The repurposing of chemical compounds as anti-*Cryptococcus* drugs

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This thesis is lovingly dedicated to my beloved husband; **Dr Abiodun Ogundeji**, and my precious children; **Oluwademilade** and **Oluwatomisin.** Thank you for everything.

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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. The successful completion of the thesis has been made possible by a joint research grant from the National Research Foundation and the University of the Free State. There are no competing financial interests.

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PAGE NUMBER

NB: The thesis consists of different chapters in manuscript format. As a result, repetition of some information could not be avoided.

CHAPTER 1:

LITERATURE REVIEW

CHAPTER 2:

THE REPURPOSING OF ASPIRIN AND IBUPROFEN AS CANDIDATE ANTI-*CRYPTOCOCCUS* DRUGS

CHAPTER 3:

COPPER ACYL SALICYLATE AS A POTENTIAL

ANTI- CRYPTOCOCCUS DRUG

CHAPTER 4:

THE REPURPOSING OF ANTI-PSYCHOTIC DRUGS,

QUETIAPINE AND OLANZAPINE AS ANTI-CRYPTOCOCCUS DRUGS

CHAPTER 1

LITERATURE REVIEW

This review has been published.

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1.1 MOTIVATION

Immunosuppressive conditions, as a result of either HIV-infection or unintended consequences of using anti-rejection drugs in organ transplant patients, have led to an increase in host vulnerability to fungal diseases. These conditions create optimal environments for opportunistic infections to occur, and thus it is not coincidental that previously non-pathogenic fungal species have become new mycotic agents. The emergence of these new mycotic agents in clinical settings is also in parallel to a rise in drug resistance, which is an inherent trait in natural selection. Therefore, it is a challenge to keep up with these agents (some of which may initiate a mode of infection that is not targeted by current antifungal drugs) given the time for development and approval period required to have a new drug on the market. And to compound this, the usage of conventional drugs such as azoles and polyenes is often times characterised by clinical failure, particularly in persons who are immunocompromised.

Thus a solution to the above could be the repurposing of existing chemical compounds (as approved by the United State Food and Drug Administration) as “new” antimicrobial agents, whose previous mechanisms of action were thought to be negative. To date, reports have emerged documenting the antifungal activity of non-traditional antimicrobial drugs, or where they enhance the activity of other traditional antimicrobial drugs. This dissertation will therefore explore literature concerning the application of non-traditional antimicrobial drugs such as the prototypical anti-inflammatory drug acetylsalicylic acid, and other compounds in controlling human fungal diseases.

1.2 INTRODUCTION

Fungi are essential for sustaining human life i.e. in their application in the preparation of food or as sources of pharmacological agents (1-4). Paradoxically, this group of microorganisms also acts as agents that mediate infectious processes that may end life in susceptible hosts. Over the years, yeasts, and in particular, species belonging to *Cryptococcus* and *Candida* genera have emerged as important disease-causing fungi (5-7). As a result, these microbes have been extensively studied, often with an attempt to understanding their biology in the context of controlling infections (8-10). Such intimate insight into their cellular pathology and physiology may reveal strategies for identifying potential drug targets.

Fungi can cause fatal diseases in individuals with apparently well-functioning immune systems, a case in point being *Cryptococcus gattii* (11, 12). Therefore, it is not surprising that the rise in the number of people with immunosuppressive conditions, primarily due to HIV, has given infectious diseases such as cryptococcosis and candidiasis a “new” lease on life. To illustrate this point, it is estimated that well over 1 million cases of cryptococcosis (considered to be an AIDS-defining illness) are reported annually (13). In addition, 10% of persons living in Asia, Africa and Latin America who are infected with HIV have oral candidiasis (14).

While there has been an interest in fungal patho-biology for more than a century, there have been many more recent studies that sought to demonstrate the usefulness of a number of chemical compounds in controlling fungal growth. The latter is primarily

informed by a number of limitations concerning the clinical usage of conventional drugs such as polyenes and azoles (which act on eukaryotic-specific drug targets and not fungi-specific drug targets) in managing fungal infections (15). These limitations include 1) a narrow therapeutic index, 2) side-effects, 3) clinical failure, more so, in immunocompromised individuals, and 4) development of drug resistance - due to either the widespread usage of drugs or poor drug-compliance by patients (14, 15). Hence, the constant need to find other compounds that may possess antifungal qualities. An interesting development in this field has been the rise in the number of texts exploring non-traditional antimicrobial agents. Importantly, some of these studies aligned their protocols to standard *in vitro* susceptibility reference methods (Clinical Laboratory Standards Institute or European Committee on Antimicrobial Susceptibility Testing). And as such, results obtained from these studies have given the scientific community a sense of the efficacy of the tested compounds, which is important in improving patient outcomes.

Further to the discussion, some of the compounds that have been tested include anti-inflammatory drugs (16), which are relatively cheap and are widely distributed across the world. It is unarguable that management of fungal infectious diseases is strongly dependent on capital resources available to a specific region (17). In a region like Sub-Saharan Africa, with its uniquely complex geo-political and socio-economic challenges, it has proven difficult for governments to provide the necessary life-saving drugs, which are often expensive. Corollary, incidences of disseminated cryptococcal diseases, for an example, have remained a major cause of morbidity and mortality

compared to developed countries (13). It is therefore pleasing to note that multi-national companies, through their drug donation programmes, have made medicines such as fluconazole readily available (18, 19). This, in conjunction with the expanded antiretroviral programme in countries like South Africa, has greatly assisted in managing fungal infections among HIV-infected populations and general public. This gesture has unfortunately also lead to unintended consequences, where such drugs can be used widely as curative agents leading to development of drug resistance. To overcome these challenges, it is time that existing chemical compounds (especially those that are cheap and widely distributed) with demonstrable antimicrobial activity and therapeutic benefit be considered, particularly in these parts of the world.

Therefore, in this text, we explore the current literature and make a case for non-traditional antimicrobial drugs, such as acetylsalicylic acid, as possible alternative antifungal drugs. The reader is also referred to excellent texts detailing the current treatment guidelines for the management of cryptococcosis (17) and candidiasis (20) as the discussion in the following sub-sections is mainly dedicated to anti-inflammatory compounds like acetylsalicylic acid. We also give the necessary attention to other compounds such as other non-steroidal anti-inflammatory drugs, natural products (plant extracts and lipids) and others in controlling human fungal diseases. Finally, in sum, we also explore a few high throughput assays were among others, small molecules, have been screened for their potential antifungal qualities.

1.3 A NEW CAREER FOR ACETYLSALICYLIC ACID AS AN ANTIFUNGAL

Definition, structure and function. Anti-inflammatory drugs, and in particular non-steroidal anti-inflammatory drugs, are a group of chemical compounds that share similar mechanisms of action (21, 22). The prototypical anti-inflammatory drug acetylsalicylic acid was discovered by chance when plant extracts were successfully employed to, among others, alleviate pain (22, 23). Since this discovery, advances in chemistry have allowed for mass production of this compound from as early as the late 1800s. The actual mode of action of this drug remained elusive until the 1970s when John Vane demonstrated that acetylsalicylic acid inhibited prostaglandin production (24). The discovery of two targeted enzymes, cyclooxygenase (COX) 1 and 2, whose products regulate important physiological and pathological processes, and the establishment that their selective inhibition could yield a specific desired outcome (i.e. control of inflammation) has led to the development of many other novel anti-inflammatory drugs, including indomethacin and ibuprofen as a few examples (Fig. 1).

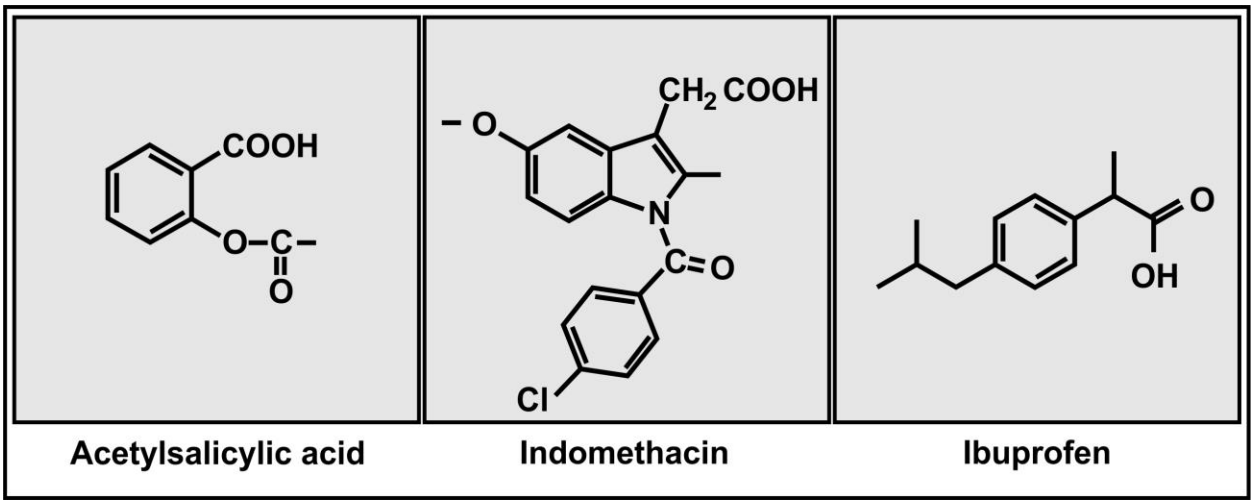


FIG 1 The schematic representation of some chemical compounds representing anti-inflammatory drugs.

While anti-inflammatory drugs have successfully been used to treat conditions such as rheumatic diseases, like any other drug they have shortcomings. For instance, one major shortcoming is gastrointestinal toxicity (22). As a result, there are efforts to design more effective anti-inflammatory drugs with little to no side effects. More encouraging is that further investigations in these compounds have implicated new mechanisms of action, which have since shed light upon additional beneficial applications (i.e. antimicrobial activity).

The discovery of antifungal activity of acetylsalicylic acid. Just over 20 years ago, the Kock group initiated bioprospecting studies aimed at testing for the presence of prostaglandins in yeasts (25). This led to a chance discovery of a mitochondrial-produced, acetylsalicylic acid-sensitive compound named 3-hydroxy eicosatetraenoic

acid, which is characterised by the hydroxyl group on the beta carbon from the carboxylic end (26, 27). Interestingly, this molecule is structurally similar to arachidonic acid (Fig. 2), which is a precursor for prostaglandins in mammalian cells. In elegant biochemical studies, Ciccoli *et al*. demonstrated that this 3-hydroxy molecule could mimic arachidonic acid and serve as a substrate to mammalian COX enzymes leading to production of prostaglandins (28).

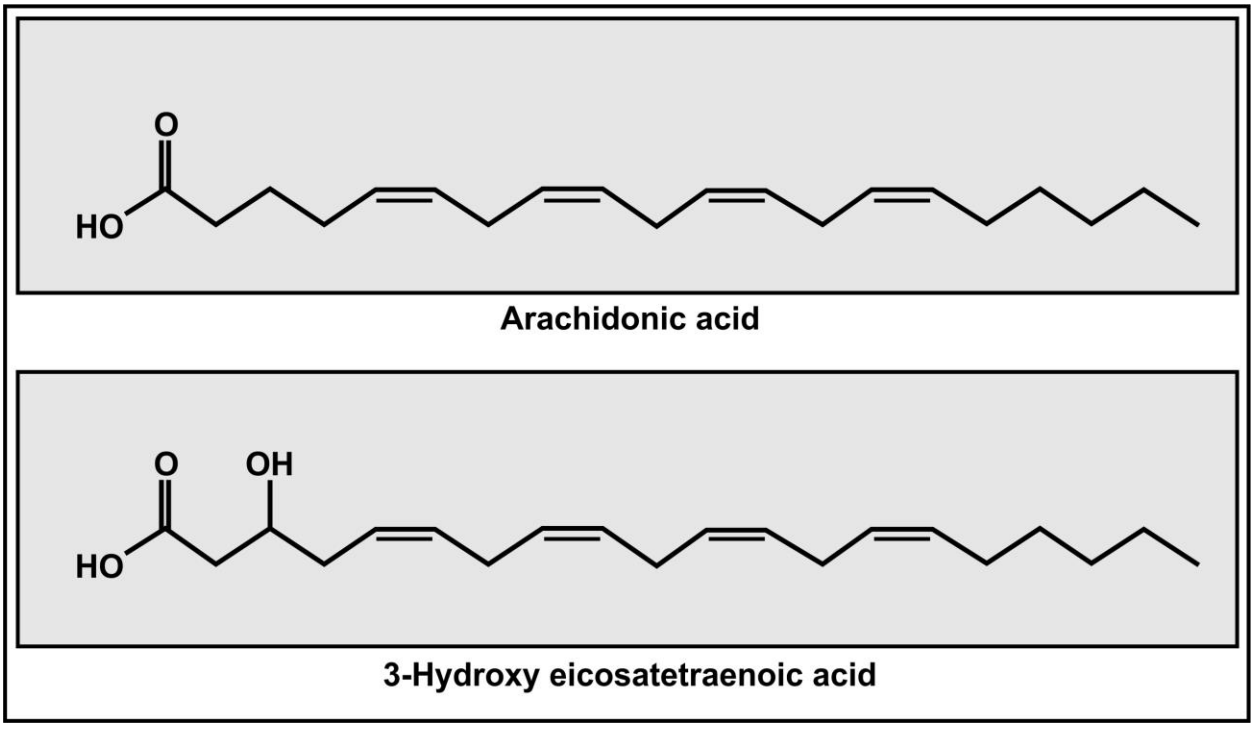


FIG 2 Chemical structures of arachidonic acid and 3-hydroxy eicosatetraenoic acid (with a hydroxyl group on the beta carbon). Although 3-hydroxy eicosatetraenoic acid is derived from fungal cells, this acetylsalicylic acid-sensitive compound serves as a substrate to mammalian cyclooxygenase enzyme leading to production of prostaglandins (28).

In yeast biology, this 3-hydroxy molecule is important for development, and in particular this molecule has been documented to be intimately involved in yeast sexual phases (29, 30). The effects of acetylsalicylic acid were at the time speculatively attributed to diminishing mitochondrial function, which was previously said to be important during the sexual phase of yeasts (31, 32).

This assertion was validated with additional biochemical studies, demonstrating the effects of acetylsalicylic acid on mitochondria. In 1999, Glasgow and co-workers showed that the active component of acetylsalicylic acid, salicylate, inhibits mitochondrial beta-oxidation (33), which is the proposed biosynthetic pathway for 3-hydroxy acids (34). In particular, this is ascribed to acetylsalicylic acid metabolites having structural similarities to the acyl-portions of the substrate and product of the 3-hydroxyacyl-CoA dehydrogenase activity of the beta-oxidation pathway. In addition, acetylsalicylic acid was shown to inhibit mitochondrial activity by uncoupling mitochondrial oxidative phosphorylation and/or inhibiting electron transport (35, 36). A recent study also showed that acetylsalicylic acid induced microbial apoptosis via accumulation of reactive oxygen species in mitochondria and cytoplasm as well as oxidation of mitochondrial NAD(P)H (37). Thus it is not surprising that the yeast sexual cycle (which is dependent on active mitochondria) is blocked by acetylsalicylic acid. This insight into the biology of yeast 3-hydroxy molecules and the effects of acetylsalicylic acid led to a hypothesis that yeast with a mitochondrion-dependent strict aerobic metabolism will be more sensitive to acetylsalicylic acid than those that can also produce energy through an alternative anaerobic glycolytic fermentative pathway in

which mitochondria are not involved (30). The hypothesis set a stage for screening microbes that would fit into the model.

Cryptococcus species are non-fermentative (38), requiring functional mitochondria to sustain growth and other energy-driven cellular processes (39). Mitochondria are reported to produce approximately 95 % of the cell's ATP (40). The high dependency of *Cryptococcus* on mitochondrial-derived ATP for survival highlights these organelles as susceptible targets for potential pharmacological intervention. To this end, a study assessing the *in vitro* susceptibility of a number of *Cryptococcus* species towards acetylsalicylic acid revealed a dose-dependent response - with growth inhibition being noted at concentrations as low as 1 mmol/mL (41).

The recommended concentrations of acetylsalicylic acid in the blood for the treatment of a number of physiological conditions in humans ranges from 20 to 30 mg/dL, which corresponds to approximately 1 to 1.5 mmol/mL (Table 1) (42). The growth of some of the *Cryptococcus* species tested was slightly inhibited at this concentration. A clear inhibition of growth of all *Cryptococcus* species tested was seen at 3 mmol/mL only, which would lead to deleterious effect in humans (42). However, this highlights the potential practical implementation of using acetylsalicylic acid in the treatment of cryptococcal infections, where acetylsalicylic acid would be used at physiological concentration in combined therapy with conventional antifungal to enhance their efficacy.

Table 1. Acetylsalicylic acid concentration range generally accepted as optimum for safe and effective therapy in the blood (42).

Physiological condition / Indication	Therapeutic dosage
Arthritis	20 mg/dL to 30 mg/dL
Rheumatic fever	20 mg/dL to 30 mg/dL
Antifungal activity against *Cryptococcus* species	18 mg/dL (approx. 1 mmol/mL)

In addition to the above, formation of cryptococcal capsules is influenced by acetylsalicylic acid treatment (43). This ability of salicylates to reduce capsule formation is a phenotypic consequence that has also been reported elsewhere in the literature (44). These authors reported that the salicylate derivative sodium salicylate reduced encapsulation in the bacterium *Klebsiella pneumonia*. This capsule chelating property of sodium salicylate could be partially reversed by addition of calcium and magnesium (44).

In order to test the wider effects of acetylsalicylic acid, another study assessing acetylsalicylic acid susceptibility among fungi was conducted (45). In brief, the tested species were categorised based on their ability to ferment. Thus some species from *Eremothecium, Ascoidea, Cryptococcus, Lipomyces, Rhodotorula, Dipodascus,* and *Dipodascopsis* constituted the fermentation negative grouping and fermentation positive grouping was made up of some species from *Candida, Pichia, Kluyveromyces, Saccharomyces, Schizosaccahromyces* and *Zygosaccharomyces*. The results of this

study revealed that indeed organisms with a strict-mitochondrion-dependent aerobic metabolism (*Eremothecium, Ascoidea, Cryptococcus, Lipomyces, Rhodotorula, Dipodascus, Dipodascopsis*) were more susceptible to acetylsalicylic acid (MIC = 1mmol/mL) compared to those that can ferment. All species with fermentation capability were shown to be resistant to acetylsalicylic acid.

The effects of acetylsalicylic acid are not limited to planktonic cells but also extend to fungal biofilms. These structured microbial communities that are linked by a matrix of exopolymeric material are of clinical significance as some community members can evade host immuno-processing and display drug resistance properties (46). Alem and Douglas (2004) reported on the ability of acetylsalicylic acid to inhibit *Candida* biofilms at concentrations as low as 1 mmol/mL. These authors further implicated acetylsalicylic acid as having the ability to exert a dual function by inhibiting prostaglandin production, which are molecules that are important for promoting development into the pathogenic hyphal stage (16). A synergistic and an indifferent outcome were reported when acetylsalicylic acid was applied in combined therapy with amphotericin B in an *in vitro* assay against *Candida* planktonic cells and biofilms (47). The latter is of clinically significance as these drugs, by acting on multiple target sites, may counter the development of drug resistance. Another derivative of salicylate, sodium salicylate, was shown to act in synergy with fluconazole (fractional inhibitory index, < 0.5) against *Candida albicans* planktonic cells (48). The findings by Scott *et al*. are encouraging as azoles are usually the first-line of drugs administered and also the ones against which resistance is more often encountered.

Possible role of anti-inflammatory antifungal drugs in controlling pathogen-induced immune reconstitution inflammatory syndrome. Another important ancillary benefit of acetylsalicylic acid, in addition to antimicrobial activity, could be the control of microbial-induced inflammatory conditions. It is well established that certain pathologies can lead to an out-of-control production of pro-inflammatory cytokines, which may overcome susceptible hosts (49, 50). The latter can be observed in the case of cryptococcal-immune reconstitution inflammatory syndrome, where, for example, the host immune system recognises pathogen antigens that were never cleared, in part, due to the fungistatic nature of drugs such as fluconazole.

Is it possible, based on the discussion above, to consider administering anti-inflammatory drugs in combined therapy with antiretroviral drugs, to restrain the out-of control inflammatory response while at the same time clearing fungal cells? On the face of this, the argument seems logical. However, the human response is always dynamic and may produce an undesired expression to such a set up. Nonetheless, these newly discovered functionalities of anti-inflammatory drugs provide a premise for new studies in the quest to improve patient outcomes.

1.4 OTHER NON-TRADITIONAL ANTIMICROBIAL AGENTS

Other non-steroidal anti-inflammatory drugs. The clinical application of non-steroidal anti-inflammatory drugs is mainly limited by their undesired side effects, namely peptic ulcers. Nonetheless, most of the anti-inflammatory drugs currently on the market were

developed with the sole aim of finding a new drug that would have fewer side effects compared to acetylsalicylic acid (22, 23). Similarly to acetylsalicylic acid, novel mechanisms of action have since been ascribed to these drugs, and thus they have found “new” careers.

Among the first reports detailing the antifungal activity of ibuprofen was a 1993 study by Sanyal and co-workers (51). In their study, these authors note that ibuprofen could be employed as an antimicrobial agent against dermatophytes, which are a group of fungi that colonise the skin due to their dependence on keratin (52). In this report, ibuprofen was shown to significantly inhibit dermatophyte growth at MIC ranging from 5 to 40 µg/mL. Based on the above, it is perhaps not surprising that these molecules (acetylsalicylic acid and ibuprofen) have been included as constituents of topical creams, where they may have an ancillary benefit of controlling skin microbiota.

In 2000, Pina Vaz and co-authors offered the first possible mode of action for ibuprofen in yeasts (53). These authors were able to show that ibuprofen achieved cell death via cell lysis. To be specific, in addition to using imaging technology, they demonstrated that ibuprofen targeted the cytoplasmic membrane causing cell-architectural structure damage that was associated with subsequent intracellular leakage. This study further demonstrated that the effects of ibuprofen are dose-dependent, where at a high concentration (10 mg/mL) *Candida* cells were killed, whereas at a low concentration, 5 mg/mL, the drug was fungistatic. Ibuprofen has also been shown to act in synergy with fluconazole when tested in combined therapy against

Candida albicans (fractional inhibitory index, < 0.5) (48). The latter is of clinically significance as these drugs, by acting on multiple target sides, may counter the development of drug resistance.

Other reports have shown that non-steroidal anti-inflammatory drugs possess antifungal activity. The list extends to, among others, diclofenac, indomethacin, piroxicam, celecoxib, etodolac, meloxicam, nimesulide (16, 54, 55).

Natural products. The usage of natural products to treat ailments is an old-age practice, which dates back to ancient civilisations (56, 57). Since time immemorial, humans and other primates have repeated looked to nature to find answers, to be specific, to discover natural products (either plant extracts or lipids i.e. fatty acids and essential oils) that may possess pharmacological qualities (56, 58). Over time, after exhaustive experimentation (as no prior knowledge existed about the product's curative properties), information regarding the performance of these products (whether they worked or not) was integrated into the local's indigenous knowledge. Interestingly, even today, a significant proportion of the population in developing countries, which is not covered by public and private health care systems, still appropriate this knowledge in order to attend to their primary health care needs (59, 60). This may also in part be attributed to the cost of conventional medicines, which are often times astronomical.

Plant extracts. By definition, a plant extract can be regarded as a natural product derivative whose chemical constituents have not been fully defined (61). Some

of these chemical constituents (phytomolecules) may be secreted as a defence mechanism to counter predation, including predation pressure exerted by microbes (62, 63). Therefore, these molecules are rather secondary metabolites, which seem to have no apparent function in the primary metabolism of the plant (62).

In many reported *in vitro* susceptibility bioassays, researchers often refer to the antimicrobial activity of crude extracts, and deem it necessary to not elucidate the full chemical composition of the active compound(s). However, it is quite crucial to pinpoint the active compound(s) that contributes to the plant extract's antimicrobial activity for purposes of mass production. But more importantly, to also demonstrate the compound's therapeutic effect in an *in vivo* study. Nonetheless, based on their chemistry, phytomolecules have been categorised into a number of classes (64, 65). Some of these include (Fig. 3):

1. Glycosides: small organic molecules in which a functional group is attached to a sugar moiety via a glycosidic bond. In plants, these molecules require an additional activation step i.e. hydrolysis, before they are utilised. An example is cyanoglycoside from cherry fruits,

2. Alkaloids: chemical compounds characterised by a nitrogen ring and may carry other elements such as oxygen and sulphur. These compounds are primarily toxic to many organisms. Examples include stimulant cocaine and caffeine,

3. Polyphenols: organic compounds characterised by repeating phenolic rings, which seem to attribute a particular function i.e. therapeutic, among others. Examples include isoflavones and tannins, and

4. Terpenes: aromatic organic compounds, which are major biosynthetic building blocks (isoprene unit) found in almost all living organisms. Examples include steroids and vitamin A.

From literature, it is clear that plant extracts exert a demonstrable pharmacological effect in humans (66, 67). And as pointed out by Ficker *et al*. the traditional uses of plants i.e. as food sources, implies their extracts would have a low toxicity profile in humans. To illustrate this point, extracts arising from edible plants such as *Zingiber officinale* (ginger), *Curcumae longae* (tumeric) and *Juglans cinerea* (butternut) have been examined and shown to cause growth inhibition (66). More to the point, the above-mentioned extracts were also shown to inhibit growth of fungal strains that were highly resistant to amphotericin B and ketoconazole. Atai and co-workers reported that ethanolic extracts of ginger exert an inhibitory effect on *Candida albicans* at concentrations as low as 2 mg/mL (68).

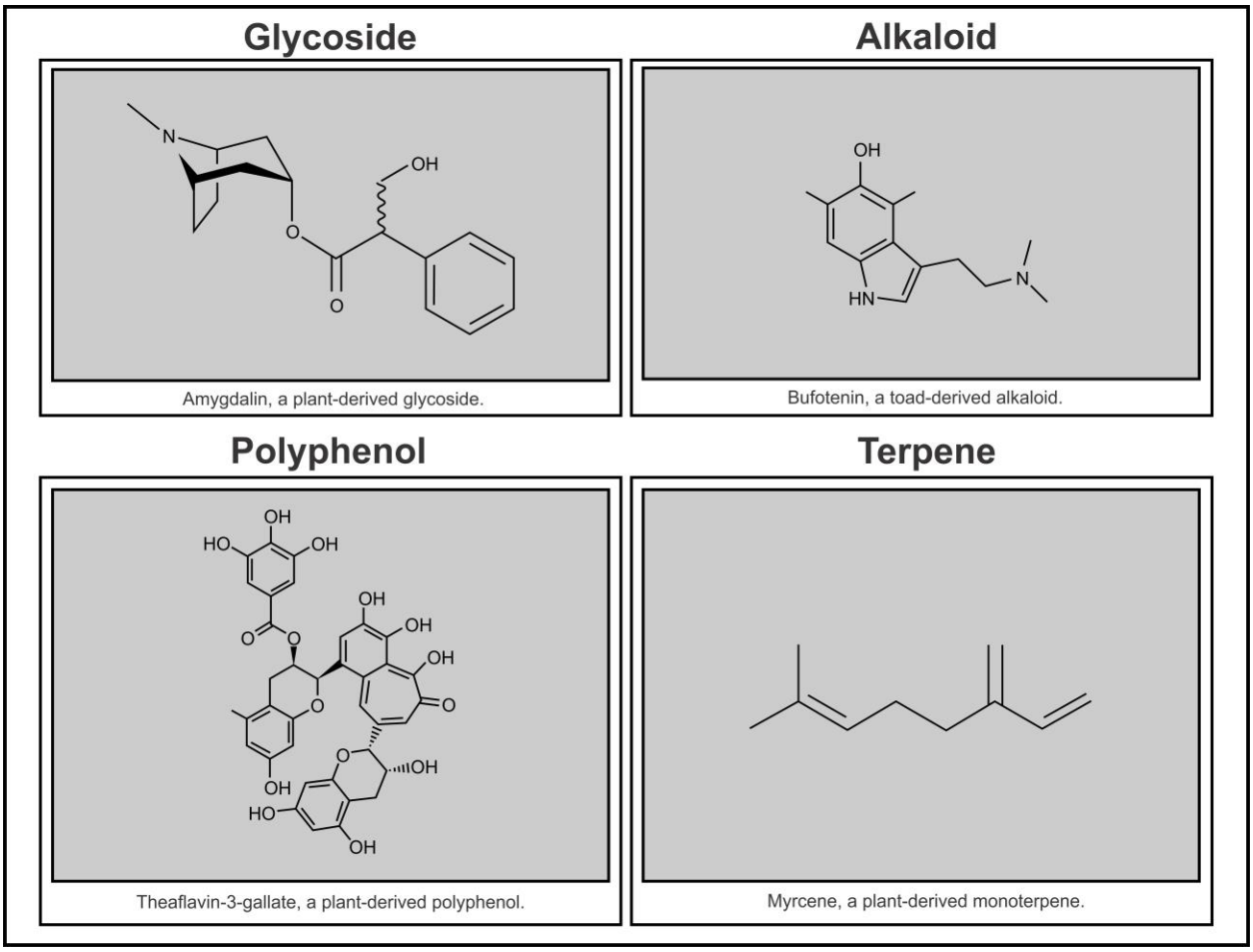


FIG 3 Some examples of plant-based extracts that possess biological activity.

