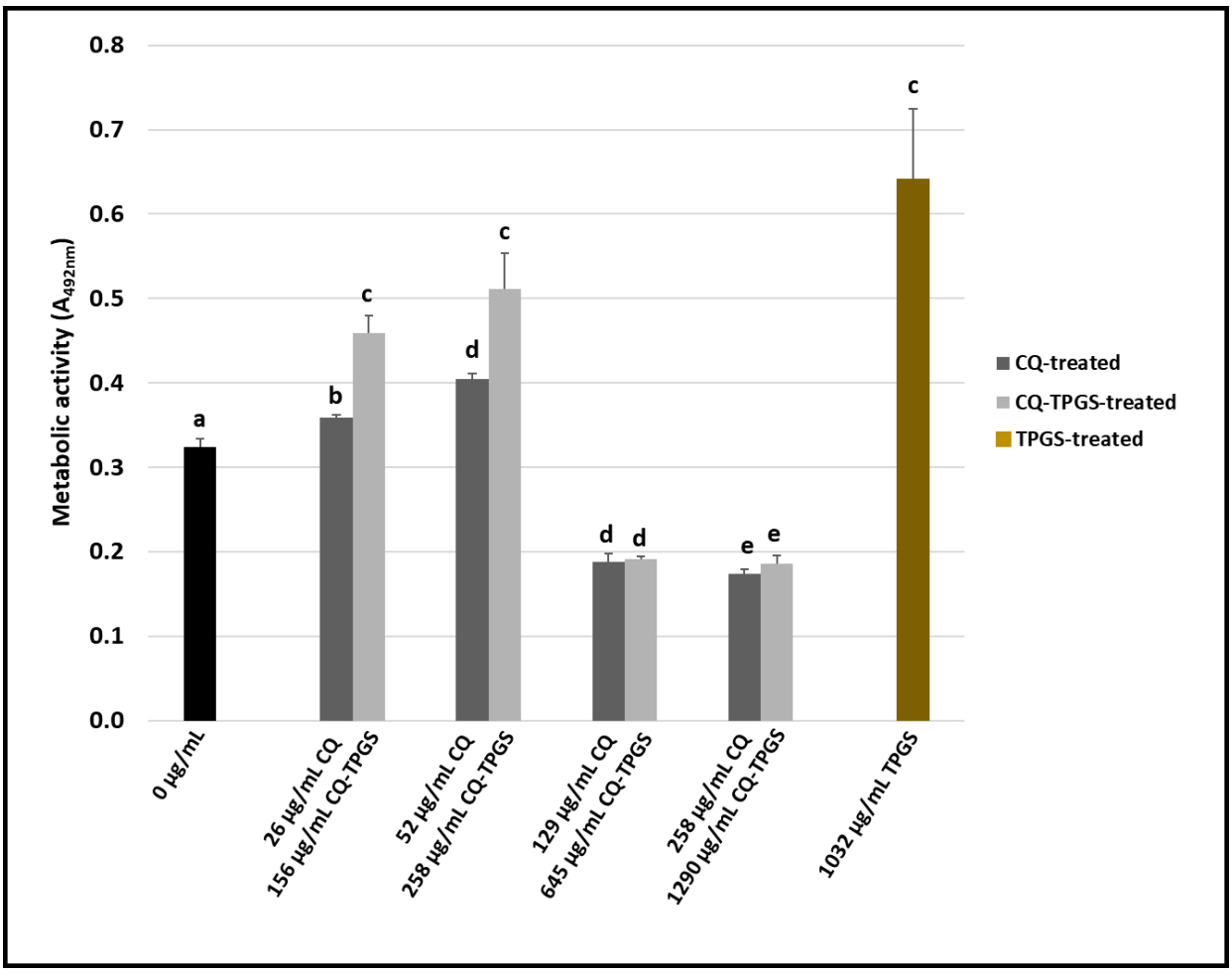

Figure 1. *Results shows the response of hCMEC/D3 cells to CQ, CQ-TPGS micelles and TPGS-alone. CQ and CQ-TPGS micelles at lower concentration as well as high concentration of TPGS led to significantly enhanced metabolic activity of hCMEC/D3 when compared to the non-treated cells. However, higher concentrations showed a significant reduction in their metabolic activity. A = absorbance; nm = nanometre; CQ = chloroquine; CQ-TPGS = chloroquine D-α-tocopheryl polyethylene glycol succinate*