An interesting extract is the polyphenolic compound curcumin, which originates from rhizomes of the plant *Curcuma longa*. This compound has been shown to inhibit growth of *Cryptococcus neoformans* and some clinical isolates of *Candida* (69). Additionally, the same study reported on the ability of this compound to limit the adherence of *Candida* cells to human buccal epithelial cells (69). This observation is important, as the adhesion of microbes to host mucosal surfaces is a prerequisite for colonisation and infection (69, 70).

An interesting application of this compound was in photodynamic therapy against clinical isolates of *Candida albicans, Candida tropicalis* and *Candida glabrata* (71). Photodynamic therapy is mainly used in cancer treatment, and has since been extended to microbes. Here, a non-toxic compound like curcumin (regarded as a photosensitizer) is activated by light of a particular wavelength leading to the production of reactive oxygen species (ROS). The resultant oxidative burst can then target cellular components (mitochondria, cell membranes, etc.) leading to cell death (72). In their study, Dovigo *et al*. reported optical properties of curcumin as a function of illumination fluence (71). In brief, their data showed that when compared with the control group, a statistically significant reduction in *Candida albicans* viability was observed after photodynamic therapy ($p < 0.05$), for both planktonic cells and biofilms.

Lipids: fatty acids and essential oils. Fatty acids and essential oils are lipid-based molecules that naturally occur in nature. As lipids, they are defined primarily on the basis of their solubility in solvents. Fatty acids exist as constituents of triglycerides or in a “free-form” where they are not attached to other molecules (73). These molecules are generally regarded as inert structural components of membrane or as sources of fuel were their catabolism results in energy generation. Some of these molecules are regarded as essential fatty acids, and they play an important role in human physiology (74). Thus these molecules are often taken in as food supplements.

In addition to the above, fatty acids have been shown to exert antimicrobial activities against microbes. Recently, Thibane *et al*. reported on the antimicrobial effects of stearidonic acid, eicosapentaenoic acid and docosapentaenioc acid: these fatty acids

reduced the metabolic activity and biofilm biomass production by *Candia albicans* and *Candida dubliniensis* (75). In another study, fatty acids (in particular stearidonic acid) acted in synergy with amphotericin B against *Candida albicans* and *Candida dubliniensis* (76). In the latter study, Thibane *et al*. point out that fractional inhibitory concentration index values of 0.25 and 0.38 were observed, which respectively correlated to 99% and 96% growth inhibition.

Another class of fatty acids i.e. 3-hydroxy fatty acids have also been documented to act as antimicrobial agents (77). These authors show that lactic acid bacterial 3-hydroxy fatty acids (3-OH 10:0, 3-OH 11:0, 3-OH 12:0 and 3-OH 14:0) exhibit antifungal activity with MIC ranging from 10 µg/mL to 100 µg/mL against species belonging to *Aspergillus, Penicillium, Kluyveromyces, Pichia* and *Rhodotorula*. These 3-hydroxy molecules may be “antimicrobial agents”, which are deliberately secreted to appropriate an environmental advantage against yeasts and molds.

Concerning essential oils, these molecules are defined as volatile aromatic organic compounds (made up of hydrocarbon molecules) that are considered to carry the “essence or scent” of a plant (78). Like fatty acids, their lipophilic nature allows them to alter the fluidity of membrane and integrity leading to cell leakage. This may be a possible mode of action for killing microbes. In their study, Pinto and co-workers showed that essential oils from *Thymus pulegioides* (thymol and carvacrol; as determined by GC and GC-MS) displayed broad-spectrum antifungal activity against clinical *Candida*, *Aspergillus* and dermatophyte species (79). These authors attributed

the killing effect on altered membrane function. Thus authors caution that considering the negative effects of these oils on fungi, which are of eukaryotic origin like humans, studies into toxicity and improved formulations, among others, should be undertaken in order to avert negative outcome when prescribed.

Other studies have also demonstrated the antifungal effect of essential oils against non-traditional pathogenic fungi i.e. those that colonise the built environment (80) and spoilage fungi of bakery products (81). Here, oils extracted from among others, included eucalyptus, cinnamon, rosemary, thyme and clove, were tested.

Other compounds

Anti-malarial drug: chloroquine. Chloroquine is primarily used as a prophylactic drug for treating malaria (82). The drug's mode of action involves binding to heme proteins and forming a complex that is toxic to the cell and ultimately targets membrane function (83). The chemosensitising properties of chloroquine have also been explored in other studies. Ma *et al*. reported that cryptococcal cells could take up residency inside macrophages and successfully replicate without evoking an immune response (84). More to this point, Webber and co-workers documented that treatment of parasitised-macrophages with chloroquine leads to accumulation of this drug inside macrophages forming iron-complexes that kill cryptococcal cells and *Histoplasma capsulatum* (85). It should now be possible to combat such cells by chloroquine treatment. This sensitising capability has also been seen when treating the usually drug-resistant *Candida* biofilms (86). By adding chloroquine in combined therapy with drugs such as azoles, biofilms

became susceptible to these conventional antifungals.

A recent paper by Huang *et al*. demonstrated via using a functional genomics approach that chloroquine achieves fungal growth inhibition via blocking thiamine transportation (87). This vitamin plays a crucial role in glucose metabolism, where cells can derive energy to support cellular processes (88).

Anti-psychotic drugs. The drug chlorpromazine is a dopamine antagonist, and works by acting on a number of receptors in the brain (89). This drug is mainly prescribed for the treatment of schizophrenia. However, a recent study has shown that chlorpromazine also possesses antifungal activity (90). This drug, together with another anti-psychotic agent trifluopherazine, was shown to inhibit fungal, bacterial and protozoan species. The inhibitory effects of these drugs were noted at concentrations ranging from 16 µg/mL to 64 µg/mL. These authors did not elucidate on the possible mode of action employed by these drugs. It is, nonetheless, conceivable that microbial death was achieved via destabilisation of the membrane integrity leading to increased membrane permeability (91).

In a recent study, Zhai *et al*. highlighted that the ineffectiveness of current antifungals may in part be due to poor central nervous system penetration, particularly in the case of disseminated cryptococcal infections (92). To circumvent this limitation, these authors sought to test the antimicrobial activity of sertraline (Zoloft®, Pfizer), which is an antidepressant drug, against *Cryptococcus neoformans*. Here, *in vitro* analysis

revealed sertraline to have a fungicidal effect as well as act in synergy with fluconazole. From the above examples, it is clear that the localisation of infection is an important consideration for drug delivery, and thus, a drug that is able to cross the blood brain barrier may be a viable treatment option in case of a disseminated cryptococcal infection.

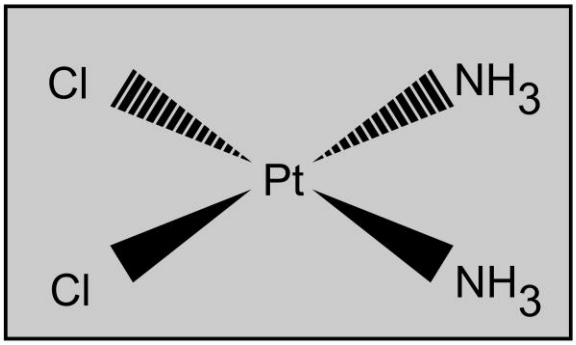
In an interesting study, Karababa *et al*. demonstrated that improved penetration or delivery of azoles could be achieved by addition of antipsychotic drugs like fluphenazine (93). This drug was shown, in elegant molecular studies, to upregulate the multidrug transporter genes in azole-resistant *Candida albicans* strains - leading to enhanced efflux of azoles.

The picture emerging above makes for a convincing case concerning the practical application of antipsychotic drugs in the treatment of fungal-infectious diseases.

Metal complexes. A metal complex is a chemical species that is formed when a ligand i.e. functional group (molecule) is attached to a central metal to form a coordination complex (Fig. 4) (94). Here, the ligand determines the capability of the metal to undergo a chemical reaction. Metal complexes have been studied quite extensively over the years, particularly in light of these metals and their ligands pronouncing strong biological activities (upon chelation) against cancer cells (95, 96). An encouraging observation about complexes is that, they are more potent and less

toxic compared to their parent compound (97).

Recently, the chelating quality of complexes has also been documented to cause microbial cell death in some studies. This is reflected in a paper by Hossain *et al*. where via using a disc diffusion method, they showed that metal complexes of copper (II), cobalt (III), rhodium (III) and platinum (IV) ions with dibasic acids displayed antifungal activity against two fungal species, *Botryodiplodia theobromae* and *Collectotrichum gloeosporioides* (96). In another study, amino acid-derived compounds and their cobalt (II), copper (II), nickel (II), and zinc (II) metal complexes were screened for their *in vitro* antifungal activity against and shown to inhibit growth of *Trichophyton longifusus*, *Candida albicans*, *Aspergillus flavus*, *Microsporum canis*, *Fusarium solani* and *Candida glabrata* (95).



Cisplatin

FIG 4 An example of a metal complex. Here, cisplatin is constituted by a metal (platinum) that is linked to four ligands i.e. [$(NH_3)_2$ and $(Cl)_2$] via dipolar bonds to form a mirror-image.

The platinum complex cisplatin has also been shown to display antifungal activity against *Candida* species (98). These complexes are reported to effect cell death by damaging the cell membrane. Conventionally, cisplatin is regarded as an anti-cancer drug and is therefore used in cancer chemotherapy (98). The application of this drug as an antifungal agent may be further limited due to inducing immunosuppression.

Molecules identified by high-throughput screening. High-throughput screening is a rapid and systematic process (primarily applied in drug discovery) where large numbers of compounds are mapped for their activity and their targets identified (99). By design the screening assays are carried out in a microtiter plate, where a logical entity like yeast cells, are added and allowed to interact with other components (drug) over time. In some cases, these targeted entities may be enzymes that catalyse

key metabolic pathways. Subsequently, the resultant response (or non-response) is then measured using an automated analysis machine that has robotic capability to, among others, mix and add reagents. At the heart of it, HTS combines statistics and experiments and, thus, a researcher should be able to sort and identify important biochemical information (hit) from the background noise (99, 100).

In recent years there have been a number of studies looking at identifying novel antifungal compounds through large screening of chemical libraries. Towards this end, we highlight a few of these. In their study, Krysan and DiDone designed a luminescent assay that detected small molecules that disrupt yeast cell integrity (101). The screened libraries (held at the Institute of Chemistry and Cell Biology-Longwood (ICCB-L)) included known compounds (Biomol, Prestwick I), commercial compounds (ChemDiv5) as well as biological extracts (NCCDG1 and ICBG6), which target among others, second messenger modulators, gene regulation agents and kinase inhibitors. Using *Saccharomyces cerevisiae* and *Candida albicans* as model yeasts; they screened over 4500 molecules and mapped the release of the intracellular enzyme adenylate kinase into the culture medium as a reporter for cell lysis. In another study, Breger and co-workers screened over 1200 compounds from ICCB-L that prolonged the survival of *Candida albicans*-infected nematodes and inhibited *in vivo* filamentation (102). Here, they used nematodes to model and explain the pathogenesis of *Candida* in mammalian cells. Based on their screening results, caffeic acid phenethyl ester and enoxacin were shown to possess antifungal activity in a murine model. By exploring if nutrient-deprived host environment may assist in clearing disseminated cryptococcal infections, Rabjohns

et al. showed in a HTS assay that a potential chemical scaffold i.e. 10058-F4 displayed a cidal effect at low micromolar range. A Library of Pharmacologically Active Compounds (LOPAC - constituted by active compounds that target lipid biosynthesis, phosphorylation, ion channels, etc.) was screened and measured residual metabolic activity using alamarBlue (profluorescent dye) (103). Further to the concept of nutrient deprivation, Dehdashti and co-authors assessed the viability of *Cryptococcus neoformans*, under these conditions, using a miniaturised ATP-content based assay involving over 1200 compounds also obtained from LOPAC (104). In the end, they were able to identify four active compounds viz. niclosamide, malonoben, 6-bromoindirubin-3'-oxime, and 5-[(-4ethylphenyl)methylene]-2-thioxo-4-thiazolidinone, and further validated their data when they reproduced the cidal effect against clinical isolates. LaFleur and co-workers were able to identify and validate antifungal potentiators that enhanced activity of clotrimazole or acted on fungal biofilms by inhibiting their metabolic activity; after screening over 120000 small molecule compounds from the NIH Molecular Libraries Small Molecule Repository (105). The library has sets of known molecules and natural products. Some of these molecules (i.e. 1,3-benzothiazole scaffold) were, in this study, shown to target biofilm formation. In an interesting study, Chaturvedi *et al*. studied the cidal effect of antifungal compounds and biocides against *Geomyces destructans* (106). *Geomyces* causes a deadly disease (white nose syndrome) in bats. After screening a SpectrumPlus compound library (made up of known compounds (including imidazoles) and natural products, they concluded that *Geomyces destructans* was susceptible to some drugs at a concentration gradient similar to that of human pathogenic fungi.

1.5 CONCLUDING REMARKS

The rise in the emergence of new mycotic agents and drug resistance drives the need to find new and/or alternative drugs. Today, a picture is emerging where non-traditional antimicrobial agents, which were previously prescribed to treat non-infectious conditions, show antimicrobial properties. It is worth our time to explore these compounds further. Our ability to also conduct high throughput screening at institutions of higher learning (not only at pharmaceutical companies), has added impetus to this cause.

In this review, we presented reports where compounds ranging from anti-inflammatory drugs to anti-psychotic drugs are documented to control fungal growth. However, due to toxicities attributed to some of these drugs, their clinical application may be limited. Nonetheless, there are on-going efforts to modify and minimize the side effects of some of these drugs. For example, new derivatives of anti-inflammatory drugs are being synthesised, and thus, these new derivatives should also be tested for their antimicrobial properties. It is also important to demonstrate if these “new” antifungals will work in host cells, and probably in some cases, in situations of advanced disease. Towards this end, the usage of animals in modelling human disease should be considered in order to clearly establish the therapeutic benefits of these chemical compounds.

Another ancillary effect of these drugs that should be explored is the application of anti-inflammatory drugs in the treatment of pathogen-induced immune reconstitution

inflammatory syndrome. The duality in function of these molecules as antifungal agents and anti-inflammatory drugs may provide an additional beneficial therapeutic outcome. To this end, studies are required to test the possible clinical outcomes of anti-inflammatory use to fungal disease.

The major limitation in providing appropriate medical treatment is the cost of drugs, which is a pertinent issue in developing countries. Therefore, the therapeutic benefit of anti-inflammatory drugs (aspirin) and natural products (plant extracts), which are relatively cheap and widely distributed, should be explored. Moreover, the possibility to take some of the above-mentioned non-traditional antimicrobial drugs as prophylactic agents should also be considered. This may be so in the case of natural products; were some are already taken in as supplements.

An important note is that some of these compounds in *in vitro* assays act in synergy with traditional antimicrobial agents, such as azoles. This is one attribute that is encouraging in that it implies these compounds can be added at low concentrations where clinically they could not exert any negative physiological outcomes. Additionally, the added effect of these chemical compounds in combination therapy enhances the efficiency of traditional antimicrobial drugs, which under normal conditions may be fungistatic. More intriguing to the above discussion, is the application of antimalarial drugs that seem to enhance immuno-processing of parasitising fungal cells in macrophages instead of enhancing the effect of another drug. It is also worth the time to

establish if clinical populations taking antimalarial drugs may be protected from fungal disease.

More to this point, the concept of also extending the conventional application of chemosensitisers and photosensitizers from treating cancer cells to combating microbial infections should be explored. Special consideration should be placed in the defined scope of enhancing the host's immune response to microbial infection. To date, reports have emerged that show natural compounds like curcumin, when chemically or optically activated, can kill microbes by releasing reactive oxygen species, which in turn, target cellular components. The latter is in some respect, similar to the cytotoxic "oxidative burst" effect, which is expressed by immune cells in response to microbial infection. By necessary extension, metals and their complexes, which are also used to treat cancer cells, should be examined for their antimicrobial properties.

Once again we point out that careful consideration should be taken when attempting such approaches - especially in the case of i) compounds that generally target eukaryotic organisms, and ii) complexes and sensitizers that may induce immunosuppression, in order to realise the desired therapeutic outcome to the exclusion of adverse effects.

1.6 PURPOSE OF PhD STUDY

With the preceding discussion providing a background and context for the studies presented in the thesis, the aims became to repurpose the following compounds as antifungal drugs that may control cryptococcal cells:

1. Aspirin and ibuprofen (anti-inflammatory drugs),
2. Copper acyl salicylate (aspirinate-metal complex), as well as
3. Quetiapine and olazanpine (anti-psychotic drugs).

It is hoped that this thesis will build on the current literature on new antifungals and further facilitate the application of these compounds in the clinical settings, more especially to better manage cryptococcal-IRIS and cryptococcal-emergent psychosis.

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CHAPTER 2

THE REPURPOSING OF ASPIRIN AND IBUPROFEN AS CANDIDATE ANTI-*CRYPTOCOCCUS* DRUGS

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2.1 ABSTRACT

The usage of fluconazole and amphotericin B in clinical settings is often limited by, among others, drug resistance development and undesired side effects. Thus there is a constant need to find new drugs to better manage fungal infections. Towards this end, this paper considers the repurposing of aspirin (acetylsalicylic acid) and ibuprofen as alternative drugs to control growth of cryptococcal cells. *In vitro* susceptibility tests, including a checkerboard assay, were performed to assess the response of *Cryptococcus* (*C.*) *neoformans* and *Cryptococcus* (*C.*) *gattii* towards the above anti-inflammatory drugs. Next, the capacity of these two drugs to induce stress as well as their mode of action to kill cryptococcal cells was determined. The studied fungal strains revealed a dose dependent response profile towards both aspirin and ibuprofen, with ibuprofen exerting greater antimicrobial action. More importantly, the MICs of these drugs did not negatively affect: 1) growth, or 2) impair the functioning of macrophages - rather they enhanced the capability of these immune cells to phagocytose cryptococcal cells. Ibuprofen was also shown to act in synergy with fluconazole and amphotericin B. The treatment of cryptococcal cells with aspirin or ibuprofen led to stress induction via activation of high osmolarity glycerol (HOG) pathway, and eventually cell death was achieved through reactive oxygen species (ROS)-mediated membrane damage. The presented data highlight the potential clinical application of aspirin and ibuprofen as candidate anti-*Cryptococcus* drugs.

Keywords. Aspirin (acetylsalicylic acid); *Cryptococcus*; Drug repurposing; Ibuprofen; Macrophage; ROS-mediated membrane damage; Synergism.

2.2 INTRODUCTION

The advent of HIV/AIDS has led to species such as *C*. *neoformans* and *C. gattii* emerging as important disease-causing microbes (1-3). To demonstrate this point, these species are reported to cause over 1 million infections worldwide, with the highest burden of infections localised in resource-poor settings (4). In their paper, Perfect et al. argued that the management of fungal diseases is strongly dependent on capital resources available to a specific region (5). Based on the latter, Sebolai and Ogundeji further argued that it is not surprising for countries in Sub-Saharan Africa, given the complex geo-political and socio-economic challenges that prevail in this region, to be unable to provide the necessary life-saving drugs, which are often expensive (6). Towards this end, a solution may be to repurpose already FDA-approved drugs that are cheap such as aspirin (acetylsalicylic acid) and ibuprofen.

We have previously reported that aspirin affected *Cryptococcus* cells in a number of ways: 1) decreased capsule shedding, 2) inhibited production and trafficking of capsule associated-3-hydroxy fatty acids, and 3) inhibited cellular growth (7). In the current study, we build further on our prior antimicrobial work by drawing a comparison between the effects of aspirin and ibuprofen. Importantly, we also attempt to elucidate the mode of action employed by the two drugs in killing cryptococcal cells. The answers to the above objectives could assist in making a case to repurpose aspirin and ibuprofen as antimicrobial drugs, which in turn, could help in the management of cryptococcal infections, more so in resource poor settings like Africa.

2.3 MATERIALS AND METHODS

Strains, cultivation and standardisation of cells. Ten clinical *Cryptococcus* strains: five *C. neoformans* strains (LMPE 028, LMPE 030, LMPE 043, LMPE 046 and LMPE 047) and five *C. gattii* strains (LMPE 045, LMPE 048, LMPE 052, LMPE 054 and LMPE 070) were used in this study. These strains were obtained from Universitas Academic Hospital, Bloemfontein, South Africa. The strains were maintained on yeast-malt-extract (YM) agar (Merck, South Africa). Following which, a loopful of cells (0.01 ml loop) was taken from a 48 h old YM agar plate and cultivated in a 250 ml conical flask, containing 100 ml of YNB (6.7 g/l; Difco Laboratories, United States) broth supplemented with 4% (w/v) glucose (Merck, South Africa) while shaking at 160 rpm at 30^{o}C. Cells were allowed to grow overnight before 0.1 ml of the old culture media was inoculated into another conical flask containing 100 ml of fresh YNB broth. The cells were grown until the mid-exponential phase and were immediately washed twice - using phosphate buffered saline (PBS; Oxoid, South Africa). Using a haemocytometer, cells were subsequently standardised to 1 x 10^{6} cells/ml in 15 ml centrifuge tubes (Becton-Dickinson Labware, United States) containing either 10 ml of RPMI-1640 media (Sigma-Aldrich, South Africa). Cells were kept on ice for further use.

For drug sensitivity testing, the strains were maintained on Sabouraud dextrose agar (Merck, South Africa) at 35^{o}C for 24 h. Following which, a loopful of cells (five distinct colonies on a 0.01 ml loop) was taken from a 24 h old agar plate and suspended in 5 ml of distilled water. The turbidity of each strain suspension was adjusted using a

spectrophotometer (Biochrom EZ Read 800 Research, United Kingdom) to a final concentration of between 0.5 x 10^5 and 2.5 x 10^5 CFU/ml (8).

A macrophage cell line RAW 264.7 was also used. This murine cell line was cultured in RPMI-1640 media (Sigma-Aldrich, South Africa) supplemented with 10% fetal bovine serum (Biochrom, Germany), 20 U/ml penicillin, 20 g/ml streptomycin (Sigma-Aldrich, USA) and 2 mM L-glutamine (Sigma-Aldrich, South Africa) in a CO_2-incubator (5%) at 37°C. The cell line (a kind donation from Prof Masoko and Mr Makola, University of Limpopo, South Africa) was originally obtained from the American Type Culture Collection (USA). Before use, cells were standardised to 1 x 10^5 cells/ml and seeded into wells of sterile, disposable 96-well flat-bottom microtitre plate(s) (Greiner Bio-One, Germany).

Drugs. Standard powders of aspirin (acetylsalicylic acid; Sigma-Aldrich, South Africa), ibuprofen (Sigma-Aldrich), fluconazole (Sigma-Aldrich, South Africa) and amphotericin B (Sigma-Aldrich, South Africa) were used in this study. Aspirin was prepared in absolute ethanol (Merck, South Africa) to yield a stock solution of 1000 µg/ml and ibuprofen was dissolved in dimethyl sulfoxide (Merck, South Africa; final stock solution of 1000 µg/ml). Fluconazole was reconstituted in distilled water (final stock solution of 1000 µg/ml) while amphotericin B was dissolved in dimethyl sulfoxide (Merck, South Africa) to yield stock concentration of 1000 µg/ml**.**

Drug susceptibility testing. The changes in minimum inhibition concentrations (MICs) in microtitre wells, including checkerboard assays, were performed using broth microdilution protocol according to method outlined by the European Committee on Antimicrobial Susceptibility Testing (8). For drug susceptibility testing, the effects of aspirin and ibuprofen were independently assessed in triplicate for each strain, in a direct comparative study using a final drug concentration gradient in RPMI-1640 media of 0.01 mM (1.80 µg/ml for aspirin and 2.06 µg/ml for ibuprofen), 0.1 mM (18.0 µg/ml for aspirin and 20.6 µg/ml for ibuprofen) and 1 mM (180 µg/ml for aspirin and 206 µg/ml for ibuprofen). These 10-fold microdilution plates (Greiner Bio-One, Germany) were incubated at 35^{o}C for 48 h. The MICs were determined spectrophotometrically after 48 h. In this study, the MIC endpoints were defined as the lowest drug concentration that resulted in a reduction in growth of 50% or more compared with the growth (i.e. optical density (OD)) in a drug-free control well (8). In anticipation of the checkerboard assay, OD readings were also measured for fluconazole (2 µg/ml, 4 µg/ml, 8 µg/ml, 16 µg/ml and 32 µg/ml) and amphotericin B (0.25 µg/ml, 0.5 µg/ml, 1 µg/ml, 2 µg/ml and 4 µg/ml), after incubating microdilution plates at 35^{o}C for 48 h.

Effect of aspirin and ibuprofen MICs on macrophage growth and function. To determine if aspirin (180 µg/ml) and ibuprofen (206 µg/ml) MICs were toxic to macrophages i.e. concentrations that led to 50% or more reduction in growth compared with growth (i.e. optical density (OD)) of a drug-free control, standardised macrophages in RPMI-1640 media (1 x 10^5 cells/ml in 100 µl) were seeded into wells and treated with aspirin (360 µg/ml in 100 µl of RPMI-1640 media) or ibuprofen (412 µg/ml in 100 µl of

RPMI-1640 media). The microtitre plates were incubated in a CO_2-incubator (5%) for 24 h at 37°C. The OD was measured using a spectrophotometer. Appropriate drug vehicle controls (ethanol and DMSO, respectively) were also included for comparison.

We also determined if macrophage exposure to aspirin or ibuprofen would alter their function i.e. capability to phagocytose cryptococcal cells (9). Here, we used the phagocytosis stain, pHrodo™ Green Zymosan A BioParticles (Life Technologies, United States). The stain only fluoresces when excited at acidic pH, such as inside the phagosome. Drug free, standardised cryptococcal cells (1 x 10^5 CFU/ml) in PBS (which has a neutral pH) were stained (1 µl of stain: 999 µl of cells) for 1 h at room temperature while slowly agitating. Next, cryptococcal cells were washed with PBS, spun down and suspended in sterile 1000 µl of PBS. A 100-µl suspension of cells was then transferred to microtitre plate wells (Greiner Bio-One, Germany) that were already seeded with macrophages (100 µl; 1 x 10^5 cells/ml in RPMI-1640). Aspirin (720 µg/ml in 100 µl of RPMI-1640 media) or ibuprofen (824 µg/ml in 100 µl of RPMI-1640 media) was added at 0 h to the *Cryptococcus*-macrophage co-culture wells and allowed to interact in a CO_2-incubator (5%) for 2 h or 6 h at 37°C. At the end of the incubation period, the induced fluorescence was measured (492 nm; ex/538 nm; em) using a Fluoroskan Ascent FL (Thermo-Scientific, United States) microplate reader, which converts logarithmic signals to relative fluorescence units. Wells with only *Cryptococcus*-macrophage (i.e. without drugs) were included for comparison. In addition, the fluorescence was also measured for macrophages alone in order to normalise the readings (9).

Checkerboard assay. A checkerboard assay was also designed (pairing: ibuprofen with fluconazole and ibuprofen with amphotericin B) in a sterile, disposable 96-well flat-bottom microtitre plate (Greiner Bio-One, Germany). These microdilution plates were incubated like-wise and at the end of the incubation period (48 h), OD readings were taken, and subsequently the fractional inhibitory concentration (FIC) index (FICI) was calculated. Fractional inhibitory concentration index was defined as $\Sigma FIC = FIC_A + FIC_B$ ([MIC of drug A in combination/MIC of drug A alone] + [MIC of drug B in combination/MIC of drug B alone]) (10). Fractional inhibitory concentration index values were determined to establish if there was synergism (≤ 0.5), no interaction (> 0.5 - 4) or antagonism (> 4).

Effect of aspirin and ibuprofen on cellular ultrastructure. For effect on outer ultrastructure, scanning electron microscopy (SEM) was performed. Material for SEM was obtained from cells i.e. non-treated cells (0 µg/ml), aspirin-treated cells (180 µg/ml) and ibuprofen-treated cells (206 µg/ml) that were growth 48 h at 30ºC. After 48 h, cells from the different experimental conditions were transferred to 1.5 ml plastic tubes (Merck). The material was prepared for SEM according to the method of van Wyk and Wingfield (11). In brief, the material was chemically fixed using sodium-phosphate-buffered 3% glutardialdehyde (Merck, South Africa) and sodium-phosphate-buffered 3% osmium tetroxide (Merck, South Africa) followed by dehydration in a graded ethanol (Merck) series. Next, the material was dried (Bio-Rad Microscience Division, England), mounted on stubs, and coated with gold using an SEM coating system (Bio-Rad

Microscience Division, England) (11). Preparations were examined using a Shimadzu Superscan SSX 550 scanning electron microscope (Japan).

For effect on inner ultrastructure, nano-scanning auger microscopy (Nano-SAM) was performed. Here, the same SEM stubs were re-coated with gold and re-examined using a nano-scanning auger microscope in SEM mode linked to Argon (Ar^+) etching according to a method previously described by Swart et al (12). Cells (from each experimental condition) were then examined with a PHI 700 Nanoprobe (Japan) equipped with SEM and Scanning Auger Microscopy (SAM) facilities. For SEM and SAM analyses in field emission, an electron gun used was set as: 2.788 A filament current; 3.56 kV extractor voltage and 175 µA extractor current. A 25 kV, 1 nA electron beam was obtained with these settings for the Auger analyses and SEM imaging. The electron beam had a diameter of 12 nm. The electron gun unit had an upper pressure of 8.7E-10 Torr and the pressure of the main chamber was 4.4E-10 Torr. Aperture A was used for all the measurements. For SEM the field of view (FOV) was 2 µm. Four (4) cycles per survey, 1 eV per step and 50 ms per step were used to obtain Auger point analyses. The Ar^+ ion sputtering gun, which the Nanoprobe was also equipped with, was set at: 2 kV beam voltage, 5 µA ion beam current and a 1 x 1 mm raster area, giving a sputter rate of 15 nm/min (12).

Effect of aspirin and ibuprofen on stress induction. In order to determine if our two test drugs could lead to stress induction, we performed a Hog-1 mitogen-

activated protein kinase (MAPK) Western blot assay (13, 14) that was complemented with a phospho-p-38 MAPK ELISA assay. For Western blot analysis, cultures were treated with 180 µg/ml aspirin, 206 µg/ml ibuprofen, 58000 µg/ml (1M) sodium chloride (NaCl; Sigma-Aldrich, South Africa) or 34 µg/ml (1 mM) hydrogen peroxide (H_2O_2; Sigma-Aldrich, South Africa) for 20 min (grown at 30^{o}C in YNB broth). Immediately after the cells were washed twice in PBS, harvested, and suspended in a Tris-EDTA buffer solution, pH 7.4 (Sigma-Aldrich, South Africa). Next, the cells were ruptured using the French press cell disrupter (Constant Cell Disruption Systems, United Kingdom) at a pressure of 36 kips per in.^{2}. The protein content was measured using the Bradford method and equal amounts of protein (50 µg) were loaded onto 12.5% SDS-PAGE gel and stained with Coomassie Brilliant Blue (Sigma-Aldrich, South Africa). The separated proteins were then transferred to Immun-Blot® PVDF membrane (Bio-Rad, United States) and incubated overnight at 4^{o}C with a rabbit primary antibody specific for Hog-1 (Santa Cruz Biotechnology, United States) and then tagged with a secondary anti-rabbit IgG horseradish peroxide-conjugated antibody (Sigma-Aldrich, South Africa) for 2 h at room temperature. The blot was developed using the Gel Doc EZ System (Bio-Rad, United States). This test was done in duplicate. To determine the phosphorylation state of our MAPK protein, we performed a phospho-p-38 MAPK ELISA assay (Sigma-Aldrich, South Africa). The assay was performed on lysates (obtained from non-treated cells, 180 µg/ml aspirin-, 206 µg/ml ibuprofen-treated cells) according to the manufacturer's instruction. A positive control, supplied by the manufacturer, was also included for referencing. The optical density (OD) readings were measured using a

spectrophotometer. The obtained OD readings were normalised using the corresponding pan-p-38 MAPK readings.

Effect of aspirin and ibuprofen on accumulation of reactive oxygen species (ROS). We first examined the effect of aspirin and ibuprofen in causing the loss of mitochondrial membrane potential, which could in part account for accumulation of ROS. Towards this end, non-treated cells and treated cells (180 µg/ml aspirin and 206 µg/ml ibuprofen) were seeded at 1x 10^6 cells/ml in sterile black 96-well flat-bottom microtitre plate (Greiner Bio-One, Germany) and incubated for 48 h at 30ºC (15). At the end of the incubation period, the mitochondrial membrane potential was measured using the dye 5,5',6,6'-terachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) according to the JC-1 Mitochondrial Membrane Potential Assay Kit instructions (Life Technologies, United States). Mitochondrial membrane potential was calculated as the ratio of the J-aggregates (healthy cells; ex = 540nm / em = 570nm) and monomeric forms (unhealthy cells; ex = 485nm / em = 535nm). The induced fluorescence was measured using a Fluoroskan Ascent FL (Thermo-Scientific, United States) microplate reader. For measurement of reactive oxygen species accumulation, cells were like-wise prepared as above. Following incubation, 10 µl of the fluorescent dye i.e. 2',7-dichlorofluorescin diacetate (DCFHDA, 1 µg/ml; Sigma-Aldrich, South Africa) was reacted with 90 µl of cells for 30 min in the dark at room temperature. The Fluoroskan Ascent FL (Thermo-Scientific, United States) microplate reader was used to measure the induced fluorescence at 485nm/535nm.

Effect of aspirin and ibuprofen on membrane function. In order to determine if membrane function was impaired or maintained after cells were challenged with aspirin and ibuprofen, we assessed membrane integrity using: 1) propidium iodide (PI) stain, 2) Toxilight® Bioassay, and 3) 4',6-diamidino-2-phenylindole (DAPI) stain. For all these experiments, cells were once again treated with 180 µg/ml aspirin and 206 µg/ml ibuprofen in sterile 96-well flat-bottom microtitre plate (Greiner Bio-One, Germany) and incubated for 48 h at 30^{o}C (15). For PI staining, cells were washed twice with PBS and 99 µl of cells (from each experimental condition) were reacted with 1 µl of PI (Life Technologies) in the dark for 1 h at room temperature. Thereafter, microscope slides were prepared in the presence of an anti-fade compound, 1,4-diazabicyclo[2.2.2]-octane (DABCO, Sigma-Aldrich, South Africa), before they were viewed using a confocal laser scanning microscope (CLSM; Nikon TE 2000; Tokyo, Japan). The Toxilight® Bioassay (Lonza Rockland, Inc., United States) was performed according to manufacturer's instructions. This assay quantitatively measures the release of adenylate kinase from cells with damaged membranes into the extracellular environment. The supernatant was collected and reacted with the Toxilight reagent in a new sterile white 96-well flat-bottom microtitre plate (Greiner Bio-One, Germany) for 15 min. The emitted light intensity was measured using a Fluoroskan Ascent FL (Thermo-Scientific, United States) microplate reader. For DAPI, cells were like-wise washed with PBS, and 99 µl of cells (from each experimental condition) were reacted with 1 µl of DAPI (Life Technologies, United States) in the dark for 1 h at room temperature. DABCO was added to prepared slides before viewing using a confocal laser scanning microscope (CLSM; Nikon TE 2000; Tokyo, Japan).

Statistical analysis. All data, unless stated otherwise, represent mean values of three biological replicates for each studied. Where appropriate, standard deviations and student *t*-tests were calculated to determine the statistical significance of data between the different experimental conditions. A *p* value equal or below 0.05 was regarded as statistically significant.

2.4 RESULTS AND DISCUSSION

Ibuprofen has greater inhibitory effect than aspirin. The need for effective antifungal drugs for the better management of cryptococcal infections necessitated this study into the activity of anti-inflammatory drugs, namely aspirin and ibuprofen. Table 1 details the effect of aspirin and ibuprofen on the metabolic activity of five *C. neoformans* strains and five *C. gattii* strains. All ten tested strains showed a dose dependent response profile towards both test drugs when compared to the drug-free control. However, a closer examination of the comparative table revealed that: 1) *C. neoformans* strains were more sensitive relative to *C. gattii* strains, and 2) ibuprofen had greater inhibitory effect than aspirin, on all strains, at each drug concentration i.e. 0.01 mM (1.80 µg/ml for aspirin and 2.06 µg/ml for ibuprofen), 0.1 mM (18.0 µg/ml for aspirin and 20.6 µg/ml for ibuprofen) and 1 mM (180 µg/ml for aspirin and 206 µg/ml for ibuprofen). The greatest growth reduction was achieved at the highest concentration for both drugs. For aspirin, 180 µg/ml is within the recommended concentration in the blood (16) while 206 µg/ml ibuprofen is four times the recommended concentration (17). However, literature

documents that ibuprofen is rapidly metabolised in plasma compared to aspirin (18). Importantly, patients who are known to have overdosed on ibuprofen – in one case consuming more than 20 times the peak plasma concentration seen after a single dose of 400 mg ibuprofen (17, 19), are reported to have experienced “no to mild symptoms” and “without experiencing sequela” (17, 20).

All subsequent tests were performed on *C. neoformans* strain LMPE 046, as this strain showed the greatest sensitivity towards all test drugs, including fluconazole and amphotericin B (Tables S1 and S2).

In the present study, it was determined that aspirin at 180 µg/ml and ibuprofen at 206 µg/ml were non-toxic to macrophages - as both drugs did not yield a 50% reduction in the growth of macrophages (Fig. 1). To the point, aspirin effected only 11% reduction in growth while ibuprofen effected a 13% reduction. Importantly, the observed drug effect was not due to drug vehicles. For this study, macrophages were chosen as a model cell line as cryptococcal cells have been reported to take up residency within these immune cells without inducing an immunological response (21). Further to the point, these drugs enhanced the capability of macrophages to phagocytose cryptococcal cells (including beads) in the presence of aspirin or ibuprofen (yielded significantly higher relative fluorescence units (RFU) values) when compared to the absence of these drugs (yielded significantly lower RFU values) after 2 h and 6 h (Fig. 2). The phagocytic capability of macrophages was also shown to increase over time

when studying the RFUs values at both 2 h and 6 h. Once more, the observed effect is not due to drug vehicles. Fig. 2 findings further support the argument that aspirin and ibuprofen, at their respective MICs, do not negatively affect macrophages as shown in Fig. 1.

Ibuprofen acts in synergy with fluconazole and amphotericin B. In our study, the MICs for fluconazole and amphotericin B were defined as 8 µg /ml and 1 µg /ml, respectively (Tables S1 and S2). Since ibuprofen was more effective than aspirin, it was subsequently paired with fluconazole or with amphotericin B (Table 2). The combined effect of all drug pairings did not yield total growth inhibition. Nonetheless, synergistic outcomes were observed. More encouragingly, all drugs (ibuprofen, fluconazole and amphotericin B) were able to effect synergism at concentrations that were lower than their individually defined MICs. The latter thus speak to the possible clinical application of ibuprofen with amphotericin B or ibuprofen with fluconazole in combined therapy. It is, however, important at the same time to point out that careful consideration should be taken when designing such studies in order to realise the desired therapeutic outcome to the exclusion of adverse effects.

When considering the effects of aspirin and ibuprofen on the outer ultrastructure of strain LMPE 046, we noted no differences with respect to how cells, across the different experimental conditions, looked i.e. degree of smoothness or roughness (Fig. 3). However, both drugs were able to drastically reduce the size ($p < 0.05$) of treated

cells relative to non-treated cells (Table S3). More to the point, non-treated cells were on average 3.96 µm (+/- 0.08) in diameter compared to aspirin-treated cells that were 2.90 µm (+/- 0.07) and ibuprofen cells that were 3.12 µm (+/- 0.08). However, when examining the inner ultrastructure of cells using NanoSAM (Fig. 4), we could differentiate treated cells from non-treated cells based on appearances. To be specific, the topography of non-treated cells appeared to be rougher at the depths of 0 nm (outer ultrastructure) and 60 nm (inner ultrastructure) compared to aspirin-treated cells and ibuprofen-treated cells, which were less rough. The observed altered organisation of the cell membrane/wall may result in cells expressing a different physiological outcome in response to aspirin- and ibuprofen-treatment.

Aspirin and ibuprofen induce stress in treated cells. The HOG pathway is well studied in yeasts (22) and the pathway is said to be activated in response to, among others, osmotic stress, high temperature and UV irradiation (23). Bahn et al. reported that phosphorylation of Hog1 MAPK activates downstream target genes to effect adaptation to environmental stressor (24). The Hog1 signalling pathway, is interestingly also reported to be responsible for the maintenance of cell membrane/wall integrity (13, 14). Thus in our study, we sought to establish if treating cells with aspirin and ibuprofen can cause membrane damage, and in turn, lead to activation of Hog1 MAPK. When exposed to NaCl, H_2O_2, aspirin and ibuprofen, the cells yielded an expected protein band of 50 kDa that was detected using an antibody specific for Hog1 MAPK (Fig. 5a). However, in order to determine the phosphorylation state and activation levels of this MAPK protein, we performed a phospho-p-38 MAPK ELISA assay (Fig. 5b). Based on our data, the MAPK levels that were induced by aspirin and

ibuprofen were highly comparable ($p > 0.05$) to levels induced by the positive control. Additionally, the levels induced by the two test anti-inflammatory drugs, including the positive control, were significantly higher ($p < 0.05$) compared to levels in non-treated cells, which provided a baseline for referencing. The latter points to activation of Hog1 MAPK possibly as a result of an assault on the membrane caused by aspirin- and ibuprofen-treatment.

Aspirin and ibuprofen kill cells via ROS-mediated damage. In addition to regulating transportation of materials needed for survival, the membrane is documented to serve as a site for energy generation (25), which is critical in respiring cells like *Cryptococcus* species. In particular, Joshi and Bakowska proposed that the membrane potential is important for maintaining the function of the respiratory chain in order to generate energy (26), and thus loss of membrane potential may prove to have dire consequences. In this part of the study, we show that aspirin- and ibuprofen-treated cells were characterised by a significant loss of membrane potential ($p < 0.01$) when compared to non-treated cells (Fig. 6a). Under normal physiological conditions, respiring cells are reported to produce ROS as part of their oxygen metabolism (27). Further to the point, it is reasonable to conclude that an increase in ROS levels may also manifest when a stressor targeting the site of respiration i.e. membrane, alters the membrane potential as well as its capacity to recycle co-factors. To demonstrate this point, we show that loss of membrane potential was associated with a concomitant increase in ROS levels (Fig. 6b). Here, aspirin- and ibuprofen-treated cells significantly ($p < 0.01$) accumulated ROS compared to non-treated cells. This accumulation of ROS

underlies the inescapable fate of cells - wherein they die as a result of oxidative damage to the membrane (Figs. 7 and 8). For both the staining experiments i.e. PI and DAPI, the microscope settings were adjusted by normalising the gain of blue, green and red lights to a unit of 1.0, in order to compare the light intensity of images across the different experimental conditions. Fig. 7a represents cells (non-treated cells, aspirin- and ibuprofen-treated cells) that were stained with PI stain. The PI was used as an exclusion stain - wherein cells with impaired membrane function were expected to accumulate the stain. The merged white and fluorescent images (middle panel) and fluorescent only images (right panel) show clear accumulation of PI inside all examined cells. Similar imaging results were obtained for other PI biological replicates. Interesting, for non-treated cells, we chose a budding cell that clearly show a mother cell with intracellular accumulation and a daughter cell that accumulated minimal amounts of PI and fluoresced less intensely. Conversely, the aspirin-treatment micrograph shows both the mother cell and daughter cell with more intracellular accumulation and fluoresced more intensely relative to the non-treated budding cell. Fig. 7b show the measurement of intracellular metabolites that leaked into the culture supernatants across the different experimental conditions. Aspirin and ibuprofen caused a significant ($p < 0.01$) accumulation of adenylate kinase in supernatants compared to non-treated cells. We also assessed membrane permeability using DAPI to determine if the stain can cross the cell membrane and reach the nuclei in order to react with the DNA (Fig. 8). Interestingly, in non-treated cells, the stain localised at the membrane and could not be transported across healthy intact membranes. However, in cells with damaged membranes (aspirin- and ibuprofen-treated cells) the stain was able to cross the

membrane and eventually reacted with the DNA as depicted in the combined white and fluorescent images (middle panel) and fluorescent only images (right panel). Similar imaging results were obtained for other DAPI biological replicates.

2.5 CONCLUSIONS

Previously registered non-antimicrobial drugs have been given a “new lease on life” following the discovery that they also possess antimicrobial qualities (28). Aspirin and ibuprofen are such drugs. This study successfully demonstrated *in vitro* anti-*Cryptococcus* activity of aspirin and ibuprofen. Also encouraging, is the finding that ibuprofen acted in synergy with fluconazole and amphotericin B. When considered in totality, this study’s findings highlighted the membrane as a target site for aspirin and ibuprofen action. Through altering membrane integrity, from disrupting membrane potential to compromising transportation across the bilayer, these two anti-inflammatory drugs may have revealed a mechanism that is efficient in killing respiring cells, although in eukaryotic organisms of lower order. It therefore becomes important to demonstrate that the repurposing of these drugs will work in higher eukaryotic host cells. Towards this end, animal studies should be performed to model the treatment of cryptococcal infections using aspirin and ibuprofen in order to establish their therapeutic benefits. The duality in function of these drugs i.e. as anti-inflammatory drugs and now as antifungal agents may provide an additional beneficial therapeutic outcome. Therefore, it will also be interesting to see the clinical effect of these drugs on inflammatory

conditions induced by pathogens such as in the treatment of pathogen-induced immune reconstitution inflammatory syndrome. It will also be important to determine if the demonstrated antimicrobial activity of aspirin and ibuprofen could be expanded to other medically important pathogens. Particularly in microbes such as *Pseudomonas* species and *Mycobacterium* species, which, like *Cryptococcus* species, are highly aerobic.

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TABLE 1. The effect of aspirin and ibuprofen on the metabolic activity of *C. neoformans* and *C. gattii.*

Species detail			**Drug response**					
		Non-treated cells	**Aspirin-treated cells**			**Ibuprofen-treated cells**		
			1.80 µg/ml	**18.0 µg/ml**	**180 µg/ml**	**2.06 µg/ml**	**20.6 µg/ml**	**206 µg/ml**
Strain name	**Strain number**	**$OD_{562\ nm}$**	**%GR**	**%GR**	**%GR**	**%GR**	**%GR**	**%GR**
Cr. neoformans	LMPE 028	0.403 (0.009)	11 (0.008)	32 (0.044)	71 (0.011)	13 (0.014)	36 (0.007)	75 (0.021)
Cr. neoformans	LMPE 030	0.411 (0.014)	10 (0.022)	34 (0.007)	72 (0.007)	12 (0.009)	37 (0.021)	74 (0.022)
Cr. neoformans	LMPE 043	0.398 (0.005)	12(0.041)	31 (0.005)	70 (0.018)	13 (0.009)	34 (0.034)	75 (0.015)
Cr. neoformans	LMPE 046	0.402(0.032)	14 (0.036)	38 (0.052)	74 (0.009)	16 (0.017)	40 (0.015)	76 (0.009)
Cr. neoformans	LMPE 047	0.401 (0.012)	13 (0.049)	35 (0.051)	73 (0.016)	14 (0.034)	37 (0.022)	74 (0.013)
Cr. gattii	LMPE 045	0.401 (0.026)	10 (0.007)	30 (0.029)	70 (0.012)	11 (0.023)	32 (0.015)	73 (0.011)
Cr. gattii	LMPE 048	0.400 (0.036)	12 (0.018)	32 (0.022)	72 (0.009)	13 (0.007)	33 (0.043)	74 (0.032)
Cr. gattii	LMPE 052	0.390 (0.009)	10 (0.021)	29 (0.014)	69 (0.021)	12 (0.016)	31 (0.012)	71 (0.013)
Cr. gattii	LMPE 054	0.401(0.015)	11 (0.009)	31 (0.008)	70 (0.007)	12 (0.030)	32 (0.006)	72 (0.016)
Cr. gattii	LMPE 070	0.394 (0.007)	10 (0.008)	30 (0.022)	70 (0.011)	11 (0.031)	31 (0.041)	71 (0.008)

Percent growth reduction was calculated as 100% - [(OD of treated cells/OD of non-treated cells) · 100%]. Values represent the mean values from three biological replicates, and values in parentheses represent standard deviations. %GR = Percentage growth reduction.

TABLE 2. The combined effect of ibuprofen and amphotericin B and ibuprofen and fluconazole of *C. neoformans* strain LMPE 046.

	Percentage (%) Growth reduction					**Fractional inhibitory concentration (FIC) index**				
Ibuprofen	**Amphotericin B**					**Amphotericin B**				
	0.25 µg/ml	**0.5 mg/L**	**1 µg/ml (MIC)**	**2 µg/ml**	**4 µg/ml**	**0.25 µg/ml**	**0.5 mg/L**	**1 µg/ml (MIC)**	**2 µg/ml**	**4 µg/ml**
52 µg/ml	41	49	60	75	79	1.03	0.91	0.45	0.32	0.30
103 µg/ml	47	57	68	74	88	0.83	0.42	0.41	0.4	0.23
206 µg/ml (MIC)	79	81	85	89	92	0.41	0.31	0.28	0.21	0.13
412 µg/ml	80	84	88	91	96	0.38	0.24	0.2	0.18	0.11
824 µg/ml	83	88	91	95	99	0.3	0.2	0.12	0.09	0.05
	Percentage (%) Growth reduction					**Fractional inhibitory concentration (FIC) index**				
Ibuprofen	**Fluconazole**					**Fluconazole**				
	2 µg/ml	**4 µg/ml**	**8 µg/ml (MIC)**	**16 µg/ml**	**32 µg/ml**	**2 µg/ml**	**4 µg/ml**	**8 µg/ml (MIC)**	**16 µg/ml**	**32 µg/ml**
52 µg/ml	46	55	62	69	77	0.96	0.48	0.45	0.41	0.35
103 µg/ml	48	61	68	73	81	0.89	0.46	0.43	0.37	0.34
206 µg/ml (MIC)	68	77	79	82	86	0.4	0.37	0.36	0.34	0.23
412 µg/ml	79	81	84	87	90	0.33	0.28	0.26	0.22	0.16
824 µg/ml	80	85	88	92	99	0.24	0.21	0.18	0.15	0.1

Shading indicates synergism. The other shaded cells represent the corresponding % growth reduction values in relation to the FICI.

TABLE 3. The effect of amphotericin B on the metabolic activity of *C. neoformans* and *C. gattii.*

Species detail			**Drug response**							
		Non-treated cells	**Amphotericin B-treated cells**							
			16 µg/ml	**8 µg/ml**	**4 µg/ml**	**2 µg/ml**	**1 µg/ml**	**0.5 µg/ml**	**0.25 µg/ml**	**0.125 µg/ml**
Strain name	**Strain number**	**OD $_{562\ nm}$**	**%GR**	**%GR**	**%GR**	**%GR**	**%GR**	**%GR**	**%GR**	**%GR**
Cr. neoformans	LMPE 028	0.393 (0.014)	95 (0.005)	84 (0.011)	73 (0.021)	66 (0.014)	56(0.014)	46 (0.007)	37 (0.014)	26 (0.007)
Cr. neoformans	LMPE 030	0.415 (0.025)	96 (0.012)	84 (0.050)	74 (0.007)	64 (0.007)	55 (0.009)	47 (0.017)	38 (0.009)	27 (0.017)
Cr. neoformans	LMPE 043	0.405 (0.015)	95 (0.040)	83 (0.015)	72 (0.005)	63 (0.018)	55 (0.012)	44 (0.014)	37 (0.014)	24 (0.014)
Cr. neoformans	LMPE 046	0.403 (0.024)	98 (0.083)	86 (0.012)	77 (0.009)	68 (0.009)	59 (0.007)	48 (0.005)	39 (0.047)	29 (0.005)
Cr. neoformans	LMPE 047	0.398 (0.015)	96 (0.049)	85 (0.051)	76 (0.012)	66 (0.018)	56(0.014)	47 (0.014)	38 (0.014)	27 (0.015)
Cr. gattii	LMPE 045	0.403 (0.016)	92 (0.016)	80 (0.015)	69 (0.013)	61 (0.014)	52(0.013)	42 (0.005)	33 (0.005)	22 (0.020)
Cr. gattii	LMPE 048	0.399 (0.016)	95 (0.008)	82 (0.021)	75 (0.008)	65 (0.009)	56 (0.018)	43 (0.016)	36 (0.016)	25 (0.033)
Cr. gattii	LMPE 052	0.386 (0.012)	94 (0.013)	79 (0.014)	71 (0.020)	64 (0.013)	53 (0.014)	41 (0.012)	34 (0.013)	21 (0.012)
Cr. gattii	LMPE 054	0.409 (0.016)	93 (0.008)	81 (0.008)	70 (0.011)	62 (0.014)	54 (0.024)	43 (0.006)	32 (0.050)	23 (0.006)
Cr. gattii	LMPE 070	0.389 (0.014)	91 (0.019)	80 (0.019)	70 (0.009)	60 (0.009)	52 (0.007)	42 (0.015)	32 (0.009)	22 (0.015)

%GR = % growth reduction. Values represent mean values of three biological replicates and values in brackets represent the standard deviation.

TABLE 4. The effect of fluconazole on the metabolic activity of *C. neoformans* and *C. gattii.*

Species detail			Drug response							
		Non-treated cells	Fluconazole-treated cells							
			128 µg/ml	64 µg/ml	32 µg/ml	16 µg/ml	8 µg/ml	4 µg/ml	2 µg/ml	1 µg/ml
Strain name	**Strain number**	**OD $_{562\ nm}$**	**%GR**	**%GR**	**%GR**	**%GR**	**%GR**	**%GR**	**%GR**	**%GR**
Cr. neoformans	LMPE 028	0.393 (0.014)	88 (0.009)	82 (0.014)	71 (0.015)	62 (0.014)	55 (0.019)	44(0.006)	41 (0.022)	37 (0.007)
Cr. neoformans	LMPE 030	0.415 (0.025)	87 (0.012)	81 (0.022)	74 (0.007)	64 (0.009)	54 (0.007)	45 (0.009)	42 (0.017)	38 (0.019)
Cr. neoformans	LMPE 043	0.405 (0.015)	90 (0.050)	83 (0.021)	70 (0.013)	61 (0.021)	55 (0.005)	45 (0.031)	42 (0.014)	37 (0.031)
Cr. neoformans	LMPE 046	0.403 (0.024)	91 (0.006)	85 (0.006)	76 (0.008)	66 (0.017)	57 (0.013)	47 (0.013)	44 (0.011)	39 (0.007)
Cr. neoformans	LMPE 047	0.398 (0.015)	89 (0.019)	84 (0.017)	74 (0.014)	65 (0.041)	56 (0.015)	45 (0.014)	43 (0.009)	38 (0.005)
Cr. gattii	LMPE 045	0.403 (0.016)	84 (0.007)	80 (0.009)	70 (0.013)	60 (0.009)	52 (0.013)	44 (0.007)	40 (0.575)	35 (0.013)
Cr. gattii	LMPE 048	0.399 (0.016)	89 (0.006)	82 (0.012)	73 (0.008)	62 (0.008)	55 (0.008)	46 (0.016)	43 (0.063)	36 (0.015)
Cr. gattii	LMPE 052	0.386 (0.012)	86 (0.014)	79 (0.017)	68 (0.015)	59 (0.014)	53 (0.010)	43 (0.014)	41 (0.042)	35 (0.016)
Cr. gattii	LMPE 054	0.409 (0.016)	85 (0.012)	81 (0.008)	70 (0.009)	61 (0.008)	50 (0.021)	44 (0.008)	41 (0.036)	34 (0.030)
Cr. gattii	LMPE 070	0.389 (0.014)	84 (0.009)	80 (0.009)	70 (0.019)	60 (0.019)	50 (0.009)	44 (0.041)	42 (0.011)	35 (0.021)

%GR = % growth reduction. Values represent mean values of three biological replicates and values in brackets represent the standard deviation.

TABLE 5. Characterisation of cell size following drug treatment. To determine the cell size, the cell diameter of a 100 cells was measured per SEM micrograph, in which a micrograph represented a different position on the SEM stub. In total 10 positions were considered.

Cell size characterisation		
Drug		**Effect on cells**
Cell treatment	**Drug concentration**	**Cell diameter**
Non-treated cells	0 µg/ml	3.96 µm (+/- 0.08)
Aspirin-treated cells	180 µg/ml	2.90 µm (+/- 0.07)
Ibuprofen-treated cells	206 µg/ml	3.12 µm (+/- 0.08).

$p < 0.05$ (non-treated cells and aspirin-treated cells) and $p < 0.05$ (non-treated cells and ibuprofen-treated cells).

FIGURE LEGENDS

FIG 1. The direct effect of aspirin and ibuprofen on macrophage (MØ) growth (A) and the corresponding expression of this effect as percentage (%) reduction in growth (B). Analysis of data revealed that aspirin at 180 µg/ml (1mM) effected only 11% reduction in growth while ibuprofen at 206 µg/ml (1mM) effected 13% reduction. The findings suggest that aspirin and ibuprofen, at tested concentrations, are non-toxic to macrophages – as they did not yield a 50% reduction in growth. Ethanol (EToH) and DMSO were included as controls.

FIG 2. The chemosensitising of macrophage (MØ) by aspirin and ibuprofen. The capability of macrophages to internalise cryptococcal cells in the absence (non-treated cells) and presence of aspirin or ibuprofen was measured using the phagocytosis stain, pHrodo® Green Zymosan A BioParticles, after 2 h (A) and 6 h (B). Addition of aspirin and ibuprofen significantly enhanced ($p > 0.05$) the capability of macrophages to internalise cryptococcal function compared to non-treated cells at both time intervals. This further indicates that the two drugs do not negatively affect macrophages as suggested in Figure 1. Ethanol (EToH), DMSO and beads were included as controls.

FIG 3. Scanning electron micrographs depicting the effect of aspirin and ibuprofen on the outer ultrastructure of cells. Cells from the different experimental conditions i.e. non-treated, aspirin-treated and ibuprofen-treated, could not be differentiated based on their degree of smoothness or roughness. However, they could be differentiated based on their cell diameter (Table S5).