When directly comparing CQ to CQ-TPGS micelles, in all the above cases, it is clear that the overall response profile of hCMEC/D3 cells to CQ was mirrored when they were exposed to CQ-TPGS micelles. However, there were some differences. For example, the CQ-TPGS micelles at 156 µg/mL or 258 µg/mL seemed to significantly improve ($p \leq 0.02$) the metabolic activity of hCMEC/D3 cells by additional 32% and 32% respectively compared to CQ at 26 µg/mL or 52 µg/mL. Nonetheless, when comparing the reduction

in the metabolic activity of the two compounds, CQ-TPGS micelles at 645 µg/mL or 1290 µg/mL appear to have a less detrimental effect on the cells with 1% and 3% less respectively compared to CQ at 129 µg/mL or 258 µg/mL, although, there were no significant differences ($p \geq 0.5$).

CQ-TPGS micelles can cross the model BBB monolayer

The bioavailability results of CQ, CQ-TPGS micelles and aspirin in the basolateral chamber are shown in Figure 2. In the absence of the hCMEC/D3 monolayer of cells lining the apical chamber, all three compounds could make their way to the basolateral chamber to varying degrees. However, there were no significant differences in their bioavailability ($p \geq 0.05$). The lining of the apical chamber with a monolayer of hCMEC/D3 induced immediate changes. For example, the control drug, aspirin, when compared to its control, only 8% of it could cross the monolayer model to reach the basolateral chamber ($p \leq 0.02$). This finding is in line with the literature, *i.e.* that aspirin poorly crosses the BBB (Kalathil et al., 2016). Moreover, when its permeability was compared to CQ or CQ-TPGS, the absorbance reading was significantly lower ($p \leq 0.05$). When considering CQ and CQ-TPGS micelles, and comparing them to their respective controls, 52% of the CQ ($p \leq 0.05$) crossed the monolayer model while 38% of the CQ-TPGS micelles ($p \leq 0.05$) crossed the monolayer model to reach the basolateral chamber. When comparing the bioavailability of CQ and CQ-TPGS micelles in the basolateral chamber, there was no significant difference ($p \geq 0.05$).