FIG 4. Nano-scanning auger micrographs depicting the effect of aspirin and ibuprofen on the outer and inner ultrastructure of cells. Cells from the different experimental conditions i.e. non-treated, aspirin-treated and ibuprofen-treated, could be differentiated based on their appearances. For examples, non-treated cells appeared to be rough with spiky protuberances compared to aspirin- and ibuprofen-treated cells, which were less rough. The images were taken at the depth of 0 nm and 60 nm following etching thin slices off the cell using an argon at a sputter rate of 15 nm/min.

FIG 5. Aspirin- and ibuprofen-treatment activates a stress response pathway. (A) Western blot analysis using antibodies specific for Hog1 MAPK. The cells were treated for 20 min and a representative is blot is shown depicting the expected protein band of 50 kDa. NaCl and H_2O_2 were included as positive controls. (B) Quantitative analysis examining the phosphorylation status of the MAPK. The p-38 phosphorylation levels of aspirin- and ibuprofen-treated cells were highly comparable ($p > 0.05$) to that of the positive control (supplied by the manufacturer), and were at the same time, significantly higher ($p < 0.05$) compared to non-treated cells.

FIG 6. Aspirin- and ibuprofen-treated cells subjected to oxidative stress. (A) Shows evidence for loss of membrane potential in aspirin- and ibuprofen-treated cells on the account of more monomeric form compared to J-aggregate. Loss of membrane potential is concomitantly associated with ROS accumulation, as the membrane potential is a driving force that leads to energy production in respiring cells. (B) Evidence for ROS accumulation in treated cells. Aspirin- and ibuprofen-treated cells accumulated more ROS compared to non-treated cells.

FIG 7. The effect of aspirin and ibuprofen of membrane function. (A) Confocal micrographs depicting cells, from different experimental conditions, stained with PI. When cells were treated with aspirin or ibuprofen, more of the PI stain accumulated inside the cells - based on the fluorescence intensity after the gain of blue, green and red light was normalised to 1.0. Left panel = white light micrographs, Middle panel = white light superimposed with fluorescent light micrographs, Right panel = fluorescent light micrographs. (B) Toxilight® Bioassay results. When exposed aspirin and ibuprofen cells significantly ($p < 0.01$) secreted more intracellular metabolites compared to non-treated cells.

FIG 8. Confocal micrographs depicting cells, from different experimental conditions, stained with DAPI. When cells were treated with aspirin or ibuprofen, the DAPI stain crossed the damaged cell membrane to reach the cell nuclei and react with DNA. However, in non-treated cells with intact cell membranes, the DAPI stain localized at the membrane and could not be transported inside the cell. Left panel = white light micrographs, Middle panel = white light superimposed with fluorescent light micrographs, Right panel = fluorescent light micrographs.

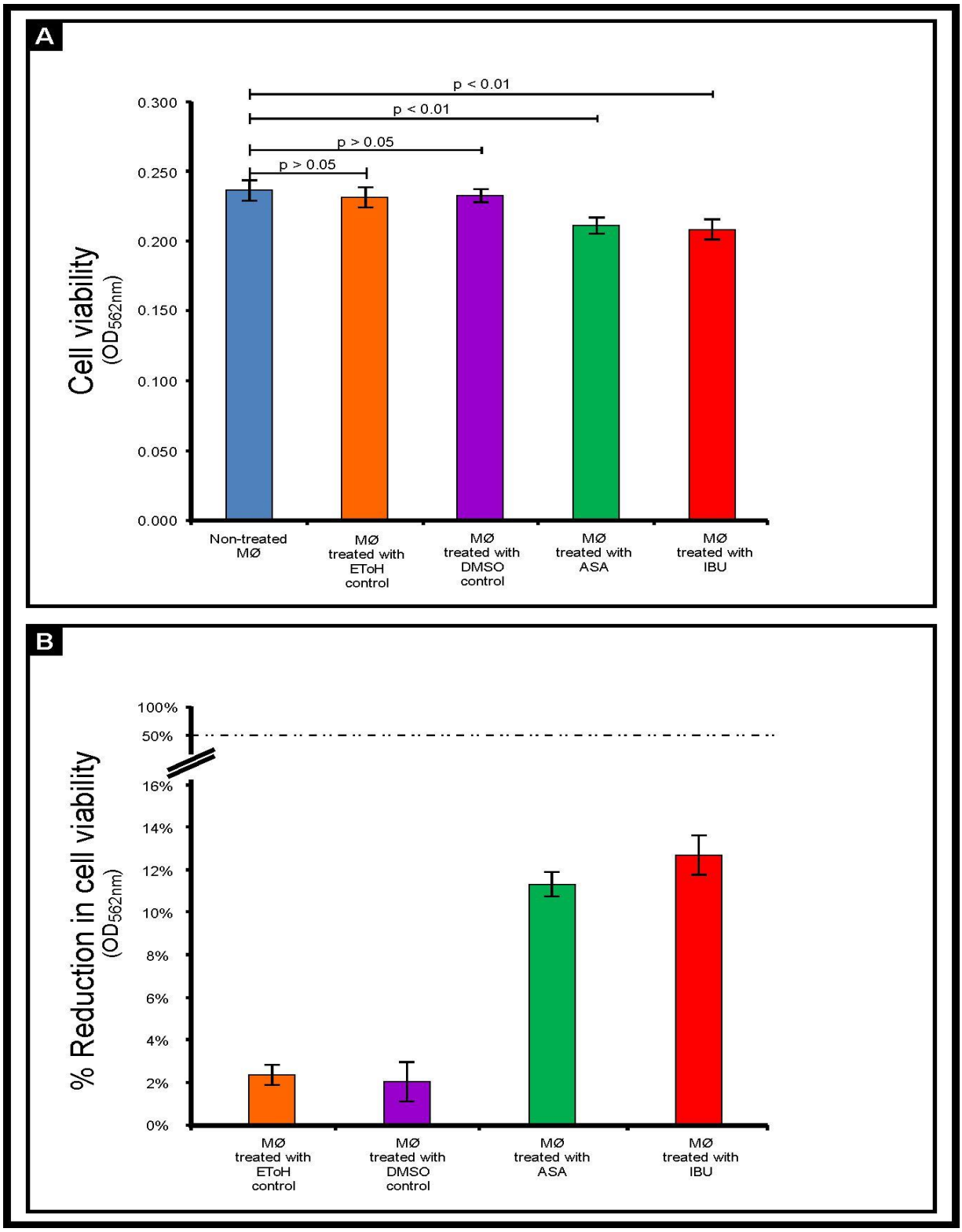
A
p < 0.01
p < 0.01
p > 0.05
p > 0.05
0.300
0.250
0.200
0.150
0.100
0.050
0.000
Cell viability
(OD562nm)
Non-treated MØ
MØ treated with EToH control
MØ treated with DMSO control
MØ treated with ASA
MØ treated with IBU
B
100%
50%
16%
14%
12%
10%
8%
6%
4%
2%
0%
% Reduction in cell viability
(OD562nm)
MØ treated with EToH control
MØ treated with DMSO control
MØ treated with ASA
MØ treated with IBU

FIG 1

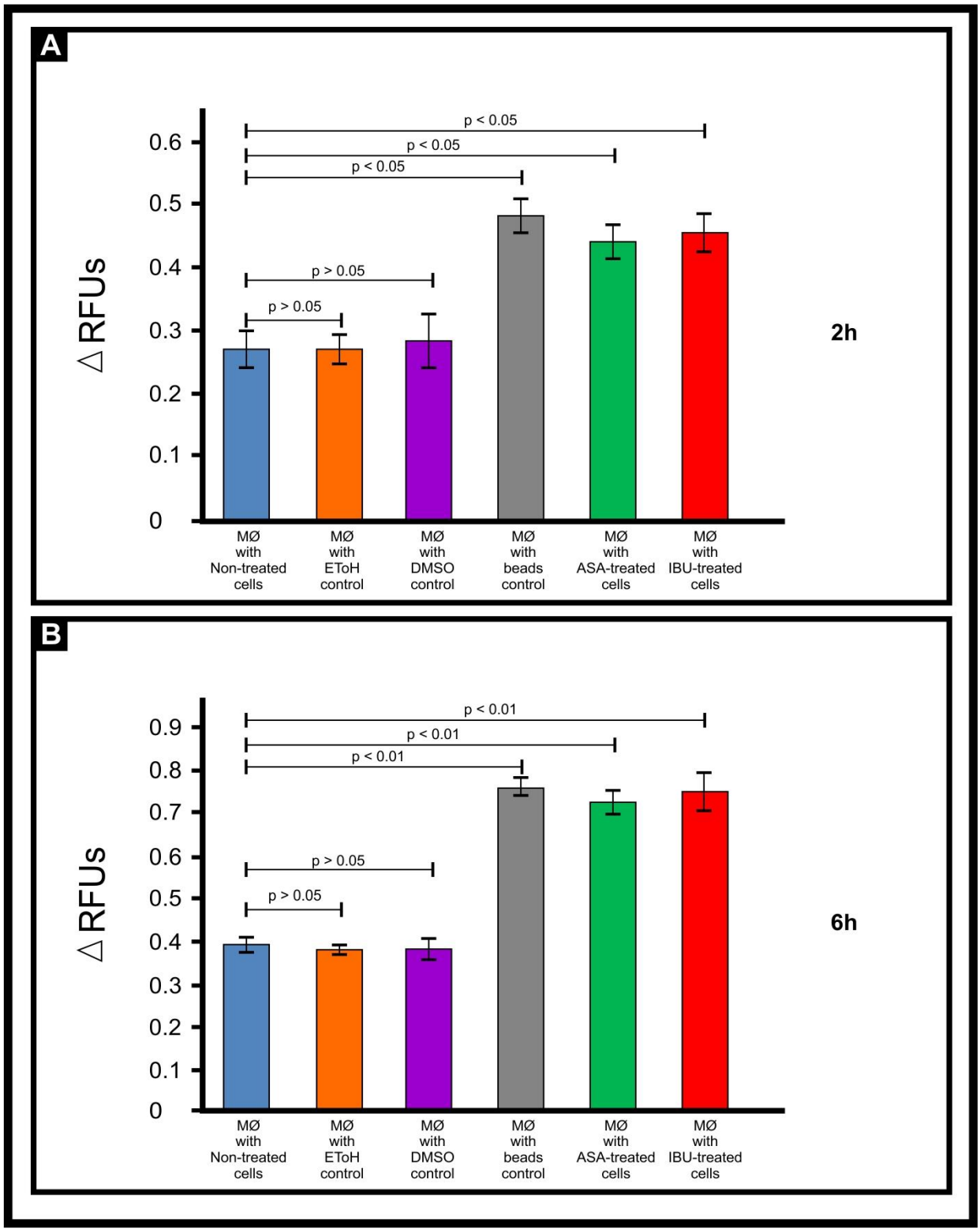
A
△RFUs
0.6
0.5
0.4
0.3
0.2
0.1
0
p < 0.05
p < 0.05
p < 0.05
p > 0.05
p > 0.05
MØ with Non-treated cells
MØ with ETOH control
MØ with DMSO control
MØ with beads control
MØ with ASA-treated cells
MØ with IBU-treated cells
2h
B
△RFUs
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
p < 0.01
p < 0.01
p < 0.01
p > 0.05
p > 0.05
MØ with Non-treated cells
MØ with ETOH control
MØ with DMSO control
MØ with beads control
MØ with ASA-treated cells
MØ with IBU-treated cells
6h

FIG 2

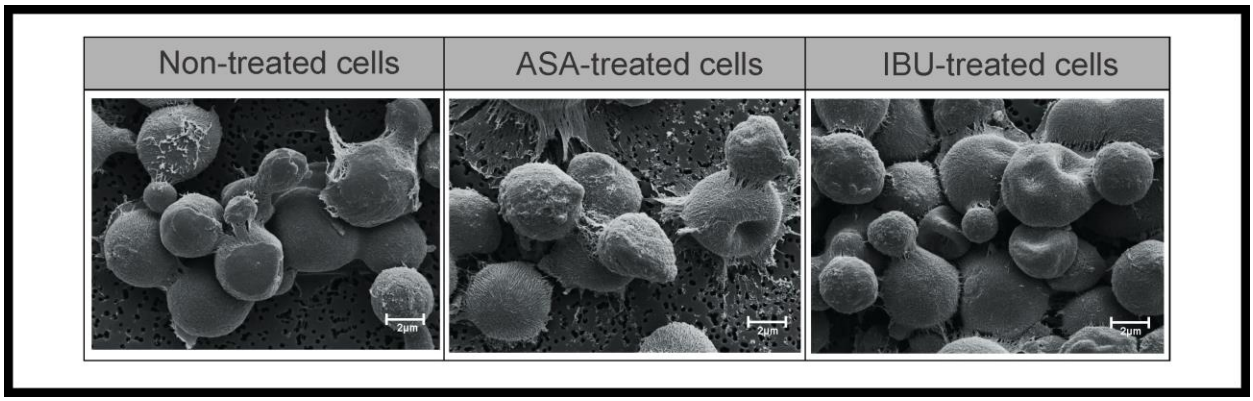
Non-treated cells
ASA-treated cells
IBU-treated cells
2µm
2µm
2µm

FIG 3

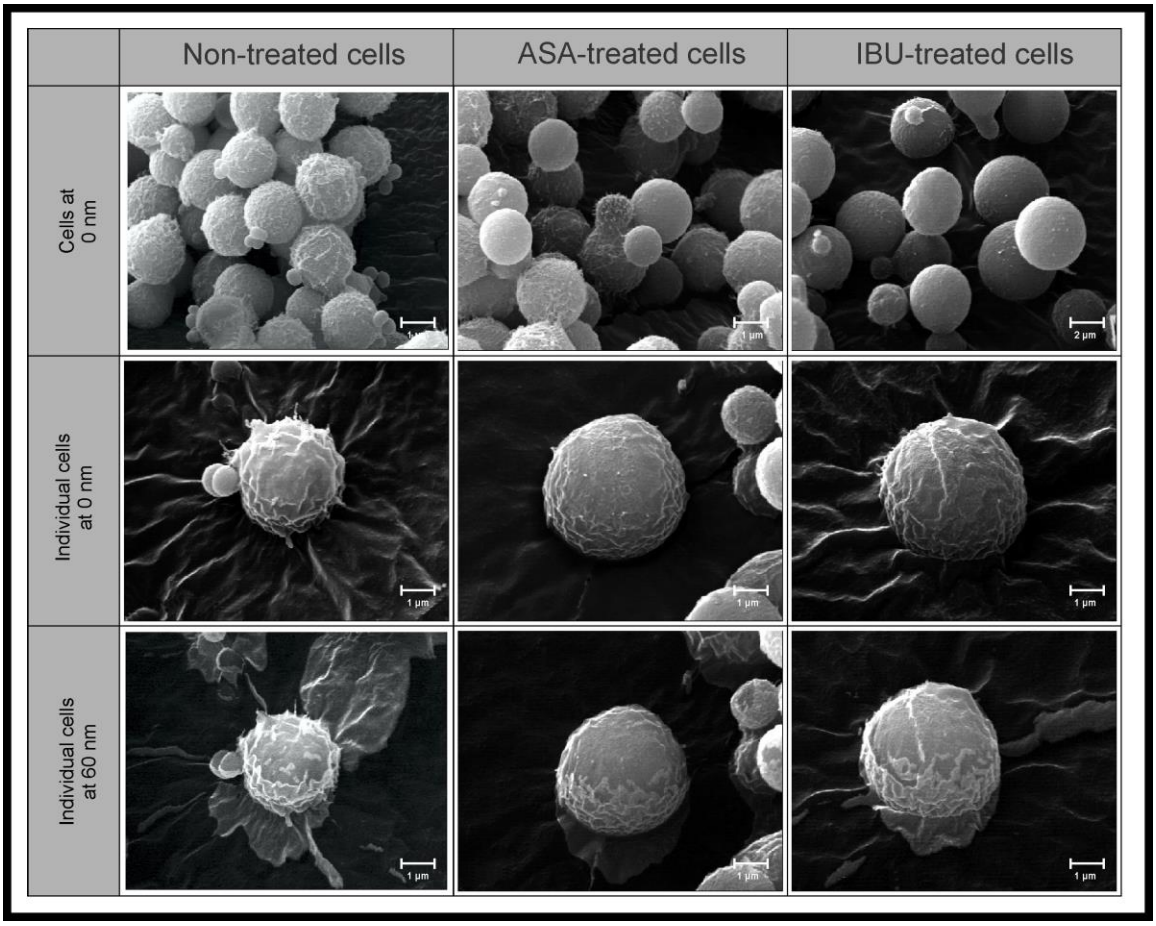
Non-treated cells
ASA-treated cells
IBU-treated cells
Cells at 0 nm
Individual cells at 0 nm
Individual cells at 60 nm
1 µm
2 µm

FIG 4

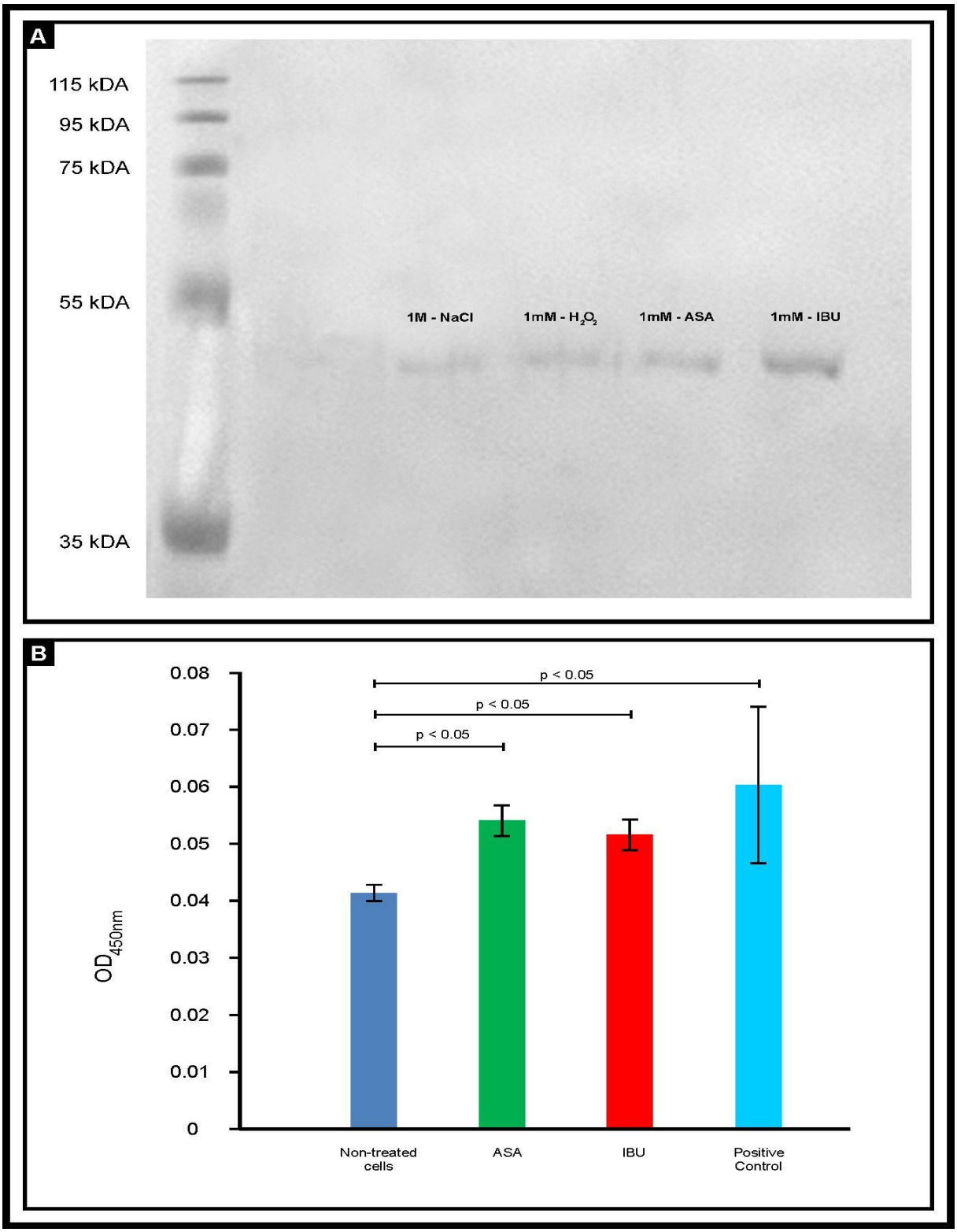
A
115 kDA
95 kDA
75 kDA
55 kDA
35 kDA
1M - NaCl
1mM - H2O2
1mM - ASA
1mM - IBU
B
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
OD450nm
p < 0.05
p < 0.05
p < 0.05
Non-treated cells
ASA
IBU
Positive Control

FIG 5

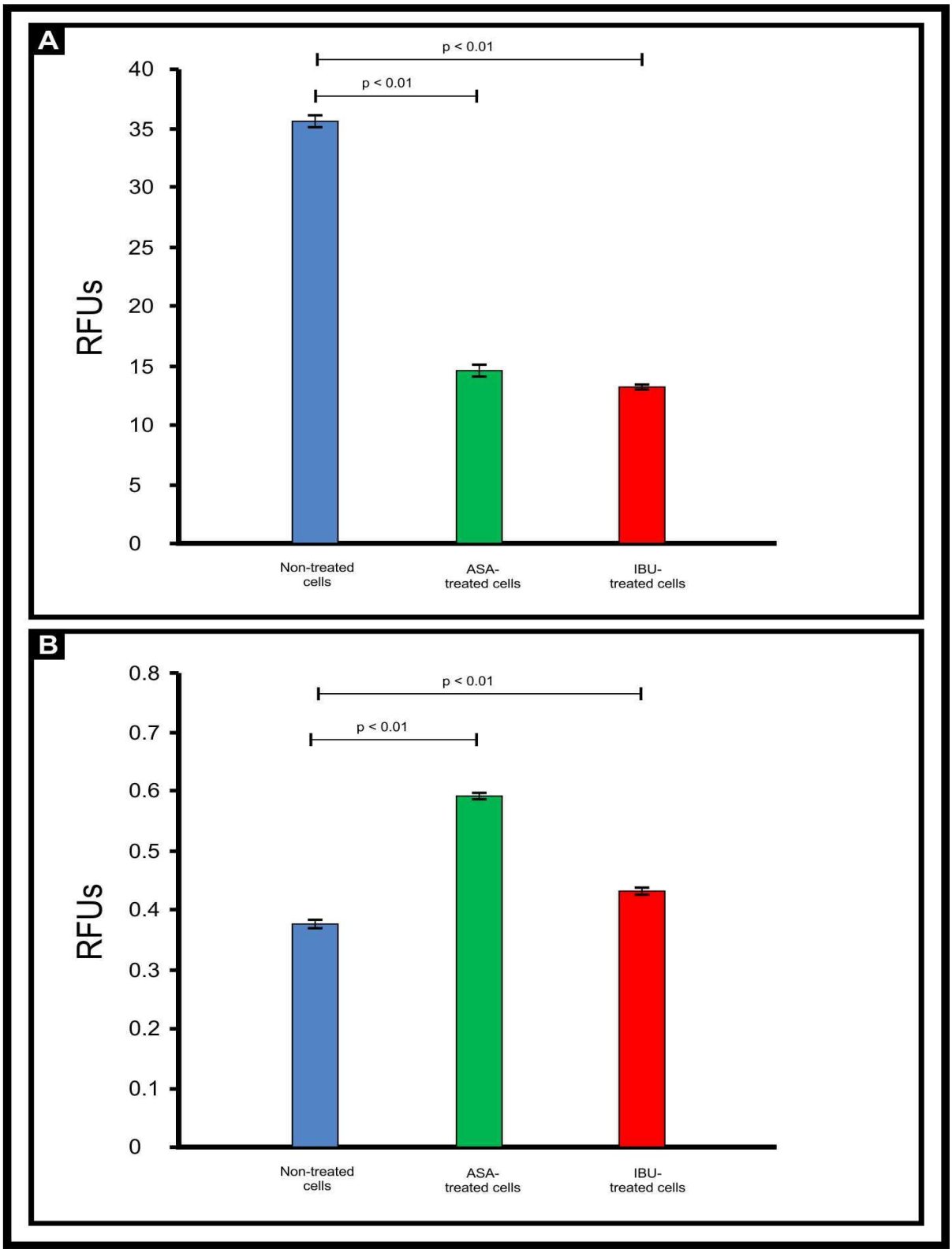
A
p < 0.01
p < 0.01
40
35
30
25
20
15
10
5
0
RFUs
Non-treated cells
ASA-treated cells
IBU-treated cells
B
p < 0.01
p < 0.01
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
RFUs
Non-treated cells
ASA-treated cells
IBU-treated cells

FIG 6

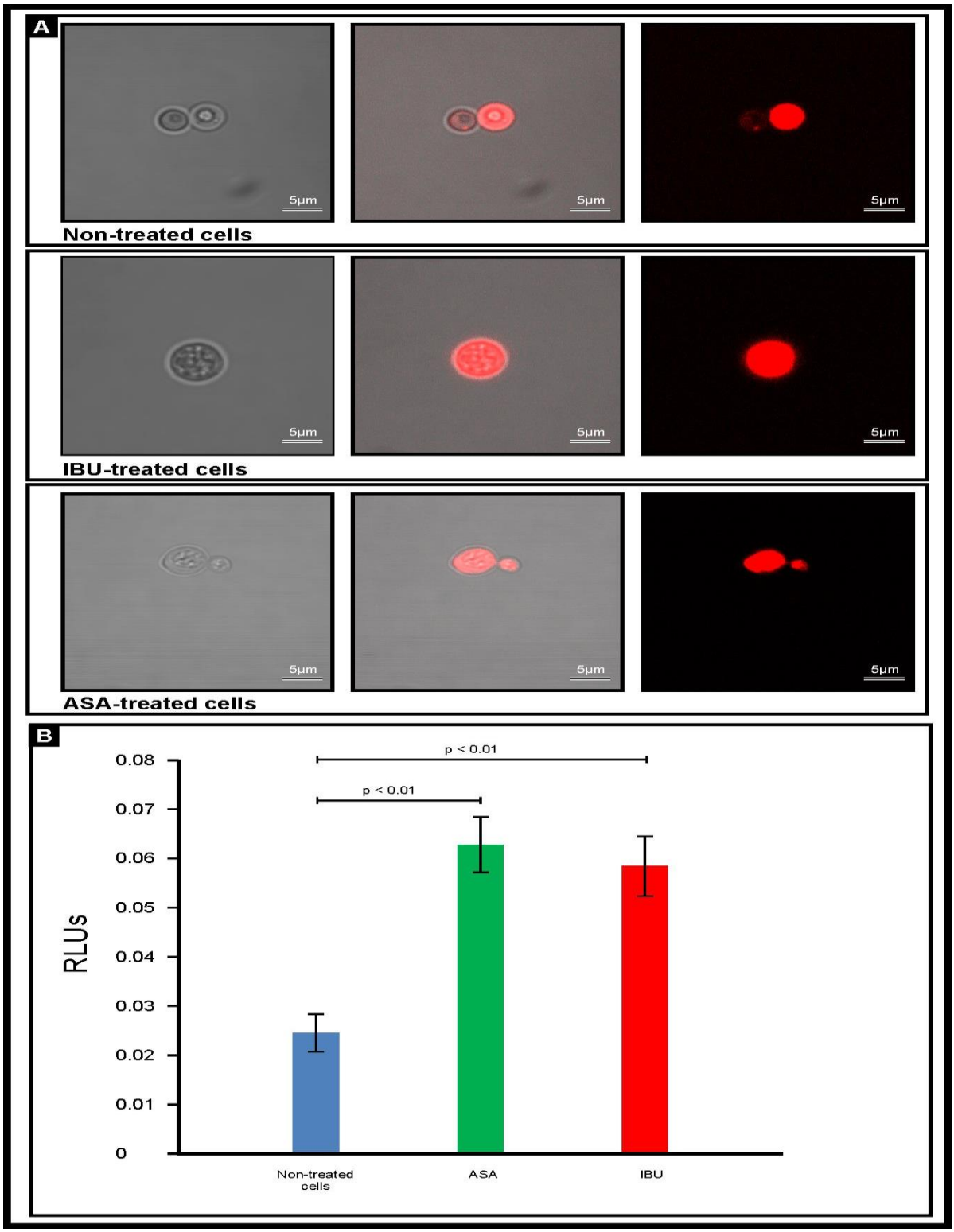
A
5µm
Non-treated cells
IBU-treated cells
ASA-treated cells
B
p < 0.01
p < 0.01
RLUs
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Non-treated cells
ASA
IBU

FIG 7

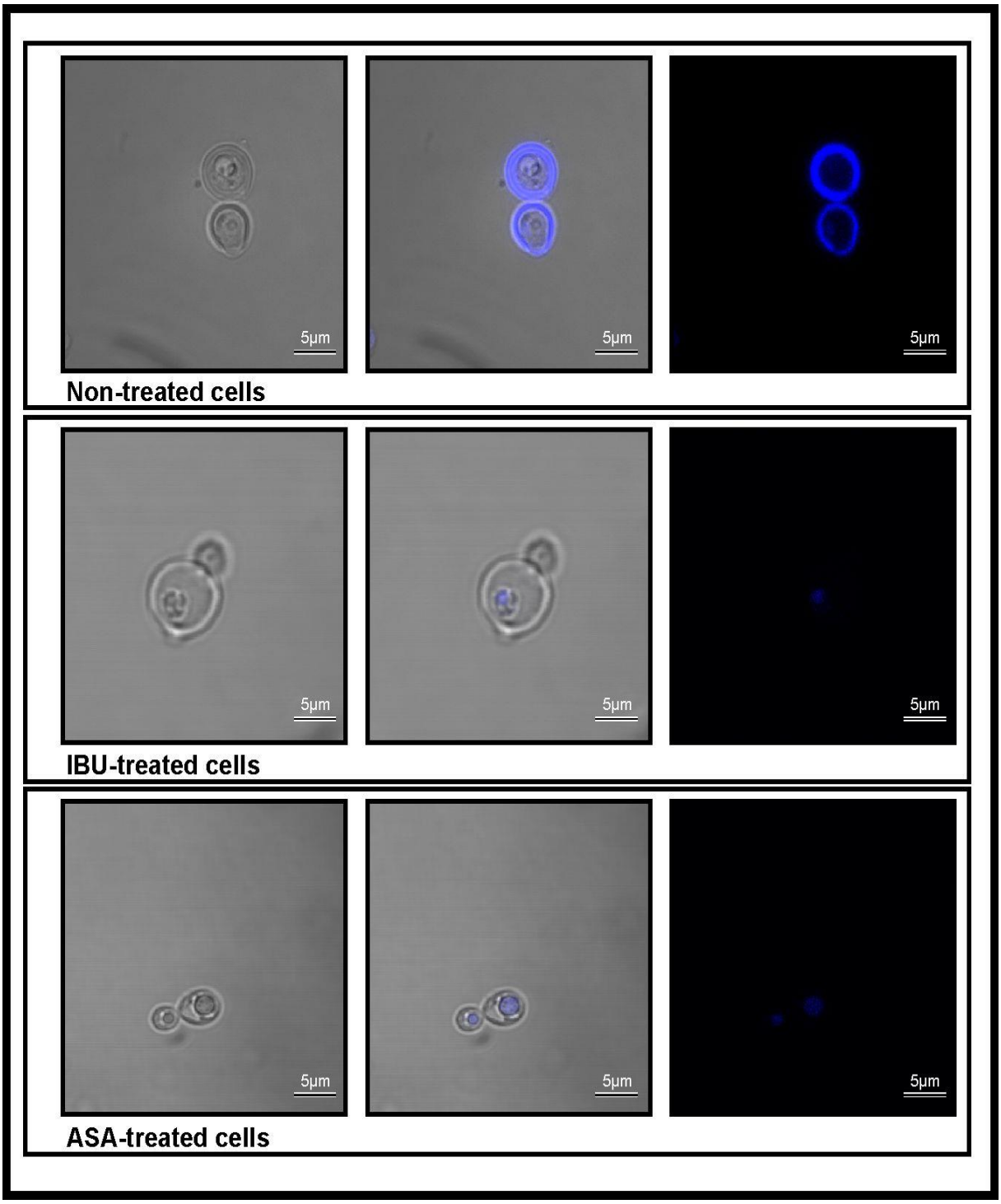
5µm
5µm
5µm
Non-treated cells
5µm
5µm
5µm
IBU-treated cells
5µm
5µm
5µm
ASA-treated cells

FIG 8

CHAPTER 3

COPPER ACYL SALICYLATE AS A POTENTIAL ANTI-*CRYPTOCOCCUS* DRUG

This chapter has been submitted for publication.

Ogundeji AO, Pohl CH, Sebolai OM. (submitted). Copper acyl salicylate as a potential anti-*Cryptococcus* drugs. Antimicrob Agents Chemother.

Candidate contribution: Co-designed the study, performed the experiments and wrote the manuscript.

3.1 ABSTRACT

The anti-fungal quality of aspirin against cryptococcal cells has been reported. However, the associated toxicity of aspirin usage in the clinical settings is of great concern. Conceivably, a derivative of aspirin could circumvent the adverse effect of aspirin. Thus, this paper reports on the effect of aspirinate-metal complex viz. Copper acyl salicylate (CAS) on the growth of cryptococcal cells. The susceptibility of *Cryptococcus* (*C.*) *neoformans* and *C. gattii* was determined. In addition, the checkerboard assay was performed to study the interaction CAS with known anti-fungals. The fungicidal mode of action against cryptococcal cells and the effect on macrophage function were also determined. CAS showed antifungal activity against all the tested strains, and also acted in synergy with fluconazole and amphotericin B. Similar to aspirin, CAS kills cells via reactive oxygen species (ROS)-mediated membrane damage. The effect of CAS did not negatively affect macrophages but rather enhanced their phagocytic function. More importantly, CAS was more effective and showed less toxicity compared with aspirin. The data presented from this study highlight the potential usefulness of CAS as an alternative anti-*Cryptococcus* drug, as this could yield a better therapeutic outcome of patients in the clinical settings.

Keywords. Aspirinate-metal complex; Copper acyl salicylate; *Cryptococcus*; Macrophages; ROS-mediated membrane damage; Synergy.

3.2 INTRODUCTION

In order to survive, microbes have had to evolve resistance against the negative effects of drugs (1). To compound this, some drugs may be ill-tolerated by patients as they also target host cells (2). For instance, amphotericin B targets renal tubules leading to renal failure (3). Thus, if these challenges are not addressed, it is clear that patients will continue to die in the foreseeable future. A possible solution may be to investigate a derivative of an already FDA-approved drug such as aspirin, as it may yield improved therapeutic outcomes.

Aspirin is a prototypical anti-inflammatory drug, which our group has shown can be repurposed as an anti-*Cryptococcus* drug (4). However, the usage of aspirin in the clinical settings can often lead to gastrointestinal toxicity (5). To overcome this, Sorenson (6) considered using an aspirinate-copper-complex (a metal complex wherein copper is bond to four aspirin ligands) as an alternative drug to circumvent the negative effects of aspirin. In their paper, they successfully demonstrated that an aspirinate-copper-complex was 30 times more effective than aspirin as an anti-inflammatory agent (6). Moreover, that aspirinate-copper-complex was pharmacologically more active in laboratory animals compared to aspirin (7, 8). Based on the above, it thus became the aim of the current study to determine if an aspirinate-copper-complex, i.e. copper acyl salicylate, could exert an anti-microbial effect on cryptococcal cells, and if the obtained results would be better than those reported by Ogundeji and co-workers on aspirin.

3.3 MATERIALS AND METHODS

Cells, cultivation and standardisation. A total of ten fungal strains collected from Universitas Academic Hospital, Bloemfontein, South Africa, were tested. These included *C. neoformans* (five strains) and *C. gattii* (five strains). The strains were stored on yeast-malt-extract (YM) agar (3000 µg/ml yeast extract, 3000 µg/ml malt extract, 5000 µg/ml peptone, 10000 µg/ml glucose, 16000 µg/ml agar; Merck, South Africa) until used. Prior to use in this study, standardised inocula were prepared in 10 ml of RPMI-1640 medium (Sigma-Aldrich, South Africa) using a final concentration of between 0.5×10^5 and 2.5×10^5 CFU/ml as described by the protocol of European Committee on Antimicrobial Susceptibility Testing (EUCAST) (9). The RAW 264.7 macrophage cell line (a kind donation by Prof Masoko and Mr Makola, University of Limpopo, South Africa) was grown at 37°C and 5% CO_2 in RPMI-1640 medium, supplemented with 10% foetal bovine serum (Biochrom, Germany), 20 U/ml penicillin, 20 g/ml streptomycin (Sigma-Aldrich, USA) and 2 mM L-glutamine (Sigma-Aldrich, South Africa). Standardised cells at 1×10^5 cells/ml were seeded into wells of a sterile, disposable 96-well flat-bottom microtitre plate (Greiner Bio-One, Germany) before use.

Drugs. Aspirinate-metal complex i.e. copper acyl salicylate (CAS) (donated from Dr Pravin S. Kendrekar, Chemistry Department, University of the Free State, South Africa), fluconazole (Sigma-Aldrich) and amphotericin B (Sigma-Aldrich) were obtained as assay powders. CAS was dissolved in absolute ethanol (Merck, South Africa); fluconazole in distilled water; amphotericin B in dimethyl sulfoxide (DMSO) (Merck) and

before use, they were further diluted in RPMI 1640 medium. Similar to the aspirin study, whereby the upper limit of drug concentrations tested was 1 mM (4), the same concentration gradient was used for CAS in this study. The final concentrations used for CAS were: 0.01 mM (8.44 µg/ml), 0.1 mM (84.4 µg/ml) and 1 mM (844 µg/ml), for amphotericin B were: 0.25 µg/ml, 0.50 µg/ml, 1 µg/ml, 2 µg/ml and 4 µg/ml, and for fluconazole were: 2 µg/ml, 4 µg/ml, 6 µg/ml, 8 µg/ml, 16 µg/ml and 32 µg/ml.

Susceptibility assay. To perform in vitro susceptibility assays, EUCAST guidelines were followed. Sterile 96-well flat-bottom microtitre plates (Greiner Bio-One, Germany) were filled with 100 µl of the drug using twice the desired final concentrations as stated above and a volume of 100 µl of the standardised cells was added. The plates were incubated at 37^{o}C for 48 h and the optical density (OD) values were measured using a spectrophotometer (Biochrom EZ Read 800 Research, United Kingdom) at 562 nm. The concentrations that led to 50% or more growth reduction for amphotericin B (8 µg/ml) or fluconazole (1 µg/ml) from the aspirin study by Ogundeji et al. (2016) were used (4). The Ogundeji et al. study and the current study were done at the same time, in the same laboratory.

Checkerboard test. CAS was paired with amphotericin B in a sterile disposable 96-well flat-bottom microtitre plate (Greiner Bio-One) and with fluconazole is a separate plate. A volume of 100 µl of the standardised cells was added to each well. The plates were incubated at 37°C for 48 h. Following the incubation period, the OD readings were

taken, and subsequently the fractional inhibitory concentration (FIC) index (FICI) was calculated as described by Ogundeji et al, 2016 (4).

Copper acyl salicylate effect on cellular ultrastructure. To perform scanning electron microscopy (SEM), we used cells from non-treated cells and CAS-treated cells (844 µg/ml) as described in the susceptibility assay were used. Preparation of cells for SEM was done as described by van Wyk and Wingfield (10). Cells were chemically fixed with sodium-phosphate-buffered 3% glutardialdehyde (Merck) and sodium-phosphate-buffered 3% osmium tetroxide (Merck) following dehydration in a graded ethanol (Merck) series. Then, the cells were dried (Bio-Rad Microscience Division, England), mounted on stubs, and coated with gold using an SEM coating system (Bio-Rad Microscience Division) (10). Cells were examined using a Shimadzu Superscan SSX 550 scanning electron microscope (Japan). Additionally, measurements of diameters of 100 cells per each experimental condition (randomly selected from different locations acquired from different stubs) were done by a ruler application that is coupled to the microscope.

Effect of copper acyl salicylate on accumulation of ROS and stress induction. We examined the effect of CAS in causing accumulation of ROS, which could in turn signal the stress signalling partway. Here, non-treated cells and treated cells (844 µg/ml CAS) were grown at 1 x 10^6 cells/ml in sterile black 96-well flat-bottom microtitre plate (Greiner Bio-One) at 37^{o}C for 48 h. After 48 h, 90 µl of cells was added

with 10 µl of the fluorescent dye i.e. 2',7-dichlorofluorescin diacetate (DCFHDA, 1 mg/ml; Sigma-Aldrich) and incubated in the dark at room temperature for 30 min. The induced fluorescence was measure at 485nm/535nm, using Fluoroskan Ascent FL (Thermo-Scientific) microplate reader.

For measurement of activation of p38, a phospho-p-38 MAPK ELISA assay (Sigma-Aldrich) was performed according to the manufacturer's instruction using cell lysates. Cell lysates were obtained following treatment of cells (non-treated cells and 844 µg/ml CAS-treated cells) with lysate buffer. The OD readings were measured using a spectrophotometer (Biochrom EZ Read 800 Research, United Kingdom).

Effect of copper acyl salicylate on macrophage growth, phagocytic and immune functions. In other to determine if CAS would have toxic effect on the macrophages, seeded standardised macrophages were treated with 100 µl of CAS (844 µg/ml) in a sterile, disposable 96-well flat-bottom microtitre plate. The non-treated macrophages were included as control. The plate was incubated at 37^{o}C in a 5% CO_2 incubator for 48 h. The OD of the cells was measured at 562 nm using a Biochrom spectrophotometer. The metabolic activity of these cells was also measured to complement the OD readings. Whereby, prepared cells as described above were reacted with 54 µL of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) (XTT; Sigma-Aldrich, South Africa) in the presence of 1 mM of menadione (Sigma-Aldrich, South Africa). Following that,

the plate was incubated in the dark inside a 5% CO_2 incubator for 3 h. The OD of the wells was measured at 492 nm using a Biochrom spectrophotometer.

The phagocytic capability of macrophages to internalise cryptococcal cells was measured using phagocytosis stain, pHrodo™ Green Zymosan A BioParticles (Life Technologies, USA). Toward this end, 1 µl of the stain were added to 999 µl of standardised cryptococcal cells in RPMI 1640 medium and incubated at 37ºC in a slow agitation manner for 1 h. The cryptococcal cells were washed twice with PBS, spun down and re-suspended in 1000 µl of fresh media that contained twice the desired concentration of CAS (844 µg/ml). A co-culture was prepared in a microtitre plate by adding 100 µl cryptococcal cells to 100 µl of seeded macrophages in a ratio of 1:1 effector-to-target. The plate was incubated at 37°C in a 5% CO_2 incubator for 6 h, After the incubation period, the induced fluorescence was then measured (492 nm; ex/538 nm; em) using a Fluoroskan Ascent FL microplate reader. The fluorescence for non-treated co-cultures was also measured as control.

The ability of CAS to illicit an immunological response was also determined. The concentrations of pro-inflammatory cytokines interferon-gamma (INF-*y*) and interleukin-6 (IL-6) concentrations were assayed using commercial ELISA kits (BioLegend, UK). A volume of 100 µl of the test drug (at twice the desired concentration) was aliquoted into the wells containing 100 µl of standardised seeded macrophages, to yield a final drug concentration of 844 µg/ml CAS. The plates were incubated at 37 ºC for 6 hours. At the end of the incubation period, the supernatant was collected and cytokines were assayed

according to the manufacturer's protocol. The OD readings measured with a Biochrom EZ spectrophotometer.

Statistical analysis. All data, unless stated otherwise, represent mean values of three biological replicates for each strain studied. Where appropriate, standard deviations and student *t*-tests were calculated to determine the statistical significance of data between the different experimental conditions. A *p* value equal or below 0.05 was regarded as statistically significant.

3.4 RESULTS AND DISCUSSION

Copper acyl salicylate has antifungal activity and act in synergy with conventional drugs. Table 1 summarises the effect of CAS on the growth of the 10 tested clinical *Cryptococcus* strains. From the table, it was observed that, as the concentration of the drug increases, there is a corresponding reduction in growth of the cells. The greatest growth reduction was achieved at 844 µg/ml, which is equivalent to 1 mM. At this concentration, the drug effected 50% or more growth reduction when compared to the drug-free control. It is interesting to note that aspirin also effected 50% or more growth reduction at 1 mM. However, comparing the effect of aspirin and CAS on the strain LMPE 046 (which is the most sensitive) at 1 mM, CAS was 5% more effective than aspirin (Fig.1). This is important, as we know from the literature that metal

complexes have strong biological activities (11, 12), and have been reported to be more effective than their parent compounds (13).

Table 2 summarises the combined effect of CAS and amphotericin B as well CAS and fluconazole. The table shows that 80% of drug pairings led to synergistic effects. More importantly, the combinations of the drugs (CAS with amphotericin B and CAS with fluconazole) led to synergism at concentrations that were lower than the individual drug concentrations that yielded 50% or more growth reduction. The significance of using lower concentrations of these drugs in the clinical settings, point towards a reduction in the risk of side effects of these drugs.

Copper acyl salicylate alters the ultrastructure of treated cells. The SEM images showed the effects of CAS on the cellular ultrastructure of strain LMPE 046, non-treated cells were also included as control (Fig. 2). Non-treated cells showed cells covered with more extracellular matrix, which could possibly be the capsule, compared to CAS-treated cells with less extracellular matrix. The effect of the drug reduced the size ($p < 0.05$) of the treated cells (3.91 µm ±0.06) compared to that of non-treated cells (4.14 µm ±0.05). The removal of the matrix point towards the exposure of the cells, thereby making them vulnerable to adverse external conditions.

Copper acyl salicylate subjects cells to oxidative stress. Figure 3 shows the effect of CAS on ROS production. The data showed that CAS-treated cells significantly ($p < 0.01$) accumulated ROS compared to non-treated cells. According to the literature, ROS are produced by respiring cells as part of their oxygen metabolism under normal physiological conditions (14). However, among other factors, exposure to drugs could lead to increase in production of ROS (15). Moreover, increase in ROS levels may serve as a stimulant in activating stress response in the stress signalling pathways, such as MAPK p38 (16). Figure 4 shows a significant increase in the levels of activation of p38 MAPK among the treated cells in the presence of CAS compared to levels in non-treated cells. In this study, the observed increase ROS accumulation was alongside associated with increase in p38 activation levels, which is an indicative of oxidative stress which could eventually lead to oxidative damage to the cell membrane (16).

Copper acyl salicylate improves the functioning of macrophages. The direct effect of CAS on the growth of macrophage was examined. Results indicate that CAS only effected 4% reduction in growth (Fig. 5A) and 6% reduction in metabolic activity (Fig. 5B) of the treated macrophages compared to non-treated macrophages. This means that, CAS at 844 µg/ml was non-toxic to the growth and metabolic activity of macrophages, as the effect of the drug did not result in 50 % reduction in growth and metabolic activity of the macrophages. Furthermore, comparing the toxic effect of CAS from this present study to the previous work on aspirin (4), we observed that the effect of CAS was less toxic to the growth of macrophages compared to aspirin, at 1 mM

(Fig.6). This is important, as it was reported in the literature that metal complexes are less toxic compared to their parent compounds (13).

We also determined if CAS would chemically sensitise macrophages, and thus enhance their phagocytic capability. Toward this end, the results presented in figure 7 showed that the presence of CAS significantly enhanced the efficiency of macrophages to internalise cryptococcal cells by 65%, compared to the non-treated cells. Furthermore, from the data on the production of cytokines (Fig. 8), it was clear that non-CAS-treated macrophages produced significantly less INF-*y* levels compared to CAS-treated macrophages ($p < 0.05$) and similarly non-CAS-treated macrophages produced significantly less IL-6 levels compared to CAS-treated macrophages ($p < 0.05$). This shows that the effect of the drug improves the immunological function of macrophages to recognise and internalise cryptococcal cells in order to be phagocytosed as shown in figure 7. Also the effect of the drug exposes the cryptococcal cells, thus making them vulnerable to the phagocytic effect of macrophages as shown in figure 2.

3.5 CONCLUSIONS

The need to find alternative drugs, more importantly with better therapeutic outcomes to combat cryptococcal infections has necessitated the current study. This study has successfully demonstrated the usefulness of CAS against cryptococcal cells. In addition

it was found to act in synergism with fluconazole and amphotericin B. Based on our results, it is clear that CAS may be an ideal drug to combat cryptococcal growth as it was more effective and less toxic than aspirin. This is crucial as the application of this drug could then possibly eliminate the issues relating to toxicity of aspirin as an alternative. Importantly, this drug did not adversely affect macrophage growth and metabolic activity, but rather enhanced macrophage immunological response and phagocytic capability. These two, are critical functions in the clearance of microbial cells. However, in order to determine if this drug can be used in future clinical settings it has to be tested in live animals. And, modelling studies will have to be conducted to determine if desired therapeutic benefits will be derived.

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TABLE 1. The effect of copper acyl salicylate (CAS) on the metabolic activity of *C. neoformans* and *C. gattii.*

Species detail			**Drug response**		
		Non-treated cells	**CAS-treated cells**		
			8.44 µg/ml	**84.4 µg/ml**	**844 µg/ml**
Strain name	**Strain number**	**OD $_{562\ nm}$**	**%GR**	**%GR**	**%GR**
Cr. neoformans	LMPE 028	0.439 (0.012)	12 (0.030)	32 (0.044)	77 (0.031)
Cr. neoformans	LMPE 030	0.446 (0.007)	13 (0.012)	34 (0.007)	76 (0.015)
Cr. neoformans	LMPE 043	0.449 (0.013)	12 (0.047)	31 (0.005)	77 (0.023)
Cr. neoformans	LMPE 046	0.469 (0.009)	15 (0.026)	38 (0.052)	81 (0.017)
Cr. neoformans	LMPE 047	0.460 (0.019)	14 (0.017)	35 (0.051)	78 (0.015)
Cr. gattii	LMPE 045	0.462 (0.016)	12 (0.037)	30 (0.029)	75 (0.029)
Cr. gattii	LMPE 048	0.464 (0.016)	13 (0.025)	32 (0.022)	76 (0.004)
Cr. gattii	LMPE 052	0.474 (0.007)	11 (0.014)	29 (0.014)	74 (0.014)
Cr. gattii	LMPE 054	0.451 (0.003)	10 (0.005)	31 (0.008)	74 (0.014)
Cr. gattii	LMPE 070	0.458 (0.003)	11 (0.013)	30 (0.022)	75 (0.099)

Percent growth reduction was calculated as 100% - [(OD of treated cells/OD of non-treated cells) · 100%]. Values represent the mean values from three biological replicates, and values in parentheses represent standard deviations. %GR = Percentage growth reduction.

TABLE 2. The combined effect of copper acyl salicylate and amphotericin B and copper acyl salicylate and fluconazole of *C. neoformans* strain LMPE 046.

	Percentage (%) Growth reduction					**Fractional inhibitory concentration (FIC) index**				
CAS	**Amphotericin B**					**Amphotericin B**				
	0.25 µg/ml	**0.5 µg/ml**	**1 µg/ml (MIC)**	**2 µg/ml**	**4 µg/ml**	**0.25 µg/ml**	**0.5 µg/ml**	**1 µg/ml (MIC)**	**2 µg/ml**	**4 µg/ml**
105.6 µg/ml	31	44	61	73	79	1.65	0.69	0.40	0.37	0.29
211 µg/ml	42	48	65	75	87	0.93	0.52	0.38	0.33	0.21
422 µg/ml	49	66	73	78	92	0.61	0.42	0.29	0.27	0.18
844 µg/ml (MIC)	60	72	76	85	95	0.42	0.34	0.23	0.20	0.12
1688 µg/ml	79	82	86	94	98	0.29	0.22	0.17	0.11	0.08
	Percentage (%) Growth reduction					**Fractional inhibitory concentration (FIC) index**				
CAS	**Fluconazole**					**Fluconazole**				
	2 µg/ml	**4 µg/ml**	**8 µg/ml (MIC)**	**16 µg/ml**	**32 µg/ml**	**2 µg/ml**	**4 µg/ml**	**8 µg/ml (MIC)**	**16 µg/ml**	**32 µg/ml**
105.6 µg/ml	26	41	58	67	76	2.06	0.65	0.43	0.40	0.34
211 µg/ml	38	49	63	74	80	1.89	0.56	0.41	0.35	0.31
422 µg/ml	45	63	72	78	85	0.64	0.47	0.37	0.29	0.26
844 µg/ml (MIC)	58	71	80	84	91	0.43	0.39	0.28	0.24	0.19
1688 µg/ml	73	80	86	88	97	0.30	0.23	0.20	0.16	0.11

The fluconazole and amphotericin B MICs were based on Ogundeji et al. 2016 study. Shading indicates synergism. The other shaded cells represent the corresponding % growth reduction values in relation to the FICI.

FIGURE LEGENDS

FIG 1. Comparison of the effect of 1 mM aspirin and CAS on strain LMPE 046. The effect of CAS led to greater growth reduction compared to aspirin. Aspirin values were taken from the previous aspirin study.

FIG 2. Scanning electron micrographs showing the effect of CAS on the ultrastructure and the size of cells. CAS-treated cells showed more extracellular matrix on their cell wall surfaces compared to non-treated cells. The treated cells were significantly smaller ($p < 0.05$) in cell diameter (CAS-treated cells = 3.91 µm +/- 0.06) when compared to non-treated cells (4.14 µm +/- 0.05).

FIG 3. Reactive oxygen species (ROS) assay results showing the effect of CAS on treated cells compared to non-treated cells. Treatment of cells with CAS led to a significant increase ($p < 0.01$) in ROS accumulation compared to non-treated cells.

FIG 4. MAPK p38 assay results showing the effect of CAS on p38 activation levels. The p-38 phosphorylation levels of CAS-treated cells were significantly higher ($p < 0.01$) compared to non-treated cells.

FIG 5. The direct effect of CAS on macrophage (MØ) growth (A) expressed as percentage (%) change in growth. (B) Expressed as percentage (%) change in metabolic activity. At tested concentrations, CAS was non-toxic to macrophages – as it did not yield a 50% reduction in growth and / or metabolic activity.

FIG 6. Comparison of the toxic effect of 1 mM aspirin and CAS on macrophages. The effect of CAS was less toxic to the macrophages compared to aspirin. Aspirin values were taken from the previous aspirin study.

FIG 7. The phagocytic efficiency of macrophages on CAS-treated and non-treated cryptococcal cells. Addition of CAS significantly enhanced ($p > 0.01$) the phagocytic capability of macrophages compared to non-treated cells.

FIG 8. The immunological response of macrophages following treatment with CAS. CAS leads to a significantly higher release of proinflammatory cytokines. (A) 74% more interferon-gamma was released in the presence of CAS, and (B) 88% more interleukin-6 was released when macrophages were exposed to CAS.