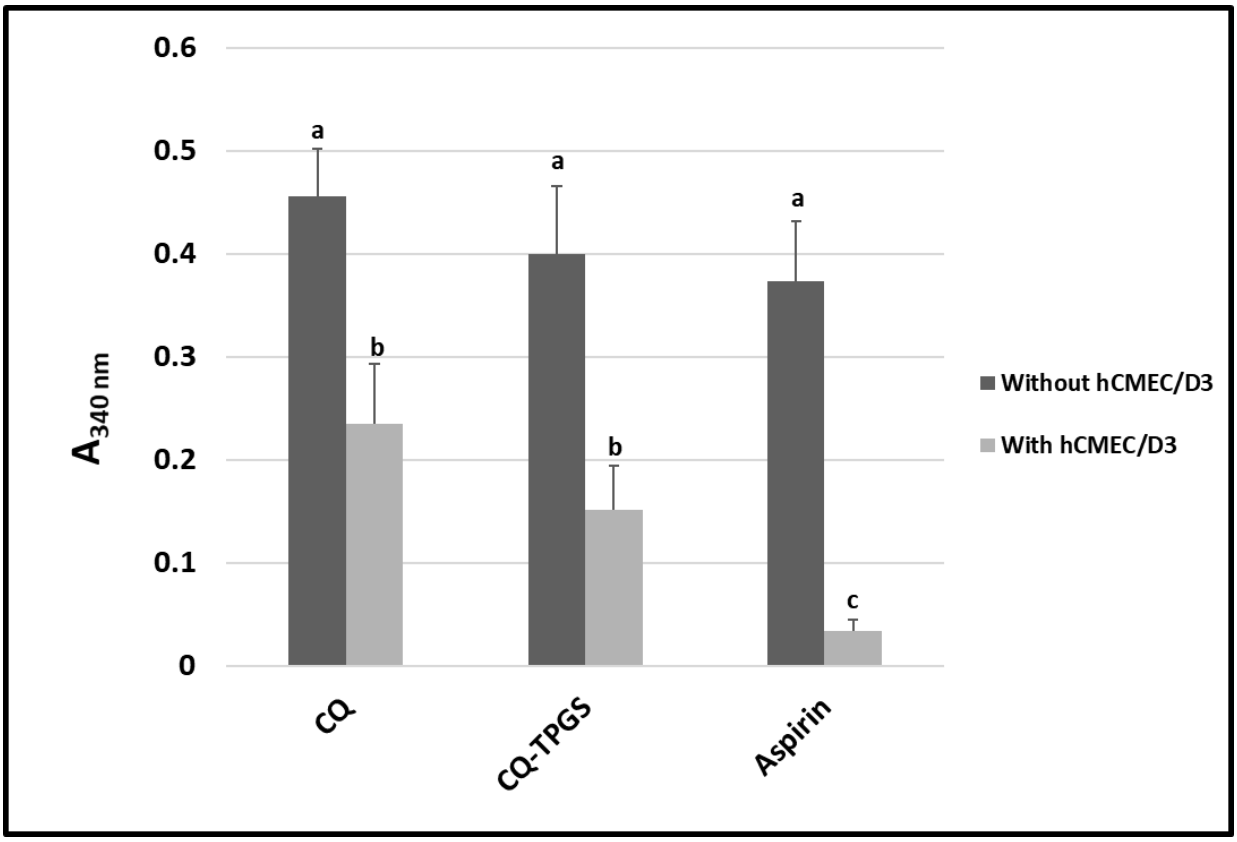

Figure 2. *Summarises the bioavailability results of CQ, CQ-TPGS micelles and aspirin in the presence and absence of hCMEC/D3 monolayer. Absence of hCMEC/D3 monolayer increased the permeability of the three drugs across the membrane insert. Nevertheless, the presence of hCMEC/D3 monolayer restricted their crossing significantly compared to their respective controls with aspirin having the most significant restriction compared to CQ and CQ-TPGS. A = absorbance; nm = nanometre; CQ = chloroquine; CQ-TPGS = chloroquine D-α-tocopheryl polyethylene glycol succinate*.

Moreover, the TEER readings before commencement (96 $\Omega.cm^2$) and after (94 $\Omega.cm^2$) the experiments were relatively close, implying that the monolayer remained tightly packed throughout the experiment. Thus, the obtained bioavailability results speak to the chemical quality of the CQ (hydrophilic) and when reformulated (hydrophilic and lipophilic). More importantly, it seems the reformulation using TPGS did not enhance the ability of CQ to cross the monolayer.

CQ-TPGS micelles can cross the model BBB monolayer to inhibit cryptococcal cells

Figure 3 summarises the effect of CQ and CQ-TPGS micelles on cryptococcal cells that were seeded in the basolateral chamber.