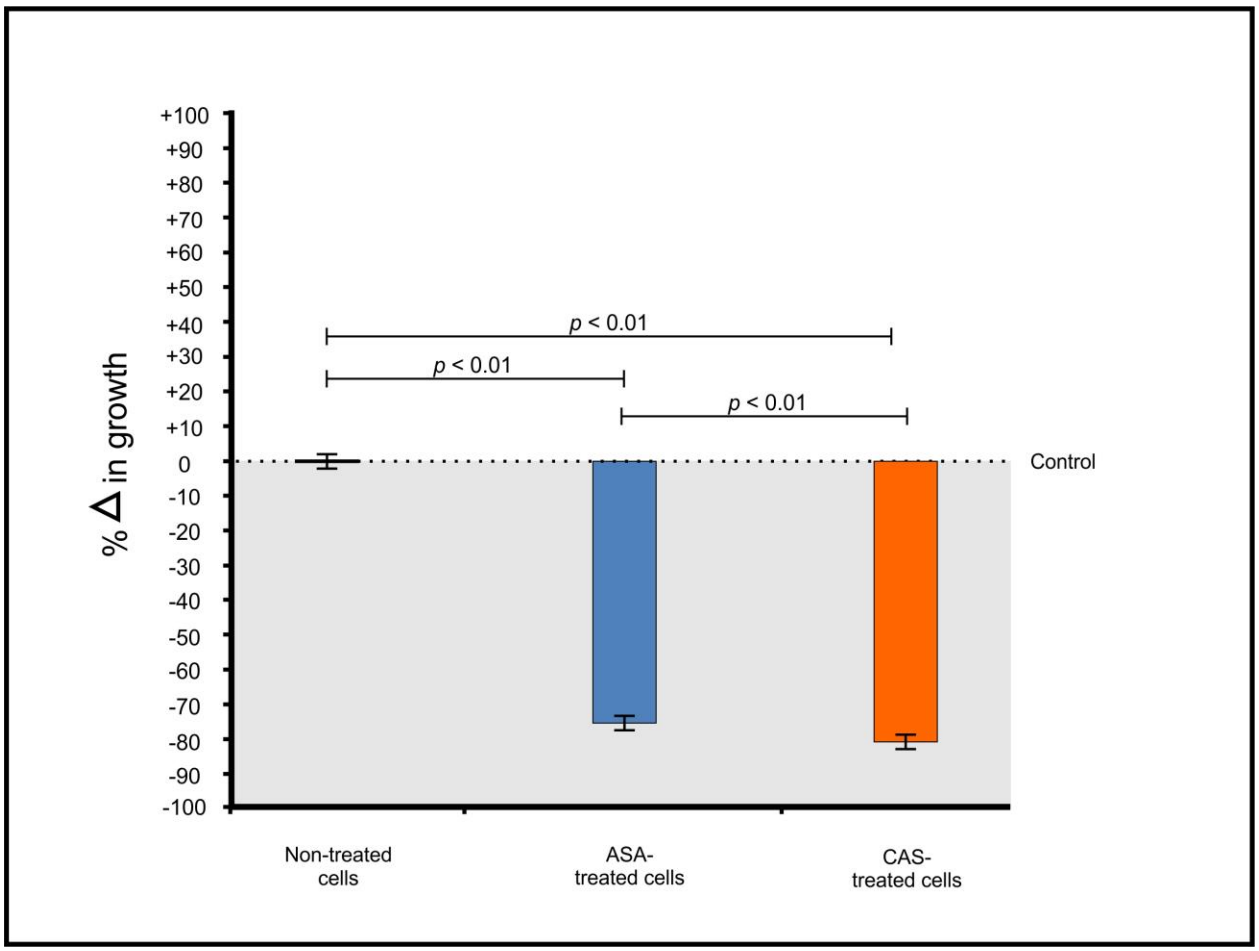

+100
+90
+80
+70
+60
+50
+40
+30
+20
+10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
% Δ in growth
p < 0.01
p < 0.01
p < 0.01
Control
Non-treated cells
ASA-treated cells
CAS-treated cells

FIG 1

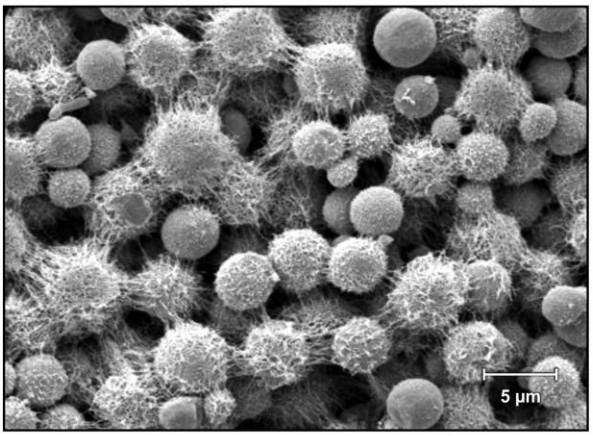
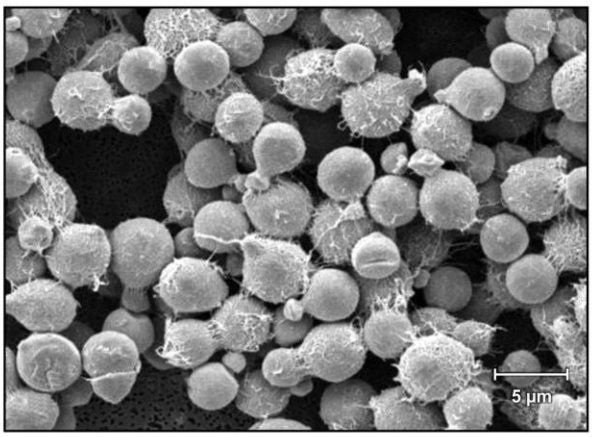
	Non-treated cells	CAS-treated cells
Ultrastructure		
Diameter	4.14 µm (+/- 0.05)	3.91 µm (+/- 0.06)

FIG 2

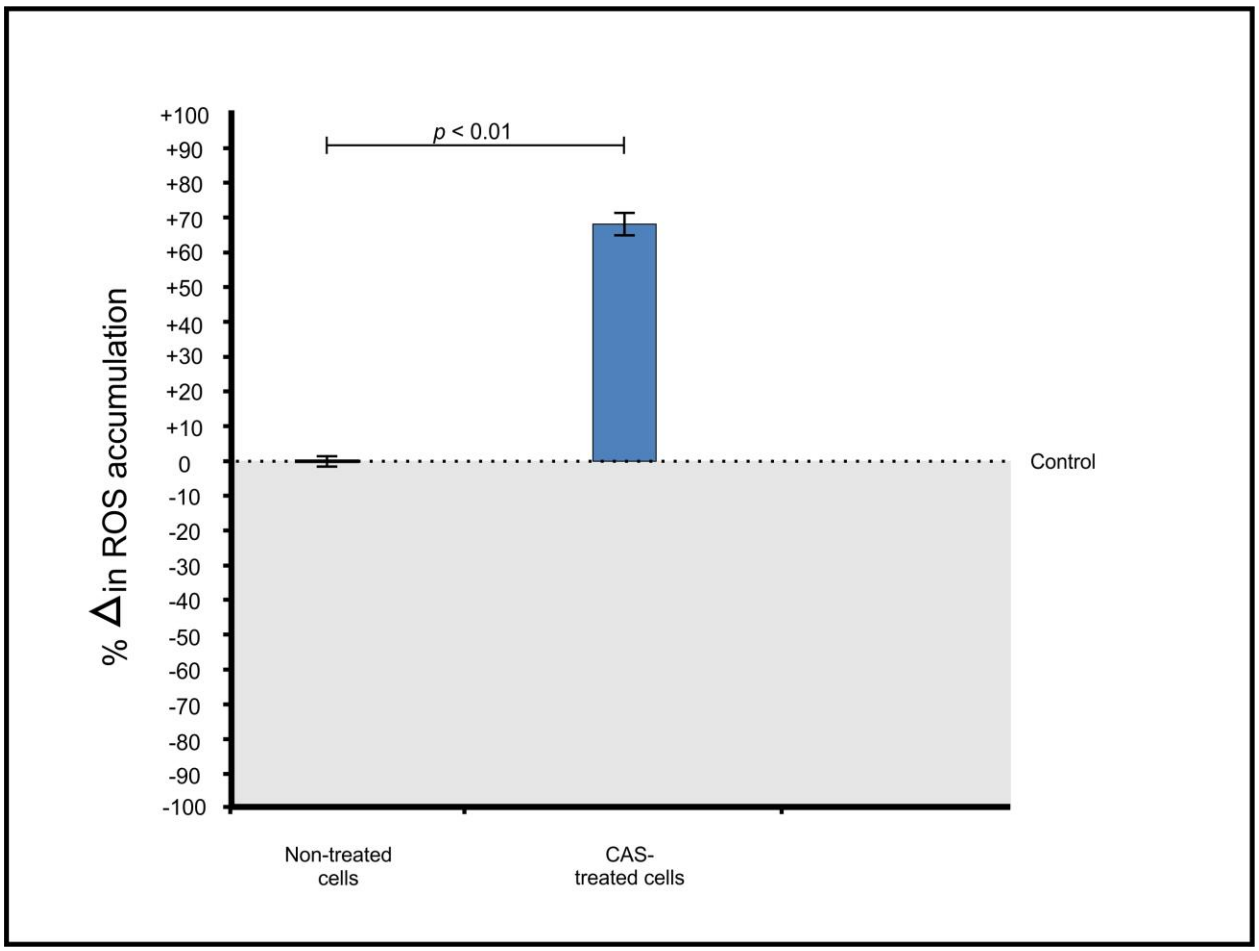

p < 0.01
+100
+90
+80
+70
+60
+50
+40
+30
+20
+10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
% Δin ROS accumulation
Control
Non-treated cells
CAS-treated cells

FIG 3

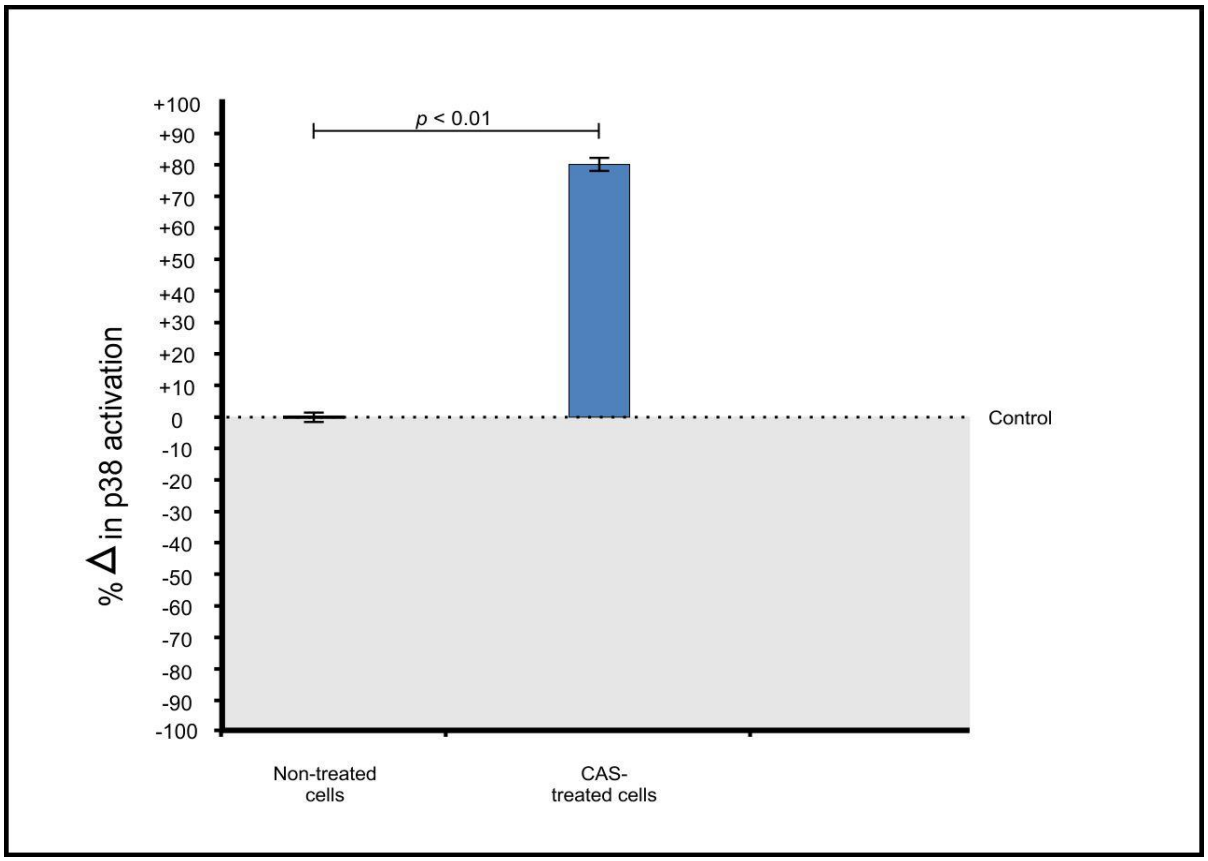

p < 0.01
+100
+90
+80
+70
+60
+50
+40
+30
+20
+10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
% Δ in p38 activation
Control
Non-treated cells
CAS-treated cells

FIG 4

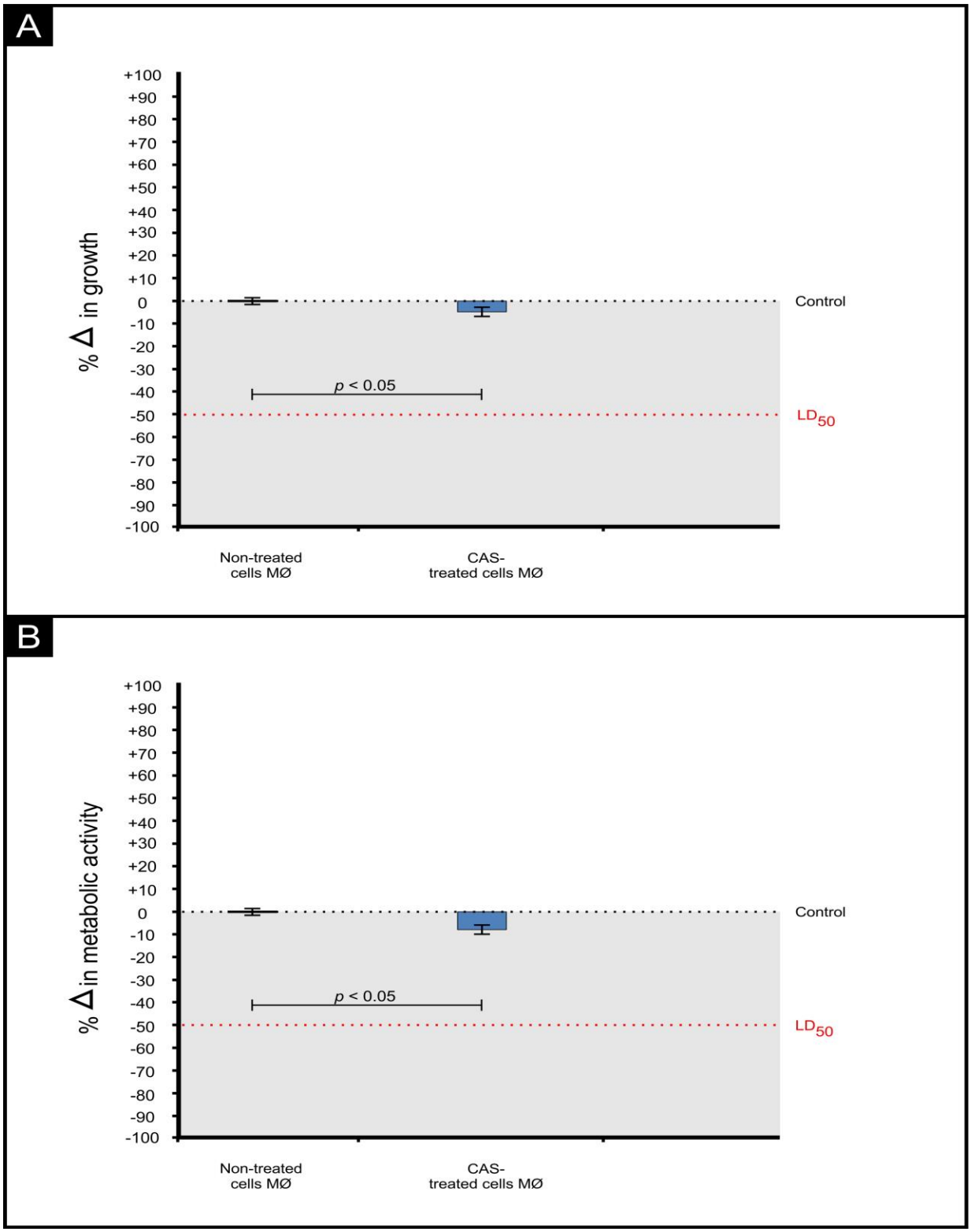

A
+100
+90
+80
+70
+60
+50
+40
+30
+20
+10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
% Δ in growth
Control
p < 0.05
LD50
Non-treated cells MØ
CAS-treated cells MØ
B
% Δ in metabolic activity
Control
p < 0.05
LD50
Non-treated cells MØ
CAS-treated cells MØ

FIG 5

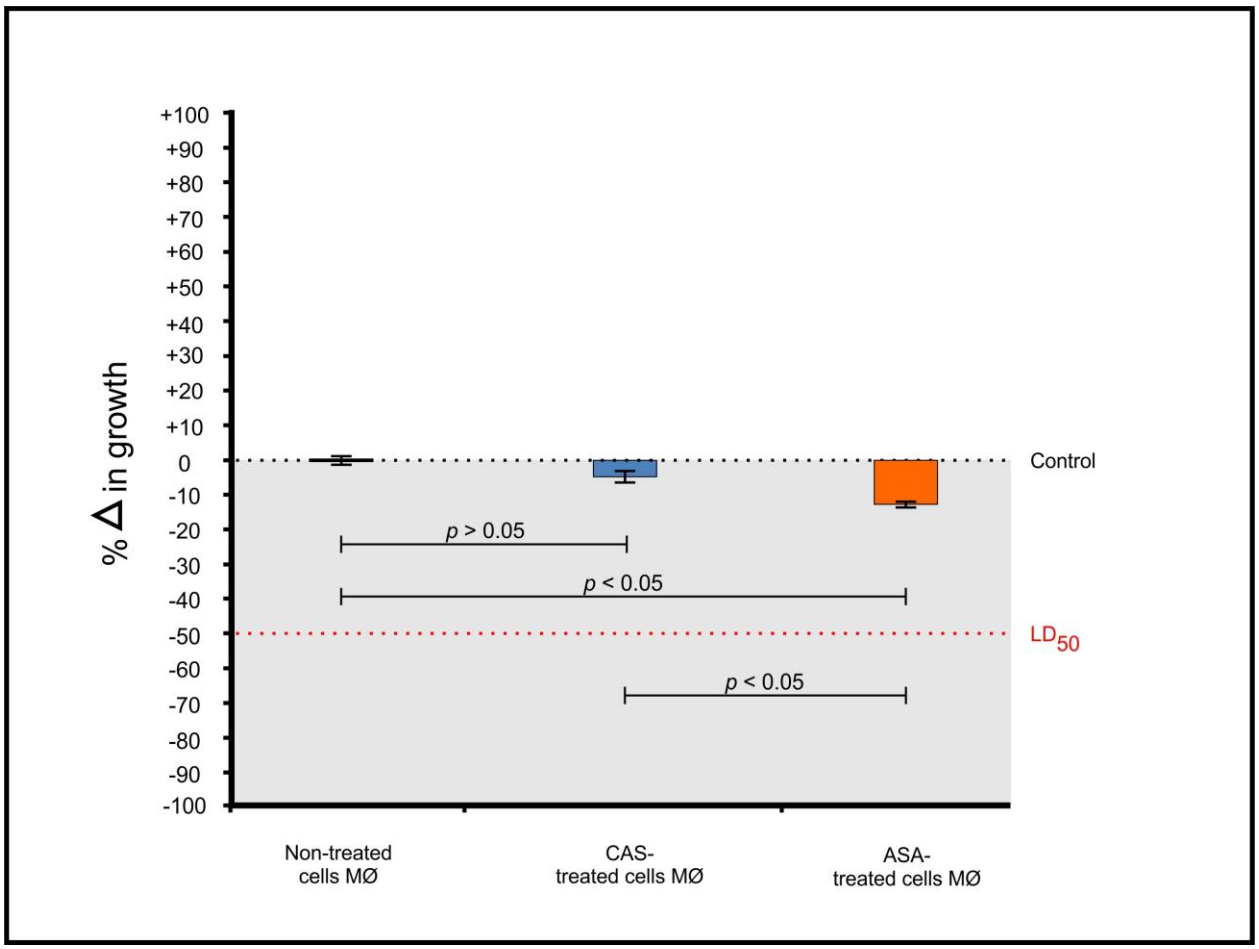

+100
+90
+80
+70
+60
+50
+40
+30
+20
+10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
% Δ in growth
Control
p > 0.05
p < 0.05
LD50
p < 0.05
Non-treated cells MØ
CAS-treated cells MØ
ASA-treated cells MØ

FIG 6

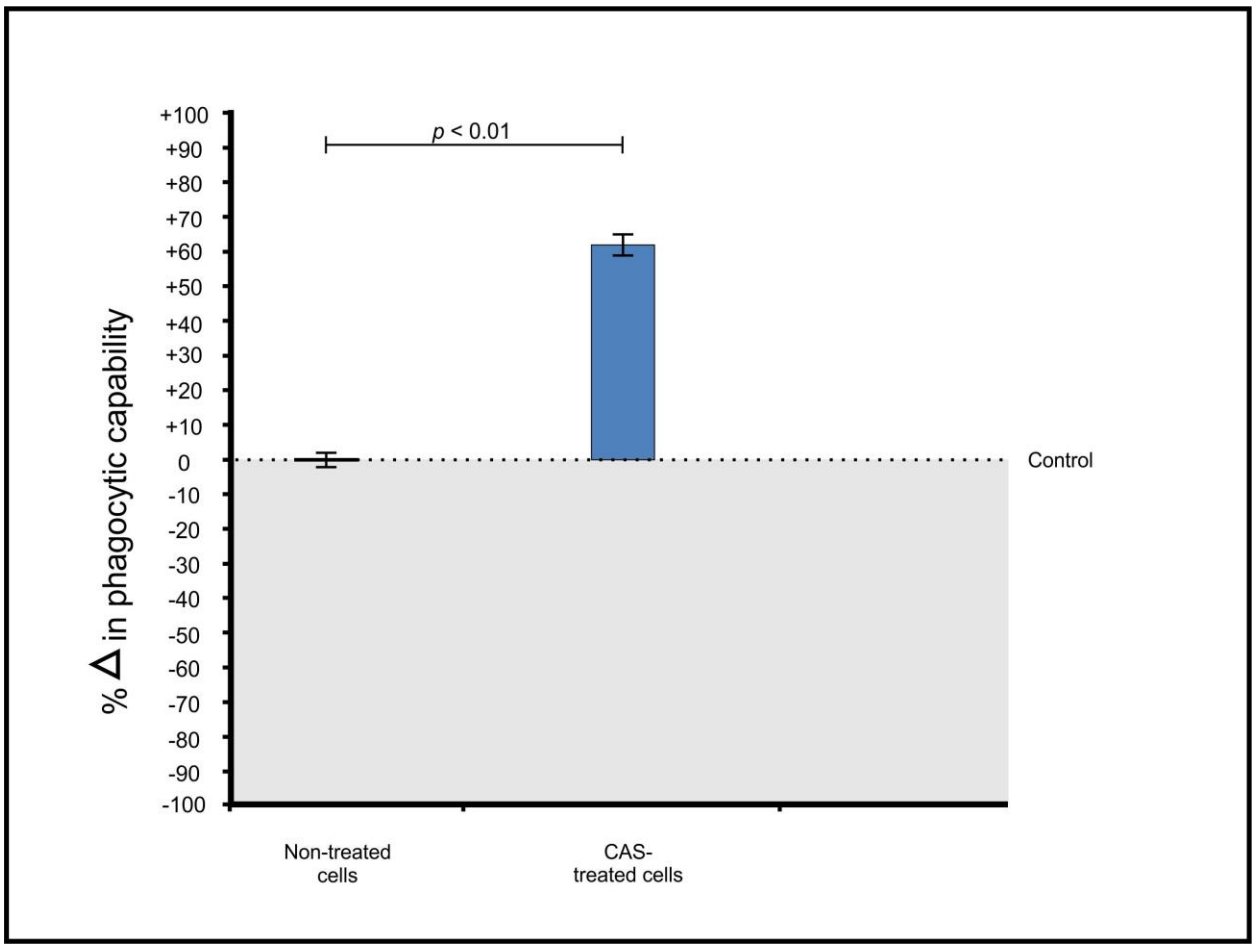

p < 0.01
+100
+90
+80
+70
+60
+50
+40
+30
+20
+10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
% Δ in phagocytic capability
Control
Non-treated cells
CAS-treated cells

FIG 7

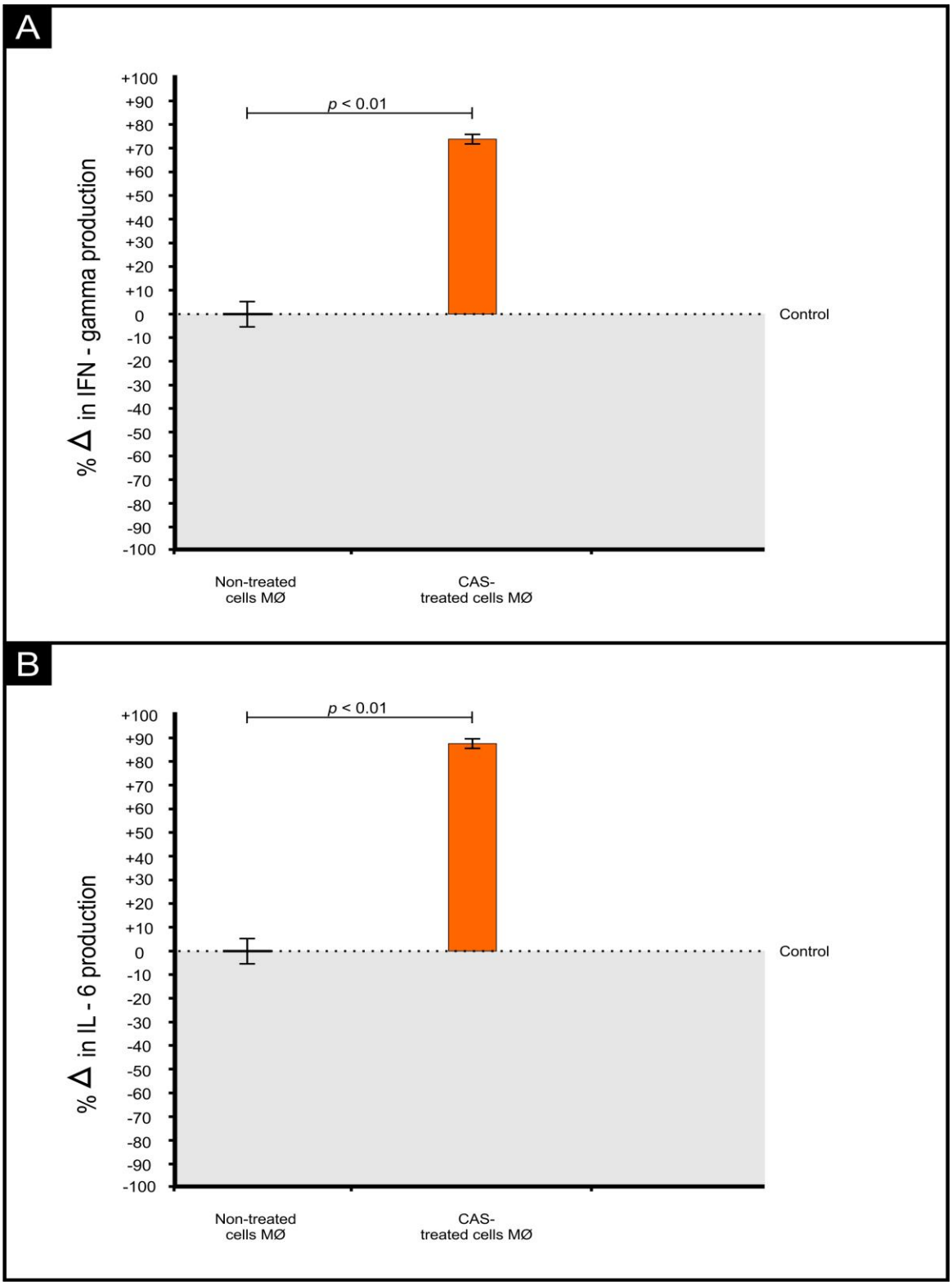

A
+100
+90
+80
+70
+60
+50
+40
+30
+20
+10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
p < 0.01
% Δ in IFN - gamma production
Control
Non-treated cells MØ
CAS-treated cells MØ
B
p < 0.01
% Δ in IL - 6 production
Control
Non-treated cells MØ
CAS-treated cells MØ

FIG 8

CHAPTER 4

THE REPURPOSING OF ANTI-PSYCHOTIC DRUGS, QUETIAPINE AND OLANZAPINE, AS ANTI-*CRYPTOCOCCUS* DRUGS

This chapter has been submitted for publication.

Ogundeji AO, Pohl CH, Sebolai OM. (submitted). The repurposing of anti-psychotic drugs, quietiapine and olanzapine, as anti-*Cryptococcus* drugs. J Antimicrob Chemo.

Candidate contribution: Co-designed the study, performed the experiments and wrote the manuscript.

4.1 ABSTRACT

The management of cryptococcal infections is often difficult. This can, in part, be attributed to the fungistatic nature of fluconazole, which may result in cells disseminating to give rise to pathogen-emergent psychosis following brain inflammation. This chance at treatment failure has necessitated the current study wherein the antimicrobial quality of anti-psychotic drugs viz. quetiapine and olanzapine, was assessed. The response of test strains towards quetiapine or olanzapine alone and in combined therapy with fluconazole or amphotericin B was measured. In addition, the mode of action of the two anti-psychotic drugs in killing cryptococcal cells was determined. At the end, the ability of these anti-psychotic drugs to chemo-sensitise macrophages was also examined. The assessed strains were shown to be susceptible to the two anti-psychotic drugs, which possibly killed them via altering their membrane function. Additionally, these anti-psychotic drugs acted in synergy with fluconazole and amphotericin B in controlling the growth of the test strains. Importantly, these drugs improved the phagocytic efficiency of macrophages and, at the same time, stimulated them to produce pro-inflammatory cytokines (interleukin 6 and interferon gamma), said to be critical in the clearance of cryptococcal cells. The minimum inhibition concentration of each anti-psychotic drugs was calculated to be within its respective recommended therapeutic range. This study's findings highlight the potential clinical application of quetiapine and olanzapine as alternative anti-*Cryptococcus* drugs, which can be used to manage the fungal burden (infection) as well as an associated symptom (psychosis).

Keywords: *Cryptococcus*; Macrophage; Olanzapine; Repurposing; Quetiapine.

4.2 INTRODUCTION

Cryptococcus (C.) neoformans is an important fungal pathogen that causes cryptococcal meningoencephalitis (1). This fatal inflammatory condition typically manifests in HIV-infected persons with a $CD4^+$ T-cell count that is less than 100 cells/µL (2). For the condition to arise, cryptococcal cells should disseminate from the lungs and cross the blood-brain barrier (3). To be specific, cells are reported to invade macrophages and, in a Trojan horse-like manner, use these immune cells to cross the blood-brain-barrier (3). In the brain, the cells compromise the ability of the brain to reabsorb the cerebrospinal fluid, leading to internal accumulation (4). The resultant intracranial pressure may leave patients with adverse neurological signs such as psychosis (2). Without treatment such a patient is expected to die within three months (5).

Although guidelines for the management of cryptococcal diseases have been published (5), management still remains difficult, more so in resource-poor countries, due to cost. In addition, the rise in drug resistance, and inability of the current anti-fungals to discriminate pathogen targets from the host's (both are of eukaryotic origin) – implies that these drugs will often lead to clinical failure (6, 7). This inability to discriminate has unfortunately also led to a stagnation in the development of new anti-fungals. To illustrate this point, it is not surprising that the last anti-fungal drug to be placed on the market was approximately 20 years ago (8).

A possible solution to overcome some of these shortcomings may be to repurpose already FDA-approved drugs that are typically prescribed to treat non-infectious conditions. Therefore, we considered repurposing two anti-psychotic drugs *viz*. quetiapine and olanzapine, as candidate anti-*Cryptococcus* drugs. The findings of this study could potentially be of clinical use in two ways: 1) kill disseminated cryptococcal cells, and 2) manage pathogen-emergent psychosis.

4.3 MATERIALS AND METHODS

Cultivation and standardisation of cells. *Fungi:* Five *C. neoformans* clinical strains (LMPE 028, LMPE 030, LMPE 043, LMPE 046 and LMPE 047) and five *C. gattii* clinical strains (LMPE 045, LMPE 048, LMPE 052, LMPE 054 and LMPE 070), which were obtained from Universitas Academic Hospital (South Africa), were used in the study. These strains were grown on yeast-malt-extract (YM) agar (3000 µg/ml yeast extract, 3000 µg/ml malt extract, 5000 µg/ml peptone, 10000 µg/ml glucose, 16000 µg/ml agar; Merck, South Africa) at 30°C for 48 hours. Five colonies (from each respective agar plate) were scraped off and suspended in 10 ml of distilled water or RPMI-1640 medium (Sigma-Aldrich, South Africa). At the end, the cells were standardised to prepare final inocula of between 0.5×10^5 and 2.5×10^5 CFU/ml according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (9). The inocula were kept on ice before use.

Immune cells: The macrophage cell line RAW 264.7, (a kind donation from Prof Masoko and Mr Makola, University of Limpopo, South Africa), was used in the study. Cells were grown using RPMI-1640 medium that was supplemented with 10% foetal bovine serum (Biochrom, Germany), 20 U/ml penicillin (Sigma-Aldrich, USA), 20 g/mL streptomycin (Sigma-Aldrich, USA) and 2 mM L-glutamine (Sigma-Aldrich, South Africa) in a 5% CO_2-incubator (Thermo Fisher Scientific, USA) at 37°C until they reached 80% confluence. Thereafter, the cells were standardised to 1 x 10^5 cells/ml and seeded into wells of a sterile, disposable 96-well flat-bottom microtitre plate (Greiner Bio-One, Germany).

Drugs. Standard powders of quetiapine (Sigma-Aldrich, South Africa), olanzapine (Sigma-Aldrich, South Africa), fluconazole (Sigma-Aldrich, South Africa) and amphotericin B (Sigma-Aldrich, South Africa) were used in this study. Quetiapine and olanzapine were prepared in dimethyl sulfoxide (Merck, South Africa) to each yield a stock solution of 1000 µg/ml. Fluconazole was reconstituted in distilled water (final stock solution of 1000 µg/ml) while amphotericin B was dissolved in dimethyl sulfoxide (DMSO) (Merck, South Africa) to yield a stock concentration of 1000 µg/ml**.** The concentrations of drug diluents, in which the stock solutions were prepared, never exceeded 1%.

Drug susceptibility testing. The testing was performed in sterile, disposable 96-well flat-bottom microtitre plates and according to EUCAST guidelines. In short, 100 µl of the standardised inoculum (between 0.5 x 10^5 and 2.5 x 10^5 CFU/ml) was aliquoted to designated wells. The cells were then treated with 100 µl of the test drug(s) at twice its desired final concentration. The following final drug concentrations were used, for quetiapine: 0.0625, 0.125, 0.25, 0.5 and 1 µg/ml and for olanzapine: 0.003125, 0.00625, 0.0125, 0.025 and 0.05 µg/ml. The plate was incubated for 48 hours at 37^{o}C. After 48 hours, the optical density (OD) of each well was measured at 562 nm using a spectrophotometer (Biochrom EZ Read 800 Research, United Kingdom). In the study, the MIC was defined as the lowest drug concentration that resulted in 50% or more growth inhibition compared to drug-free control.

All subsequent tests, which are detailed below, were carried out on the one fungal strain that showed the greatest sensitivity towards all test drugs. In addition, fluconazole and amphotericin B MICs were based on those reported by Ogundeji et al. (10). The Ogundeji et al. study and the current study were done at the same time, in the same laboratory. Thus in this study, the MIC for fluconazole was considered as 8 µg/ml while for amphotericin B it was 1 µg/ml.

Four checkerboard assays were prepared i.e. quetiapine-fluconazole, quetiapine-amphotericin B, olanzapine-fluconazole and olanzapine-amphotericin B in sterile, disposable 96-well flat-bottom microtitre plates. These drug combinations were used to

treat the standardised inoculum. The plates were incubated for 48 hours at 37°C. At the end of the incubation period, OD readings were taken, and subsequently the fractional inhibitory concentration (FIC) index (FICI) was calculated. Fractional inhibitory concentration index (that is, the sum of the FICs [ΣFIC]) was defined as FIC_A + FIC_B, where FIC_A is the MIC of drug A in combination/MIC of drug A alone and FIC_B is the MIC of drug B in combination/MIC of drug B alone (10). Fractional inhibitory concentration index values were determined to establish if there was synergism (≤ 0.5), no interaction (> 0.5 - 4) or antagonism (> 4).

Effect of quetiapine and olanzapine on cellular ultrastructure. Cells for scanning electron microscopy (SEM) was obtained from 48-h old non-treated cells (0 µg/ml), quetiapine-treated cells (at determined MIC of 0.5 µg/ml) and olanzapine-treated cells (at determined MIC of 0.025 µg/ml). These cells were prepared as detailed for drug sensitivity testing assay. They were aspirated and separately transferred to 1.5 ml Eppendorf tubes (Merck, South Africa). The cells were then prepared for SEM according to van Wyk and Wingfield (11). In short, the cells were chemically fixed using sodium-phosphate-buffered 3% glutaraldehyde (Merck) and sodium-phosphate-buffered 3% osmium tetroxide (Merck, South Africa) followed by dehydration in a graded ethanol (Merck, South Africa) series. Following that, the cells were dried (Bio-Rad Microscience Division, England), mounted on stubs, and coated with gold using an SEM coating system (Bio-Rad Microscience Division, England) (11). Preparations were examined using a Shimadzu Superscan SSX 550 scanning electron microscope (Japan). In addition, the diameters of 100 cells per each experimental condition (randomly selected

from different locations acquired from different stubs) were measured using a ruler application that is coupled to the microscope.

Effect of quetiapine and olanzapine on membrane function. Cells were prepared as detailed for drug sensitivity assay. Non-treated cells were included as control. For Toxilight® bioassay, the supernatant (20 µl) was collected from the wells and separately transferred to wells of a sterile, white 96-well flat-bottom microtitre plate (Greiner Bio-One, Germany). Next, 100 µl of the Toxilight reagent (Lonza, USA) was added to all the wells. The plate was incubated for 5 min at 37 ^{o}C. To quantify the amount of adenylate kinase released from cells with damaged membranes, the bioluminescence generated from each well was measured using a Fluoroskan Ascent FL (Thermo-Scientific, USA) microplate reader, which converted logarithmic signals to relative luminescence units.

For propidium iodide (PI) staining assay, the plate was briefly agitated in order to re-suspend the cells in the cultivation media. Next, 99 µl of the re-suspended cells (from indicated wells) were separately transferred to a sterile, black 96-well flat-bottom microtitre plate (Greiner Bio-One, Germany). Following this, 1 µl of the PI (Life Technologies, USA) stain was added to designated wells to initiate the reaction with cells. The plate was immediately incubated in the dark for 30 min at 37^{o}C. To measure the amount of PI that permeated through damaged membranes, the stain was excited at 485 nm and the corresponding emitted fluorescence signal was read at 538 nm using the Fluoroskan Ascent FL microplate reader.

Effect of quetiapine and olanzapine on macrophages.

Effect on macrophage growth. Standardised macrophages were first seeded and grown overnight in a sterile, disposable 96-well flat-bottom microtitre plate. The next day, the media was aspirated and fresh media (100 µl) was added to the wells. The cells were then challenged with either 100 µl of twice the desired final concentration of quetiapine (1 µg/ml) or olanzapine (0.05 µg/ml). The plate was then incubated for 48 hours at 37ºC in a 5% CO_2 incubator. The OD of the cells was measured at 562 nm using a Biochrom spectrophotometer. The non-treated cells were included as control.

To complement the OD readings, the metabolic activity of these cells was also measured. In short, a duplicate plate was prepared as stated above. After a 48 h incubation period, the cells were reacted with 54 µL of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) (XTT; Sigma-Aldrich, South Africa) in the presence of 1 mM of menadione (Sigma-Aldrich, South Africa). The plate was accordingly incubated in the dark in a 5% CO_2 incubator. Three hours after initiating the tetrazolium reaction, the OD of the wells was finally measured at 492 nm using a Biochrom spectrophotometer. The non-treated cells were included as control.

Effect on immunological function. To characterise the immunological response of macrophages to cryptococcal cells, in the presence of test drugs, ELISA assays were performed. In short, standardised, seeded macrophages were incubated overnight in a 5% CO_2 incubator. The next day, the media was aspirated and fresh media (100 µl) was

added to the wells. Standardised cryptococcal cells in 100 µl of RPMI 1640 medium containing twice the desired final concentration of either quetiapine (1 µg/ml) or olanzapine (0.05 µg/ml) were added to macrophage wells to prepare co-cultures (1:1 effector-to-target ratio). Cells were allowed to interact for 6 h before ELISA assays were performed. Co-cultures prepared in the absence of test drugs were included as controls. The supernatant was then aspirated and used for interferon-gamma (IFN-γ; BioLegend, USA) and interleukin-6 (IL-6; BioLegend, USA) ELISA assays. Each ELISA assay was performed according to its manufacturer's instructions. At the end, all plates were read at 450 nm on the Biochrom spectrophotometer. Where applicable, concentrations were extrapolated from the constructed standard curve.

Effect on macrophage phagocytic function. The ability of macrophages to internalise cryptococcal cells was measured using the phagocytosis stain, pHrodo^{TM} Green Zymosan A BioParticles (Life Technologies, USA). This stain only fluoresces when excited at acidic pH, such as inside the lumen of phagolysosomes. Standardised cryptococcal cells in 999 µL of RPMI 1640 medium, were reacted with 1 µl of the stain in 1.5 ml Eppendorf tubes for 1 h at 37^{o}C while slowly agitating. Next, the cryptococcal cells were washed twice with PBS, spun down and suspended in 1000 µl of fresh media that contained twice the desired final concentration of either quetiapine (1 µg/ml) or olanzapine (0.05 µg/ml). A 100 µl suspension of cells was then immediately aliquoted to wells that contained seeded macrophages (100 µl) to prepare a co-culture (1:1 effector-to-target ratio) in a microtitre plate. The plate was incubated for 6 h at 37°C in a 5% CO_2 incubator. At the end of the incubation period, the induced fluorescence

was measured (492 nm; ex/538 nm; em) using a Fluoroskan Ascent FL microplate reader. Fluorescence was also measured for non-treated co-cultures i.e. co-cultures without drugs.

In addition, the ability of cytokine standards i.e. IFN-γ (500 pg/ml) and IL-6 (500 pg/ml) to activate macrophages to internalise cryptococcal cells was also measured. Here, cytokines substituted the test drugs stated above. Likewise, the cryptococcal cells were stained and the co-culture prepared similarly. The plate was also handled in the same manner before taking measurements on the plate reader. Fluorescence was also measured for co-cultures without cytokines.

Statistical analysis. All data, unless stated otherwise, represent mean values of three biological replicates. Where appropriate, standard deviations and student *t*-tests were calculated to determine the statistical significance of data between the different experimental conditions. A *p* value equal or below 0.05 was regarded as statistically significant.

4.4 RESULTS

Quetiapine and olanzapine possess anti-fungal activity and act in synergy. All ten *Cryptococcus* strains (five *C. neoformans* and five *C. gattii*) showed a dose-dependent

growth reduction pattern towards the two test anti-psychotic drugs when compared to their respective drug-free controls (Tables 1 and 2). The MIC of quetiapine was defined as 0.5 µg/ml while that of olanzapine was 0.025 µg/ml. At these respective concentrations, both drugs effected a 50% or more growth reduction when compared to their respective drug-free controls. More importantly, each defined MIC was within the recommended therapeutic range in the blood, for each respective drug (12, 13). Outside the therapeutic dosage, greater growth reduction could be achieved. However, this is not ideal as this will result in ill-tolerance by patients. The strain *C. neoformans* LMPE 046 was the most sensitive towards all test drugs, including fluconazole and amphotericin B (10). Thus, all subsequent results were based on the response of this one fungal strain at the above-mentioned MICs.

When paired with either fluconazole or amphotericin B, each anti-psychotic drug could effect a synergistic outcome (Tables 3 and 4). However, no drug combination, within the FIC index, yielded total reduction of fungal growth. Nonetheless, a two-fold downward shift in the concentration (of each test drug – including fluconazole or amphotericin B), that effected a 50% or more growth reduction was observed. The latter is critical as it implies a lower dosage may be required to yield the same desired outcome – this could assist in minimising adverse effects that are expressed at higher concentrations.

Quetiapine and olanzapine treatment compromises fungal cell wall function. A close examination of SEM images revealed the topography of quetiapine-treated cells and olanzapine-treated cells to be different in appearance when compared to that of non-treated cells (Figure 1). Non-treated cells have more web-like extracellular matrixes on their cell wall surfaces when compared to treated cells, which had less web-like structures. The observed reduction in matrixes, may compromise the defences of cryptococcal cells and may leave them vulnerable to macrophage action. In addition, the treated cells were significantly smaller ($p < 0.05$) in cell diameter (quetiapine-treated cells = 3.59 µm +/- 0.08; olanzapine-treated cells = 3.94 µm +/- 0.07) when compared to non-treated cells (4.51 µm +/- 0.07) (Figure 1).

When considering the permeability assay results, it was evident that drug-treated cells significantly ($p < 0.05$) leaked intracellular metabolites, specifically adenylate kinase, into the culture media (Figure 2) and accumulated the PI stain in the cytoplasm (Figure 3), compared to non-treated cells. Both these results (Figures 2 and 3) speak to cell walls losing their ability to control the trafficking of molecules in and out of the cells. It reasonable to conclude that the rupture sites on the cell walls, as seen from the topography of quetiapine-treated cells in Figure 1, may be the site where molecules are leaked.

Quetiapine and olanzapine treatment improves macrophage function. While it was important to demonstrate *in vitro* susceptibility, it was equally important in this study to test the effect(s) of the anti-psychotic drugs on macrophages, which are central for “driving” disseminated infections. Thus, the two test anti-psychotic drugs were chosen particularly because of their lipophilic nature (14, 15), a quality that may assist these drugs to cross into the lumen of macrophages – thus influence the functioning of macrophages.

It was first sought to determine if the two drugs, at their determined MICs, may negatively affect the growth and metabolic activity of macrophages (Figure 4). When compared to non-treated macrophages, the drug-treated macrophages showed a reduction in both growth (Figure 4A) and metabolic activity (Figure 4B). However, the observed reduction (both growth and metabolic activity) was not significant to reach levels required to effect a lethal dosage (LD_{50}) – wherein 50% of the macrophages would be adversely affected. This finding implies that a mammalian host would not experience negative effects when exposed to the test drugs. This is in line with the determination that the defined MICs, for both drugs, were within the recommended range.

The primary function of macrophages is to sense and resolve threats such as invading cryptococcal cells through the process of phagocytosis (16). It therefore became important to assess how these drugs may influence the functioning of

macrophages. First, the production of pro-inflammatory cytokines by macrophages when challenged with cryptococcal cells, in the presence of quetiapine or olanzapine, was assayed. The obtained results showed that macrophages produced significantly ($p < 0.05$) more IFN-γ (Figure 5A) and IL-6 (Figure 5B) when treated with respective anti-psychotic drugs compared to when they were not treated. These pro-inflammatory cytokines are reported in literature to recruit macrophages and enhance their phagocytic action – functions that are pivotal in the clearance of infecting cells (17). Second, it was sought to determine if the test drugs may also sensitise macrophages to internalised and trap more cryptococcal cells inside phagolysosomes compared to in the absence of either drug. Here, it was determined that both drugs significantly ($p < 0.05$) enhanced the ability of macrophages to internalise more cells (Figure 6). To be specific, quetiapine enhanced internalisation by 65% (Figure 6A) while olanzapine enhanced it by 63% (Figure 6B) when compared to the internalisation of cryptococcal cells by macrophages in the absence of quetiapine or olanzapine. For reference purposes, the internalisation of cryptococcal cells by macrophages was also assayed in the presence of either IFN-γ or IL-6 (Figures 6A and B). As expected, the two cytokines significantly ($p < 0.05$) enhanced internalisation of cryptococcal cells when compared to macrophages not treated/stimulated with cytokines (Figure 6). It was, thus, interesting to note that the response of macrophages to the test drugs mirrored the macrophage response to the two cytokines.

4.5 DISCUSSION AND CONCLUSION

The need for alternative anti-fungal drugs, to better manage disseminated cryptococcal infections, necessitated the current study. In advanced cases, these infections can leave a patient with an altered mental state. At the moment, conventional drugs such as fluconazole and amphotericin B are routinely used in South Africa to control cryptococcal infections (18). Fluconazole is made available through a drug donation programme in South Africa. An unintended outcome of this good will act is that this drug is indiscriminately used in clinical settings, which has led to increased resistance.

Therefore, in the current study, anti-psychotic drugs were considered as suitable candidate drugs. This choice was motivated by the idea that these drugs may have an ancillary benefit of managing pathogen-emergent psychosis and at the same time control the fungal burden. The concept of repurposing drugs is not new. Over the years, drugs which were primarily purposed to manage non-infectious conditions have been shown to also possess anti-microbial activity (19). In some of these studies, researchers followed standard protocol for assessing *in vitro* susceptibility (i.e. EUCAST or CLSI), which provided insight into the effectiveness of repurposed drugs. One such study concerned aspirin, which is traditionally used as an anti-inflammatory drug (10). These researchers reported that aspirin affected cryptococcal growth inhibition at a MIC that was within the recommended dosage in the blood.

The current study successfully demonstrated that quetiapine and olanzapine have anti-*Cryptococcus* activity and possibly killed cells by compromising their

membrane integrity. It was significant to note that this activity was displayed at concentrations, for each drug, which were within the recommended dosage in the blood. Additionally, they acted in synergy with conventional drugs. Another interesting finding was that the drugs chemically sensitised macrophages (much like cytokines) to phagocytose more cryptococcal cells.

It now becomes critical to also demonstrate if these drugs, which targeted eukaryotic membranes, would yield the desired outcome without attacking the host membranes. Toward this end, the effectiveness of these drugs should be determined in laboratory animals.

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Table 1. The effect of quetiapine on *C. neoformans* and *C. gattii* strains.

Species detail		**Drug response**					
		Non-treated cells	**Quetiapine (µg/ml)**				
			0.0625	**0.125**	**0.25**	**0.5 (MIC)**	**1**
Name	**Number**	**OD $_{562\ nm}$**	**%GR**	**%GR**	**%GR**	**%GR**	**%GR**
C. neoformans	LMPE 028	0.535 (0.021)	24 (0.005)	33 (0.011)	44 (0.011)	54 (0.006)	76 (0.008)
C. neoformans	LMPE 030	0.536 (0.005)	22 (0.012)	34 (0.050)	45 (0.017)	55 (0.012)	75 (0.012)
C. neoformans	LMPE 043	0.534 (0.016)	23 (0.040)	33 (0.015)	44 (0.015)	55 (0.008)	75 (0.005)
C. neoformans	LMPE 046	0.534(0.008)	26 (0.083)	35 (0.012)	47 (0.009)	57 (0.032)	79 (0.014)
C. neoformans	LMPE 047	0.533 (0.013)	23 (0.049)	34 (0.051)	45 (0.013)	56 (0.016)	74(0.009)
C. gattii	LMPE 045	0.532 (0.016)	22 (0.016)	30 (0.015)	43 (0.008)	53 (0.024)	72(0.023)
C. gattii	LMPE 048	0.530 (0.040)	25 (0.008)	33 (0.021)	45 (0.014)	56 (0.009)	76 (0.006)
C. gattii	LMPE 052	0.530 (0.008)	22 (0.013)	33 (0.014)	43 (0.009)	54 (0.014)	73 (0.005)
C. gattii	LMPE 054	0.531(0.005)	22 (0.008)	31 (0.008)	42 (0.015)	54 (0.011)	74 (0.006)
C. gattii	LMPE 070	0.534 (0.017)	21 (0.019)	30 (0.019)	43 (0.012)	53 (0.012)	72 (0.017)

Percent growth reduction was calculated as 100% - [(OD of treated cells/OD of non-treated cells) · 100%]. Values represent the mean values from three biological replicates, and values in parentheses represent standard deviations. %GR = Percentage growth reduction.

Table 2. The effect of olanzapine on *C. neoformans* and *C. gattii* strains.

Species detail		Drug response					
		Non-treated cells	Olanzapine (µg/ml)				
			0.003125	0.00625	0.0125	0.025 (MIC)	0.05
Name	**Number**	**OD $_{562\ nm}$**	**%GR**	**%GR**	**%GR**	**%GR**	**%GR**
C. neoformans	LMPE 028	0.533 (0.009)	15 (0.015)	28 (0.013)	43 (0.021)	56 (0.014)	63 (0.014)
C. neoformans	LMPE 030	0.531 (0.014)	16 (0.014)	27 (0.040)	41 (0.007)	54 (0.007)	62 (0.009)
C. neoformans	LMPE 043	0.538 (0.005)	18 (0.008)	28 (0.005)	42 (0.005)	55 (0.018)	64 (0.012)
C. neoformans	LMPE 046	0.532(0.032)	19 (0.042)	30 (0.022)	44 (0.009)	58 (0.009)	67 (0.007)
C. neoformans	LMPE 047	0.531 (0.012)	18 (0.009)	29 (0.041)	43 (0.012)	56 (0.018)	65 (0.014)
C. gattii	LMPE 045	0.531 (0.026)	13 (0.018)	24 (0.017)	40 (0.013)	52 (0.014)	64 (0.013)
C. gattii	LMPE 048	0.530 (0.036)	15 (0.009)	26 (0.023)	42 (0.008)	55 (0.009)	66 (0.018)
C. gattii	LMPE 052	0.530 (0.009)	14 (0.011)	25 (0.015)	41 (0.020)	54 (0.013)	63 (0.014)
C. gattii	LMPE 054	0.531(0.015)	13 (0.009)	24 (0.013)	40 (0.011)	52 (0.014)	63 (0.024)
C. gattii	LMPE 070	0.534(0.007)	14 (0.021)	25 (0.009)	40 (0.009)	54 (0.009)	64 (0.007)

Percent growth reduction was calculated as 100% - [(OD of treated cells/OD of non-treated cells) · 100%]. Values represent the mean values from three biological replicates, and values in parentheses represent standard deviations. %GR = Percentage growth reduction.

Table 3. Combined effects of quetiapine and amphotericin B and quetiapine and fluconazole on *C. neoformans* strain LMPE 046.

	Percentage (%) growth reduction					**Fractional inhibitory concentration (FIC) index**				
Quetiapine (µg/ml)	**Amphotericin B (µg/ml)**					**Amphotericin B (µg/ml)**				
	0.25	**0.5**	**1 (MIC)**	**2**	**4**	**0.25**	**0.5**	**1 (MIC)**	**2**	**4**
0.0625	23	41	59	68	80	2.21	0.61	0.41	0.35	0.28
0.125	35	48	62	74	88	1.91	0.59	0.4	0.3	0.22
0.25	48	56	70	79	92	0.63	0.44	0.32	0.28	0.16
0.5 (MIC)	61	68	79	83	95	0.4	0.24	0.31	0.23	0.13
1	79	81	85	91	98	0.39	0.2	0.24	0.17	0.07
	Percentage (%) Growth reduction					**Fractional inhibitory concentration (FIC) index**				
Quetiapine (µg/ml)	**Fluconazole (mg/L)**					**Fluconazole (µg/ml)**				
	2	**4**	**8 (MIC)**	**16**	**32**	**2**	**4**	**8 (MIC)**	**16**	**32**
0.0625	21	39	54	65	75	2.23	0.62	0.42	0.37	0.3
0.125	30	45	59	71	79	2.01	0.55	0.4	0.32	0.25
0.25	46	51	68	77	83	0.86	0.44	0.38	0.34	0.21
0.5 (MIC)	59	64	74	80	89	0.48	0.38	0.31	0.25	0.19
1	72	79	81	89	92	0.31	0.28	0.21	0.19	0.1

The fluconazole and amphotericin B MICs were based on Ogundeji et al. 2016 study. Shading indicates synergism. The other shaded cells represent the corresponding % growth reduction values in relation to the FICI.

Table 4. Combined effects of olanzapine and amphotericin B and olanzapine and fluconazole on *C. neoformans* strain LMPE 046.

	Percentage (%) growth reduction					**Fractional inhibitory concentration (FIC) index**				
Olanzapine (µg/ml)	**Amphotericin B (µg/ml)**					**Amphotericin B (µg/ml)**				
	0.25	**0.5**	**1 (MIC)**	**2**	**4**	**0.25**	**0.5**	**1 (MIC)**	**2**	**4**
0.003125	19	35	59	69	79	2.69	0.82	0.42	0.33	0.22
0.00625	29	43	62	73	86	2.24	0.65	0.39	0.28	0.18
0.0125	45	56	68	78	90	0.71	0.43	0.33	0.23	0.16
0.025 (MIC)	58	64	75	83	93	0.44	0.35	0.25	0.2	0.12
0.05	69	77	80	88	96	0.32	0.29	0.23	0.18	0.09
	Percentage (%) Growth reduction					**Fractional inhibitory concentration (FIC) index**				
Olanzapine (µg/ml)	**Fluconazole (µg/ml)**					**Fluconazole (µg/ml)**				
	2	**4**	**8 (MIC)**	**16**	**32**	**2**	**4**	**8 (MIC)**	**16**	**32**
0.003125	16	32	53	67	75	2.89	0.89	0.41	0.35	0.24
0.00625	27	40	57	70	80	2.41	0.71	0.39	0.32	0.21
0.0125	42	52	64	75	86	0.75	0.44	0.32	0.25	0.19
0.025 (MIC)	57	61	72	80	90	0.46	0.38	0.29	0.22	0.14
0.05	67	75	78	85	93	0.37	0.3	0.25	0.2	0.11

The fluconazole and amphotericin B MICs were based on Ogundeji et al. 2016 study. Shading indicates synergism. The other shaded cells represent the corresponding % growth reduction values in relation to the FICI.

FIGURE LEGENDS

FIG 1. The effects of quetiapine and olanzapine on the ultrastructure of treated cryptococcal cells as well as on cell size. For comparison, a SEM micrograph of non-treated cryptococcal cells and their measured cell sizes are included. ECM = extracellular matrix, Rcap = raptured capsule.

FIG 2. Toxilight® Bioassay results of treated- and non-treated cryptococcal cells. When exposed to quetiapine and olanzapine, cells significantly ($p < 0.01$) secreted more intracellular metabolites (adenylate kinase) compared to non-treated cells.

FIG 3. Propidium iodide (PI) assay results of treated- and non-treated cryptococcal cells. When exposed to quetiapine and olanzapine, cells significantly ($p < 0.01$) accumulated more PI compared to non-treated cells.

FIG 4. The graph illustrates the effect of quetiapine and olanzapine on macrophage (MØ) growth (expressed as percentage change in growth) (A) as well as their effect on metabolic activity (expressed as percentage change in metabolic activity) (B). When considering the results shown in A and B, it is evident that the two test drugs did not negatively affect macrophages.

FIG 5. Effect of quetiapine and olanzapine on the immunological response of macrophages. Drug treatment induced challenged macrophages to produce significantly ($p = 0.01$) more pro-inflammatory cytokines i.e. interferon gamma (A) and interleukin 6 (B) when compared to non-treated macrophages.

FIG 6. Effect of quetiapine (A) and olanzapine (B) in chemosensitising macrophage (MØ) to phagocytose cryptococcal cells. Drug treatment sensitised challenged macrophages to significantly ($p < 0.01$) internalise cryptococcal cells when compared to non-treated cells. A similar response i.e. increased internalisation, was observed when macrophages were stimulated (challenged) with interferon gamma (A) and interleukin 6.