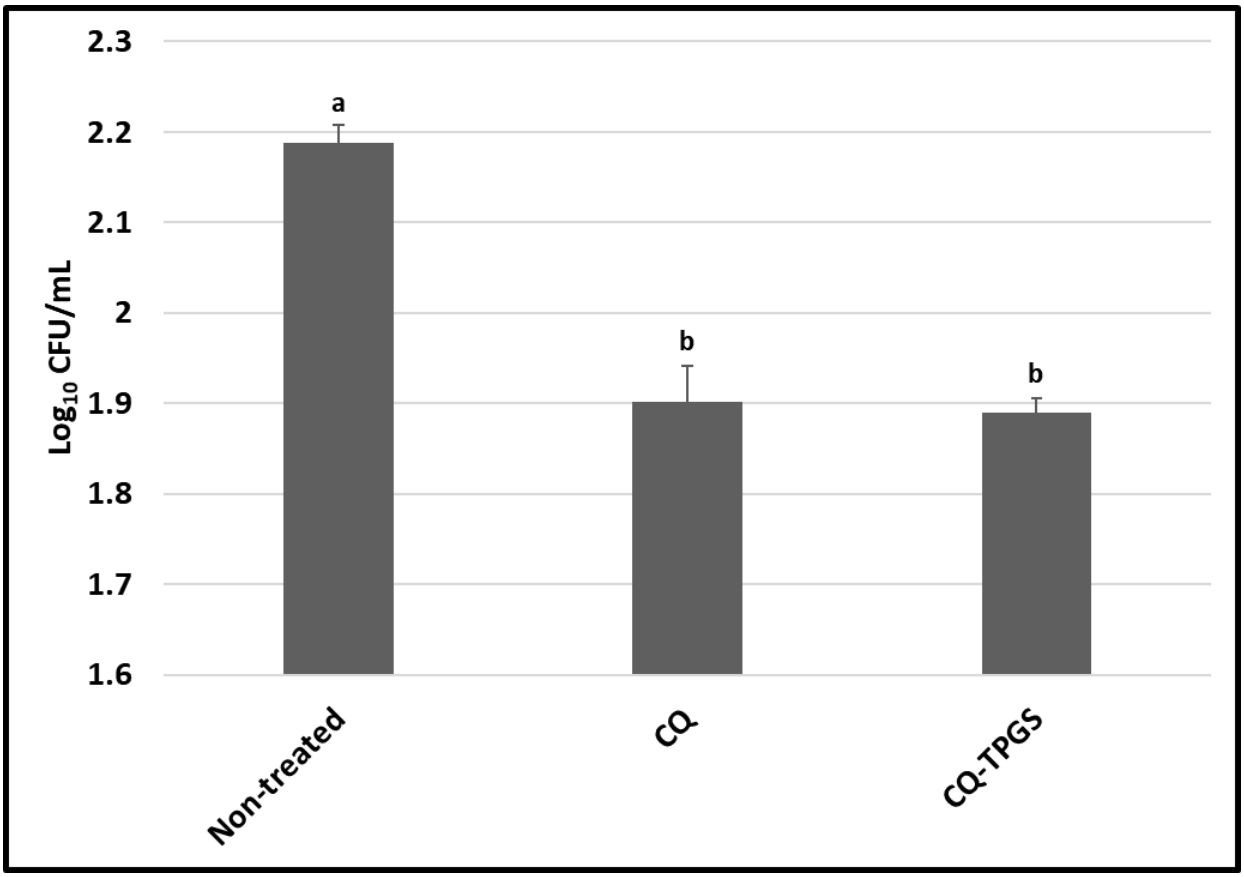

Figure 3. *Effect of CQ and CQ-TPGS micelles on cryptococcal cells seeded in the basolateral chamber of BBB model. CQ and CQ-TPGS micelles significantly inhibited the growth of cryptococcal cells with CQ having a more inhibition compared to CQ-TPGS even though it was not significant. CQ = chloroquine; CQ-TPGS = chloroquine D-α-tocopheryl polyethylene glycol succinate; CFU/mL = colony forming unit per millilitre.*

In line with Figure 2's results, it is reasonable to assume that the two drugs could reach the seeded cryptococcal cells in the basolateral chamber. When comparing the CFU counts of cells not exposed to either drug (non-treated cells; 0 µg/mL), it was clear that drug exposure significantly reduced the CFU counts when the cells were exposed to

either CQ (51% ($p \leq 0.03$)) or CQ-TPGS micelles (48% ($p \leq 0.05$)). Importantly, there was no significant difference ($p \geq 0.7$) in the CFU counts obtained when directly comparing CQ to CQ-TPGS micelles.

4.5 DISCUSSION

In this study, an *in vitro* system that imitates the BBB was developed using transwell plates that were overlaid with a monolayer of hCMEC/D3 endothelial cell line. hCMEC/D3 cells are widely known to yield low TEER values compared to other immortalised cell lines; they are still able to retain the most critical functional attributes of primary brain endothelial cells. For example, they can express the requisite tight junction proteins, i.e. occludin 1 and claudin 7 (Vu et al., 2009; Weksler et al., 2005). Therefore, even though the above model cannot completely replicate the *in vivo* BBB, the model has been used successfully to study drug transport across the BBB (Ahmed et al., 2019; Berezowski et al., 2004; Cucullo et al., 2008; Meng et al., 2017).

CQ is an antimalarial drug that has widely been used beyond its scope, including in the treatment of glioma cancer by enhancing the cytotoxic effects of temozolomide which is a brain condition (Golden et al., 2014; Hori et al., 2015). Importantly, CQ has been reported to be well-tolerated by patients (Weyerhäuser et al., 2018). Thus, it was not surprising to note that the drug, and when reformulated, did not negatively affect the metabolic activity of the model endothelial cells. Given the chemistry of TPGS, lipophilic and water-soluble (Guo et al., 2013; Muthu et al., 2012), it was theorised it would enhance

the transport of CQ across the artificial endothelium barrier (Ahmed et al., 2019; Guo et al., 2013; Meng et al., 2017). However, this was not the case in this study. To enhance the transport of CQ, perhaps a better nano-carrier can be considered.