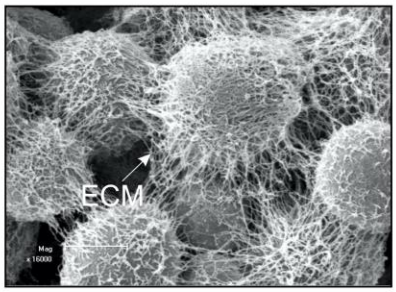
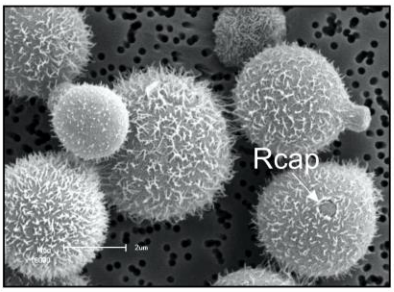
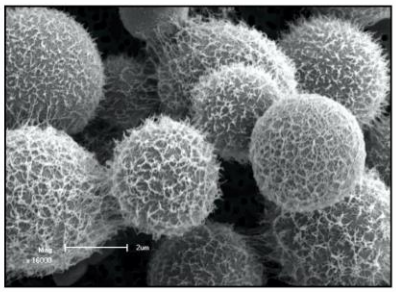
	Non-treated cells	Quetiapine-treated cells	Olanzapine-treated cells
Ultrastructure			
Diameter	4.51 µm (+/- 0.07)	3.59 µm (+/- 0.08)	3.94 µm (+/- 0.09)

FIG 1

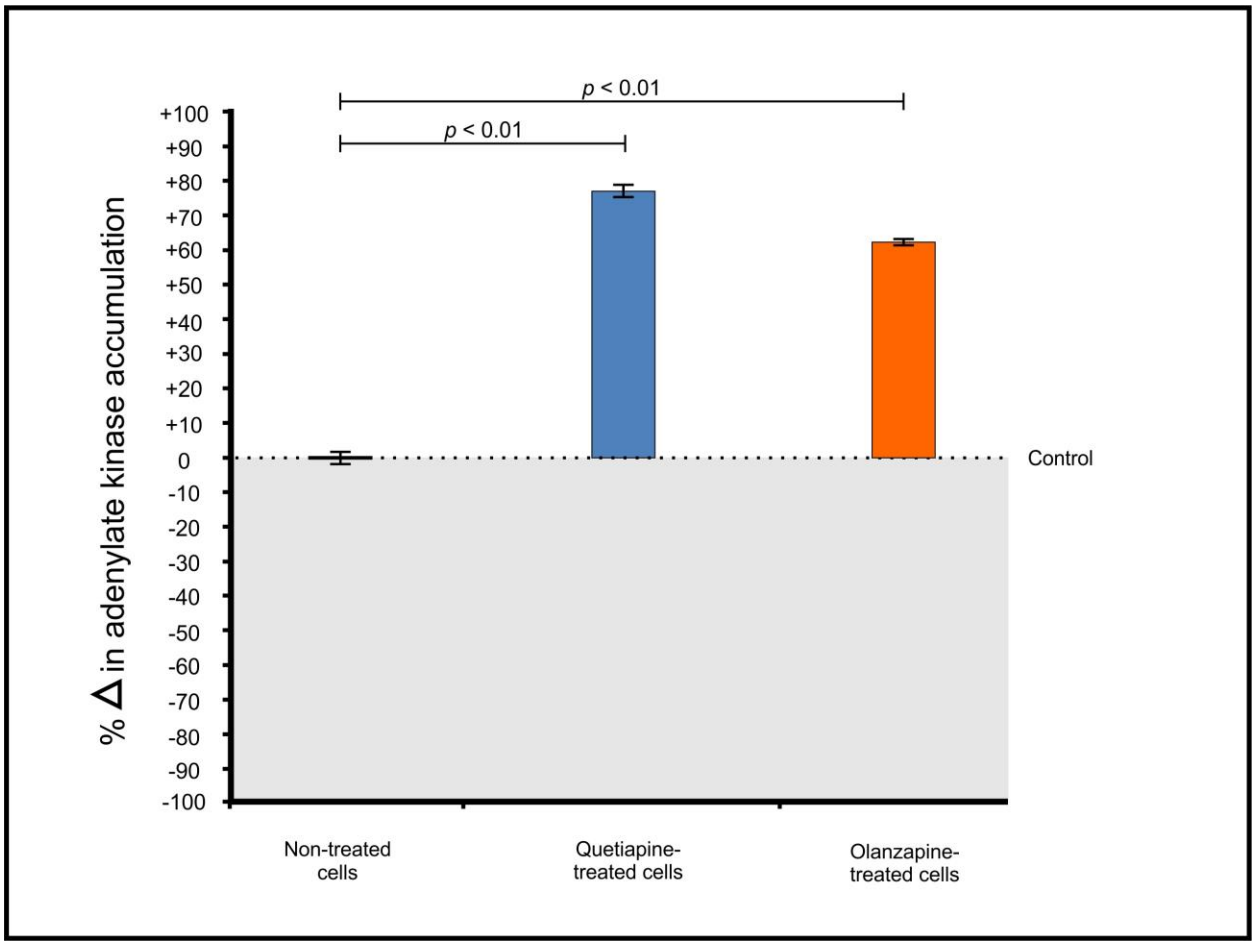

p < 0.01
p < 0.01
+100
+90
+80
+70
+60
+50
+40
+30
+20
+10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
% Δ in adenylate kinase accumulation
Control
Non-treated cells
Quetiapine-treated cells
Olanzapine-treated cells

FIG 2

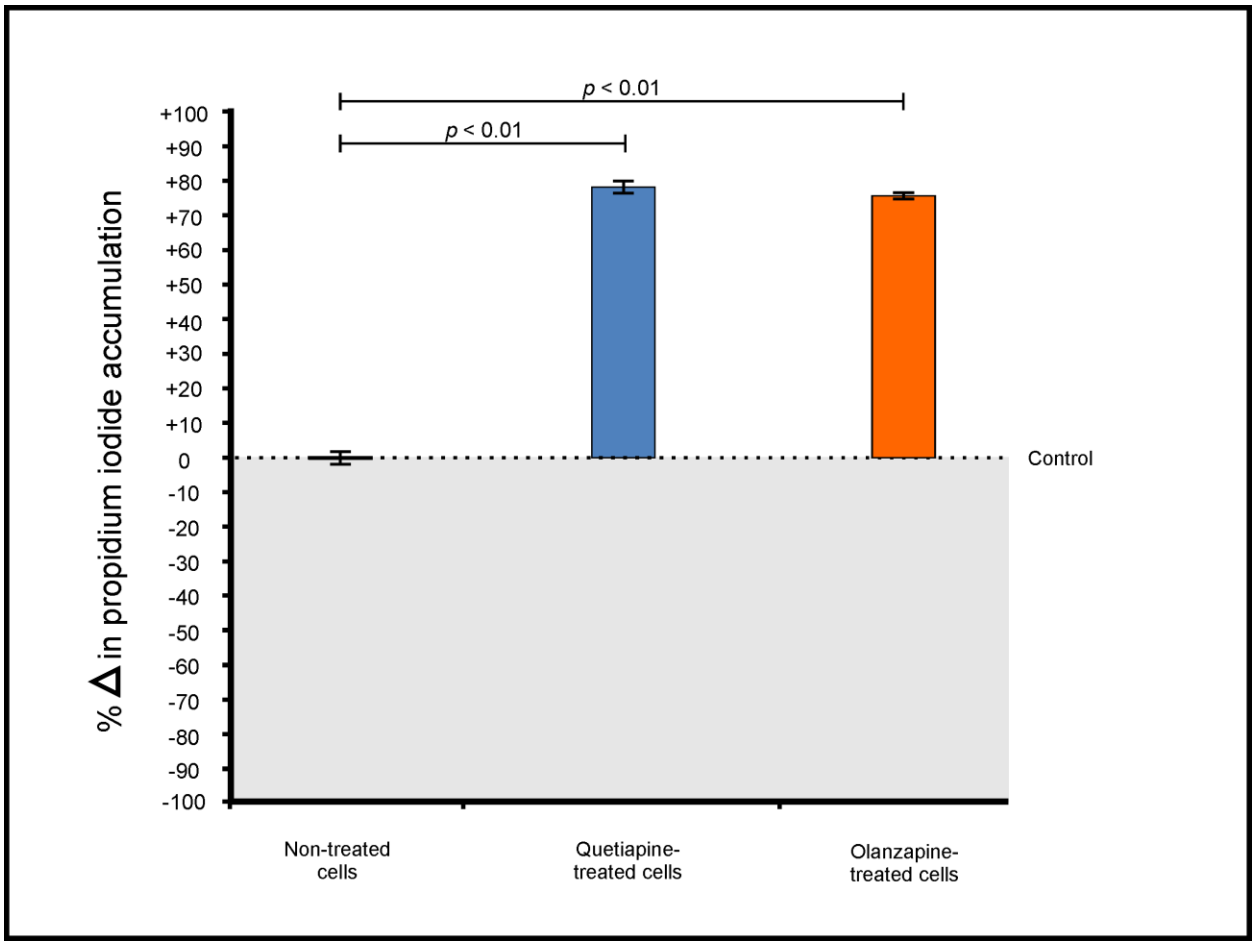

p < 0.01
p < 0.01
+100
+90
+80
+70
+60
+50
+40
+30
+20
+10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
% Δ in propidium iodide accumulation
Control
Non-treated cells
Quetiapine-treated cells
Olanzapine-treated cells

FIG 3

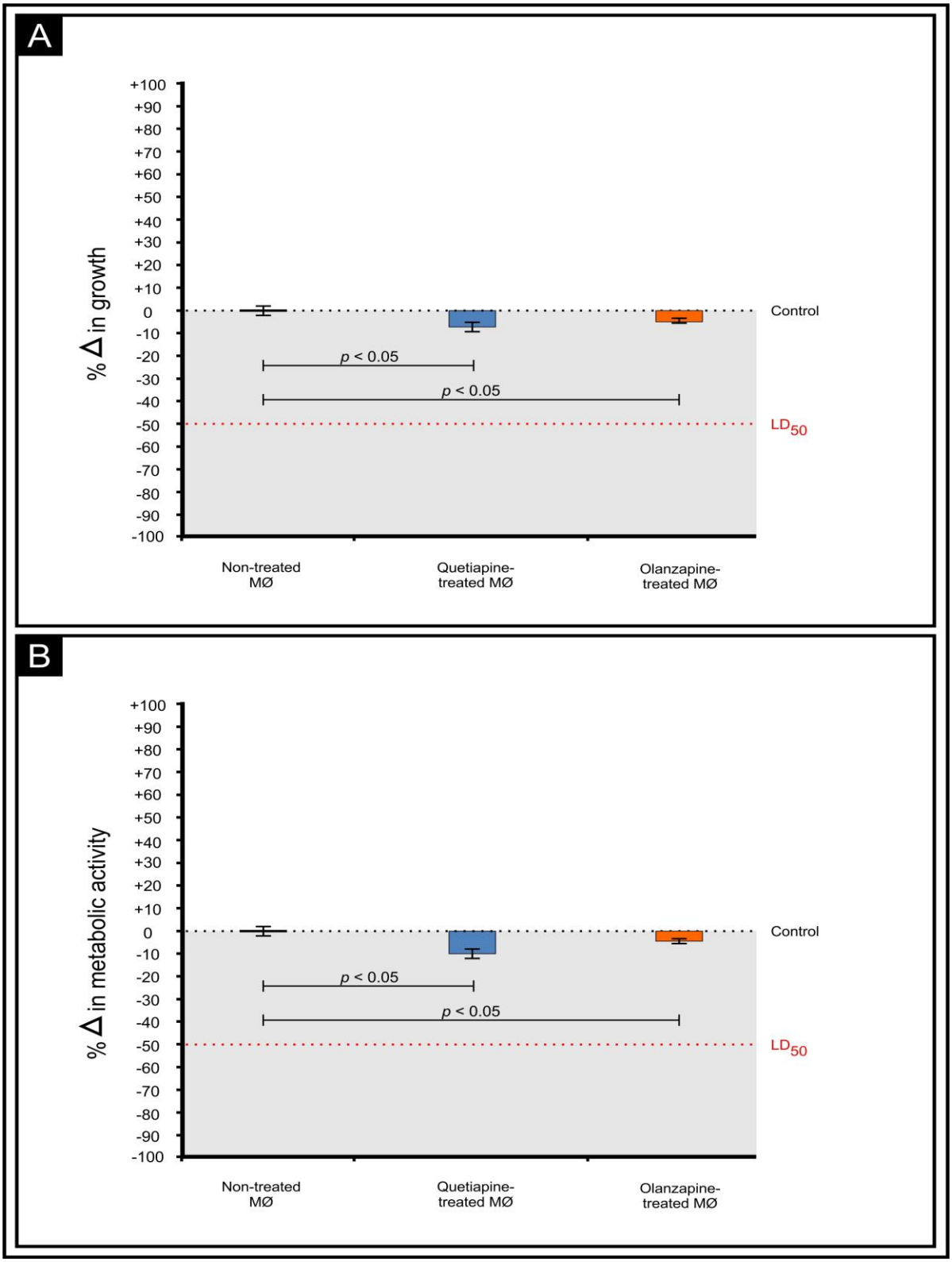

A
+100
+90
+80
+70
+60
+50
+40
+30
+20
+10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
% Δ in growth
Control
p < 0.05
p < 0.05
LD50
Non-treated MØ
Quetiapine-treated MØ
Olanzapine-treated MØ
B
+100
+90
+80
+70
+60
+50
+40
+30
+20
+10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
% Δ in metabolic activity
Control
p < 0.05
p < 0.05
LD50
Non-treated MØ
Quetiapine-treated MØ
Olanzapine-treated MØ

FIG 4

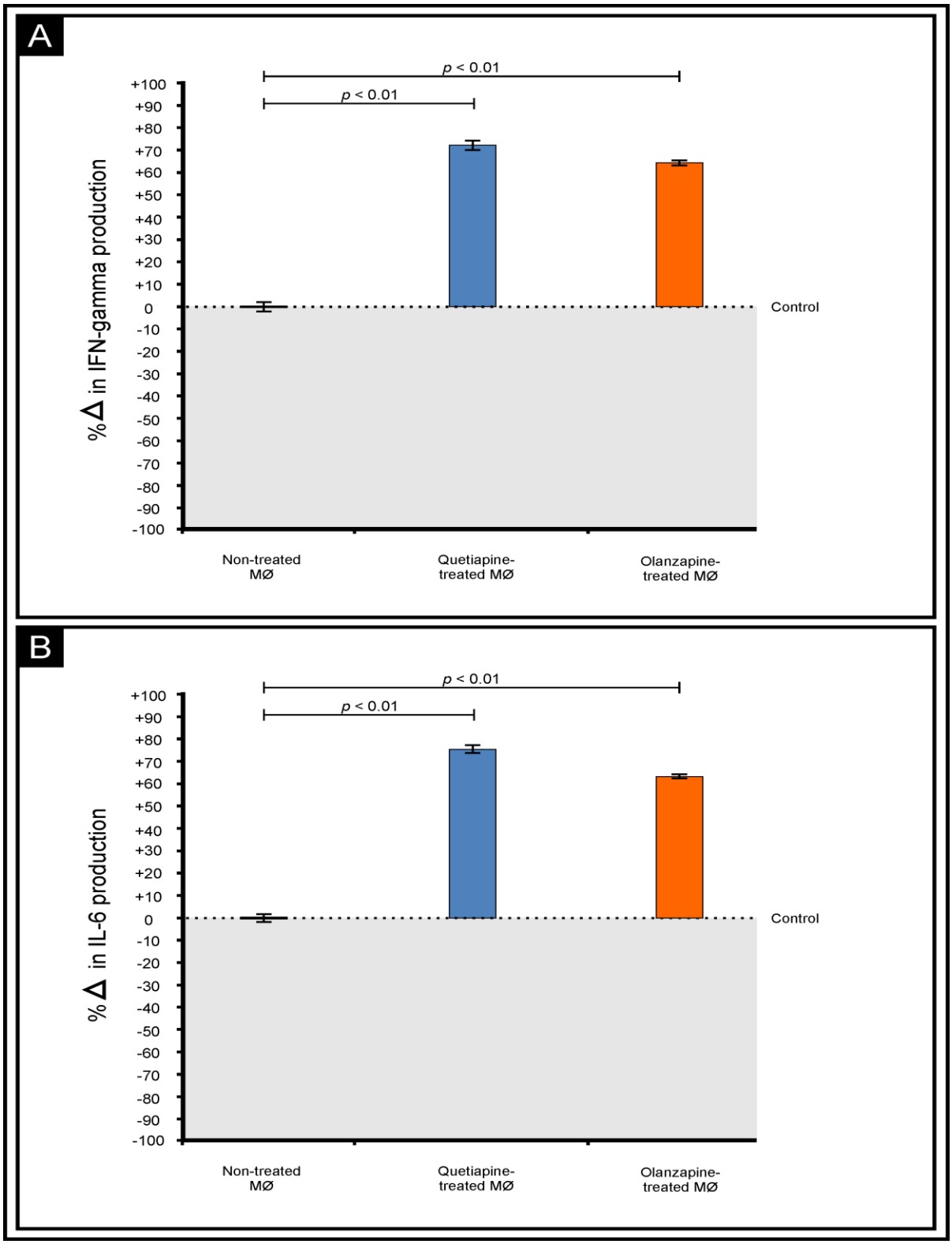

A
p < 0.01
p < 0.01
+100
+90
+80
+70
+60
+50
+40
+30
+20
+10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
% Δ in IFN-gamma production
Control
Non-treated MØ
Quetiapine-treated MØ
Olanzapine-treated MØ
B
p < 0.01
p < 0.01
% Δ in IL-6 production
Control
Non-treated MØ
Quetiapine-treated MØ
Olanzapine-treated MØ

FIG 5

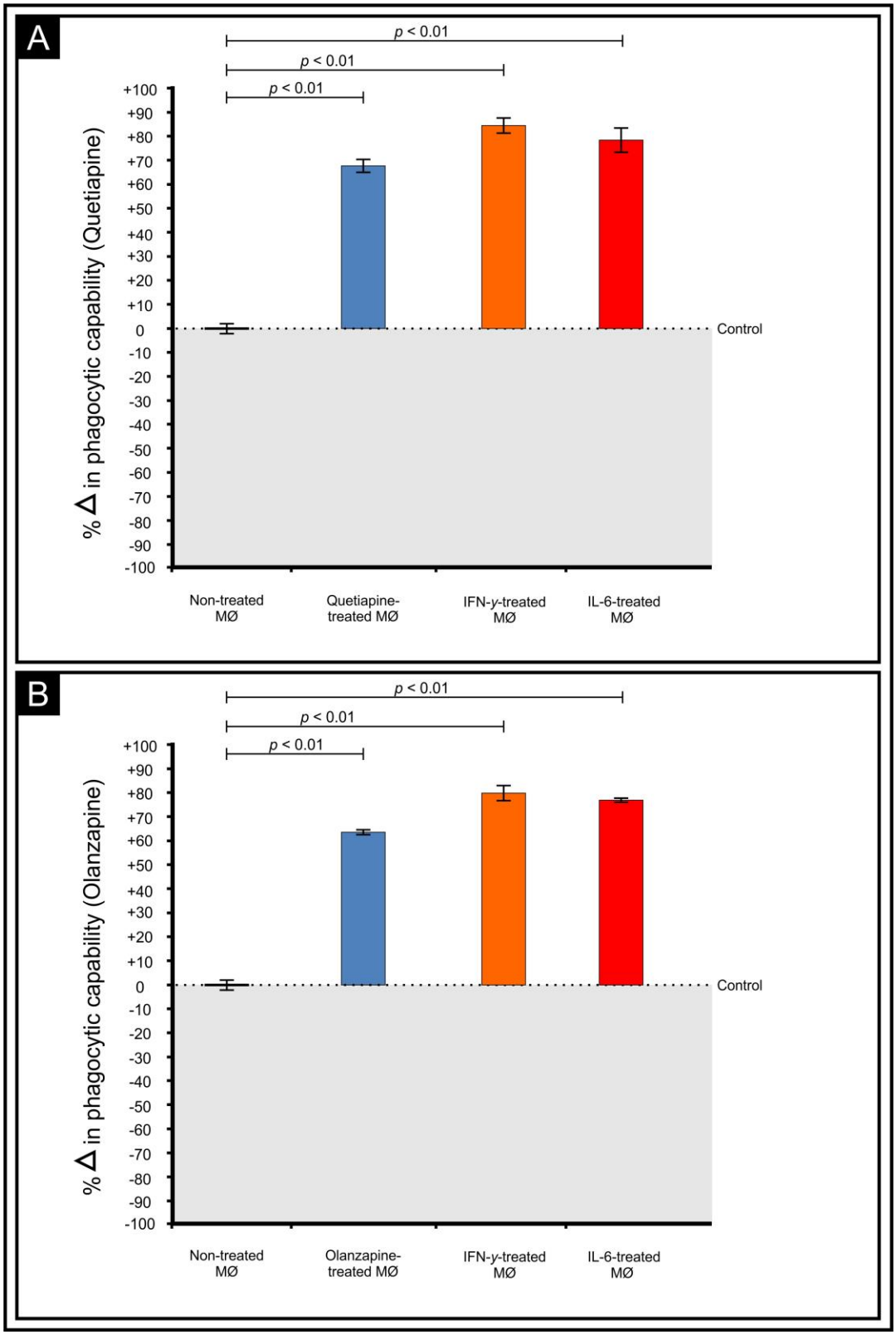

A
p < 0.01
p < 0.01
p < 0.01
% Δ in phagocytic capability (Quetiapine)
+100
+90
+80
+70
+60
+50
+40
+30
+20
+10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
Control
Non-treated MØ
Quetiapine-treated MØ
IFN-γ-treated MØ
IL-6-treated MØ
B
p < 0.01
p < 0.01
p < 0.01
% Δ in phagocytic capability (Olanzapine)
Control
Non-treated MØ
Olanzapine-treated MØ
IFN-γ-treated MØ
IL-6-treated MØ

FIG 6

THESIS SUMMARY

The manifestation of disseminated cryptococcal infection in HIV-infected individual is a life-threatening infection, with 70% mortality rate in sub-Saharan Africa. People are dying because of the complications around the management. Thus, there is a need for alternative drugs for better management of HIV-associated cryptococcal infection. This thesis successfully demonstrated *in vitro* anti-*Cryptococcus* activity of: 1) anti-inflammatory drugs (aspirin and ibuprofen), 2) aspirinate-metal complex (CAS), and 3) anti-psychotic drugs (quetiapine and olanzapine). In this thesis, EUCAST guidelines were followed closely and this provides a sense of effectiveness, which is very important for improving patient outcomes.

Chapter 2 focused on repurposing aspirin and ibuprofen as alternative anti-cryptococcal drugs. The major findings from this part of the thesis show that, all the tested fungal strains revealed a dose dependent response profile towards aspirin and ibuprofen. Compared to aspirin, ibuprofen exerts greater antimicrobial action. More importantly, the MICs of both drugs did not negatively affect the functioning of macrophages - rather they enhanced the phagocytic capability of macrophages to internalize more cryptococcal cells. Ibuprofen was also shown to act in synergy with fluconazole and amphotericin B at lower concentrations than individual concentrations tested. Our findings revealed the mode of action employed by aspirin and ibuprofen which is via oxidative damage.

Chapter 3 focused on a derivative of aspirin viz. copper acyl salicylate (CAS). CAS possess anti-fungal activity against cryptococcal cells and acted in synergy with fluconazole and amphotericin B at lower concentrations than their individual concentrations tested. CAS also kills cells via reactive oxygen species (ROS)-mediated membrane damage. The effect of CAS did not negatively affect macrophages but rather enhance their phagocytic function. Comparing with aspirin, CAS led to more growth reduction and showed less toxicity.

Today, one of the main challenges in the management of disseminated cryptococcal infections is the secondary complications such as psychosis. Therefore, chapter 4 considers repurposing two anti-psychotic drugs i.e. quetiapine and olanzapine as suitable candidate anti-*Cryptococcus* drugs. The *in vitro* susceptibility results revealed that quetiapine and olanzapine have anti-*Cryptococcus* activity and kill cells by compromising their membrane integrity. Importantly, the concentrations of drug tested were within the recommended dosage in the blood. Additionally, they acted in synergy with conventional drugs at concentrations that were lower than their defined MICs. It was also interesting to find that these two drugs chemosensitised macrophages, just like cytokines, which in turn, increase the appetite of macrophages against cryptococcal cells.

The presented data from this thesis has highlighted the potential clinical application of aspirin, ibuprofen, CAS, quetiapine and olanzapine as candidate anti-

Cryptococcus drugs. More encouragingly, all the drugs were able to effect synergism at reduced concentrations, which in turn can minimize the issues of side effects. These compounds employed a killing mechanism that was efficient against cryptococcal cells, although of lower eukaryotic origin. Therefore, it is important to demonstrate the effectiveness of these drugs in higher eukaryotic host cells. Towards this end, animal studies should be used as model to establish their therapeutic benefits in higher eukaryotic hosts. The duality of these compounds should also be assessed, which may provide additional beneficial therapeutic outcomes such as to manage pathogen-emergent psychosis and out-of-control inflammatory responses. Lastly, it is also important to determine if the antimicrobial activities of these compounds also occurs in other medically important pathogens.

Key words: Cryptococcal infection; aspirin; ibuprofen; copper acyl salicylate; reactive oxygen species; quetiapine; olanzapine; fluconazole; macrophages; psychosis; *In vitro* susceptibility; phagocytose.

PROEFSKRIFOPSOMMING

Die verskeinsel van verspreide *Cryptococcus* infeksie in MIV-besmette individue is 'n lewensgevaarlike infeksie, met ‘n sterftesyfer van 70% in sub-Sahara Afrika. Mense sterf as gevolg van komplikasies rondom die bestuur. So, daar is 'n behoefte aan alternatiewe medisyne vir beter bestuur van MIV-verwante krypto infeksie. Hierdie proefskrif het suksesvol in vitro anti-*Cryptococcus* aktiwiteit van: 1) anti-inflammatoriese middels (aspirien en ibuprofen), 2) aspirinaat-metaalkomplekse (KAS), en 3) anti-psigotiese middels (quetiapien en olanzapien) gedemonstreer. In hierdie proefskrif is EUCAST- riglyne gebruik wat ’n gevoel van doeltreffendheid daarstel, wat baie belangrik is vir die verbetering van die pasiëntuitkomste.

Hoofstuk 2 fokus op heraanwending van aspirien en ibuprofen as alternatiewe anti-*Cryptococcus* middels. Die belangrikste bevindings van hierdie deel van die proefskrif toon dat, al die getoetse gisstamme 'n dosis afhanklik reaksieprofiel teenoor aspirien en ibuprofen vertoon het. In vergelyking met aspirien, het ibuprofen ‘n groter antimikrobiese aksie uitgeoefen. Nog belangriker, die minimum inhiberende konsentrasie (MIK) van beide middels het die funksionering van makrofage nie negatief beïnvloed nie - eerder versterk hulle die fagositiese vermoë van makrofage om meer *Cryptococcus* selle te internaliseer. Dit is ook aangetoon dat ibuprofen in sinergie met flukonasool en amfoterisien B optree, teen laer konsentrasies as getoetste individuele konsentrasies. Ons bevindinge het getoon dat die antimikorobiese werking van aspirien en ibuprofen is via oksidatiewe skade.

Hoofstuk 3 fokus op 'n afgeleide van aspirien nl. koper asielsalisilaat (KAS). KAS besit anti-fungale aktiwiteit teen *Cryptococcus* selle en tree op in sinergie met flukonasool en amfoterisien B teen laer konsentrasies as hul individuele getoetste konsentrasies. KAS dood ook selle via membraanskade a.g.v. reaktiewe suurstof spesies (RSS). Die effek van KAS het nie 'n negatiewe invloed op makrofage nie, maar verhoog eerder hul fagositiese funksie. In vergeleke met aspirien, het KAS gelei tot meer groei vermindering en minder toksisiteit.

Huidiglik is een van die belangrikste uitdagings in die bestuur van verspreide *Cryptococcus* infeksies, die sekondêre komplikasies soos psigose. Daarom oorweeg hoofstuk 4 die heraanwending van twee anti-psigotiese middels, en wel quetiapien en olanzapien, as geskikte kandidaat anti-*Cryptococcus* middels. Die in vitro vatbaarheidsresultate het aangetoon dat quetiapien en olanzapien anti-*Cryptococcus* aktiwiteit het en selle dood deur hul membraanintegriteit te beskadig. Wat belangrik is, is dat die konsentrasies van middels wat getoets is, binne die aanbevole dosis in die bloed is Daarbenewens tree hulle in sinergie op met konvensionele medisyne, teen konsentrasies wat laer as hul gedefinieer MIK was. Dit was ook interessant om te sien dat hierdie twee middels makrofage chemosensiteer, net soos sitokiene, en so die aptyt van makrofage vir *Cryptococcus*-selle verhoog.

Die data wat aangebied word in hierdie proefskrif het die potensiële kliniese toepassing van aspirien, ibuprofen, KAS, quetiapien en olanzapien as kandidaat anti-

Cryptococcus middels uitgelig. Al die middels was in staat om sinergisme te bewerkstellig teen verlaagde konsentrasie, wat op sy beurt die kwessies van newe-effekte kan verminder. Hierdie verbindings meganismes gebruik wat doeltreffende was om *Cryptococcus*-selle, van 'n laer eukariotiese oorsprong, te dood. Daarom is dit belangrik om die doeltreffendheid van die middels in hoër eukariotiese gasheerselle demonstreer. Dit ten doel moet dierestudies gebruik word as model om hul terapeutiese voordele in hoër eukariotiese gashere te vestig. Die dualiteit van hierdie verbindings moet ook beoordeel word, wat bykomende voordelige terapeutiese uitkomste kan lewer soos om patogeen-ontluikende psigose en onbeheerde inflammatoriese response te bestuur. Laastens is dit ook belangrik om te bepaal of hierdie verbindings se antimikrobiese aktiwiteit uitgebrei kan word na ander medies belangrike patogene.

Sleutelwoorde: *Cryptococcus*-infeksie; aspirien; ibuprofen; koper asielsalisilaat; reaktiewe suurstof spesies; quetiapien; olanzapien; flukonazool; makrofage; psigose; *In vitro* vatbaarheid; fagositose.