Despite the above challenge, the obtained data shows that nonetheless, CQ-TPGS micelles were able to cross the barrier and reach the basolateral chamber to kill the cryptococcal cells. It is possible that CQ may have killed the cryptococcal cells by oxidative inactivation. It is important to point out that the clinical application of anti-malarial such as CQ may be limited by a dose-dependent haemolysis that can be experienced in persons with the glucose 6-phosphate dehydrogenase deficiency (Peters and van Noorden, 2009) or in patients with underlying heart conditions as seen in covid-19 clinical trials (unpublished data).

In conclusion, the high fatality rate caused by cryptococcal meningitis, particularly in sub-Saharan African, cannot only be attributed to the infection itself but also the lack of effective treatment options available. It is now of interest to determine the effectiveness of CQ-TPGS micelles in controlling the growth of this neuropathic pathogen in the brain of laboratory animals relative to CQ-alone.

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CHAPTER 5

GENERAL CONCLUSION AND PERSPECTIVES

There is a significantly low number of patents in the field of drug development for the treatment of cryptococcosis, indicating that efforts to alleviate this unmet needs remain insufficient. Without sufficient fundings to drive innovations and research, patients will continue to face the challenge of inadequate assistance. Given the existing situation characterised by scarce fundings and little interest for technological advancement, drug repurposing may be the immediate greatest alternative for cryptococcosis treatment. Additionally, the repurposing of existing drugs with new indications should be encouraged in pharmaceutical companies as therapeutics for fungal diseases, that are urgent demand for global health.

In the last decade, significant progress has been achieved to the development of repurposed drugs for the control of fungal infections. A number of compounds have produced promising data but the developmental efforts are stuck in the preclinical stage. In the preclinical development of repurposing drugs for fungal treatment, the additional relevant issues that should be taken into consideration is the need to increase their bioavailability to the central nervous system.

The high cost and prolonged timeline of new drug discovery and development are significant roadblocks to creating therapies for infectious diseases. In this study, chloroquine (CQ) and primaquine (PQ) were screened to control the growth of *Cryptococcus* using several approaches. These included susceptibility testing, photodynamics therapy (PDT) and a susceptibility testing that included a model blood-brain barrier (BBB) set up. The results provide the survey of the inhibition of

cryptococcal growth by existing drugs CQ and PQ. From a drug repurposing point of view, the drugs chosen in the thesis showed *in vitro* potential as anti-*Cryptococcus* drugs. However, there were some limitations. For example, some conclusions were drawn based on the results of a limited number of *C. neoformans* strains. Given that susceptibility assay was carried out on both *C. neoformans* and *C. gattii*, it would have been ideal to include *C. gattii* throughout the thesis. Moreover, more strains should have been considered. This would assist to better define the statistical significance of the data - for appropirate conclusions to be drawn on both isolates. Another area of this thesis to consider is the use of a liver cell line – as it is a more acceptable option for toxicity study instead of a macrophage cell line.

Transwell culture setup was utilised to mimic the BBB. This system is ideal and generally accepted for drug transport study due to its low-cost and straightforward fabrication among other advantages. However, there are considerable limitations to this system that should be taken into account. For instance, the absence of a three-dimensional structure present *in vivo* and the absence of endothelial exposure to physiological shear stress that regulates the differentiation of the endothelial cells into a BBB phenotype. The above drawbacks may reduce the reliability of their assumptive value for human responses. Over time, advanced understanding of the processes regulating barrier functions has provided the basis for biotechnological advancements. This has birthed the development of more realistic and sophisticated *in vitro* BBB models such as 3D printing technology which is now increasingly used among researchers. This technology allows for the rapid *in vitro* reconstruction of

intrinsic structures and components of the targetted tissue with high precision. In chapter four, it may have also been prudent to determine the levels of metalloproteinase in *Cryptococococcus* after exposure to the drugs. This is an enzyme that has been shown to help this pathogen across the BBB effectively. Thus, an experiment that explores impairment of this enzyme (production or activity) would be of great value.

Taken together, the thesis provides a foundation for successive work targeted at optimising the antifungal properties of CQ and PQ. This may lead to future research that may directly impact patient outcomes.