COPPER ACYL SALICYLATE HAS POTENTIAL AS AN ANTI-*CRYPTOCOCCUS* ANTI-FUNGAL AGENT

Running title: The effects of aspirinate-metal complex on cryptococcal cells

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ABSTRACT

The *in vitro* anti-fungal activity of aspirin against cryptococcal cells has been reported. However, its undesired effects may limit its clinical application. Conceivably, a derivative of aspirin could overcome this challenge. Toward this end, this paper considered the usage of an aspirinate-metal complex viz. copper acyl salicylate (CAS) as an anti-*Cryptococcus* anti-fungal agent. Additionally, the paper examined the influence of this compound on macrophage function. The *in vitro* susceptibility results revealed that cryptococcal cells were vulnerable (in a dose-dependent manner) to CAS, which may have effected growth inhibition by damaging cryptococcal cell membranes. Interestingly, when used in combined therapy with fluconazole or amphotericin B, synergism was observed. Furthermore, CAS did not negatively affect the growth as well as the metabolic activity of macrophages rather it sensitised these immune cells to produce INF-*y* and IL-6, which, in turn, may have aided in the phagocytosis of cryptococcal cells. When compared to our aspirin data, CAS was noted to be more effective in killing cryptococcal cells (based on susceptibility results) and less toxic towards macrophages (based on growth inhibition results). Taken together, it is reasonable to conclude that CAS may be a better anti-*Cryptococcus* drug that could deliver better therapeutic outcomes when compared to aspirin.

Keywords. Copper acyl salicylate; *Cryptococcus*; Macrophages; Membrane damage; Synergy.

INTRODUCTION

Although it has become easy to diagnose cryptococcal infections, even in resource poor settings (1-4), treatment has largely remained difficult. This is due to current anti-fungal drugs being too expensive to some regions of the world. Importantly, the usage of some anti-fungal drugs is limited by their unintended side effects (5). A classical example is that of amphotericin B (administered to treat disseminated cryptococcal infections in patients with HIV), which is also known to target host renal tubules (5, 6). Given the risks associated with this drug and considering that in South Africa, 13 out of 100 people of the total population is living with HIV (7), it is reasonable to extrapolate that a significant portion of this population group is at danger of dying from this opportunistic fungus. Because of these challenges, there is an urgency to find better management strategies to control cryptococcal infections.

Our group has actively sought to identify and demonstrate "new" applications of "old" drugs - typically those that are FDA-approved and are currently purposed to treat non-infections conditions like inflammation; as anti-*Cryptococcus* drugs. In one of our recent papers, we successfully demonstrated that the prototypical anti-inflammatory drug aspirin, can be repurposed as an anti-*Cryptococcus* anti-fungal agent (8). However, the usage of aspirin in clinical settings can often lead to gastrointestinal toxicity (9). In order to derive the maximum therapeutic benefits out of aspirin, Sorenson considered preparing an aspirinate-copper-complex (a complex wherein copper was bound to four aspirin ligands) as an alternative to aspirin (10). Copper complexes are traditionally

known to be more effective and less toxic when compared to their individual parental compounds (11, 12). To illustrate this point, the Sorenson study noted that aspirinate-copper-complex was 30 times more effective than aspirin as an anti-inflammatory agent. Moreover, that aspirinate-copper-complex was pharmacologically more active in laboratory animals when compared to aspirin (10). Based on the above, it thus became the aim of the current study to determine if an aspirinate-metal complex i.e. copper acyl salicylate, could also exert anti-microbial effect on cryptococcal cells and importantly, if it would yield better results than those reported by Ogundeji and co-workers on aspirin (8).

MATERIALS AND METHODS

Cells, cultivation and standardisation. A total of ten clinical fungal strains (obtained from Universitas Academic Hospital, Bloemfontein, South Africa) were tested. These included five *C. neoformans* strains (LMPE 028 (*C. neoformans* var. *neoformans*), LMPE 030 (*C. neoformans* var. *neoformans*), LMPE 043 (*C. neoformans* var. *neoformans*), LMPE 046 (*C. neoformans* var. *neoformans*) and LMPE 047 (*C. neoformans* var. *neoformans*)) and five *C. gattii* strains (LMPE 045 (*C. neoformans* var. *gattii*), LMPE 048 (*C. neoformans* var. *gattii*), LMPE 052 (*C. neoformans* var. *gattii*), LMPE 054 (*C. neoformans* var. *gattii*) and LMPE 070 (*C. neoformans* var. *gattii*)). Moreover, a reference strain for *C. neoformans* viz. H99 (LMPE 150) and for *C. gattii* viz. R265 (LMPE 109) were included for comparison purposes. The strains were streaked 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) and incubated for 2 days

at 30°C. Following this, five colonies were selected and suspended in 10 ml of distilled water. Next, a standardised inocula (0.5 x 10^5 and 2.5 x 10^5 CFU/ml) was prepared as described by European Committee on Antimicrobial Susceptibility Testing (EUCAST) (13). In addition, a murine macrophage cell line (RAW 264.7; ATCC accession number TIB-71); a kind donation by Prof Masoko and Mr Makola, University of Limpopo, South Africa) was also used. The cell line was initially obtained from ATCC. The cells were grown (37°C and 5% CO_2) in RPMI-1640 medium (Sigma-Aldrich, South Africa) that was supplemented with 10% of foetal bovine serum (Biochrom, Germany), an antibiotic cocktail of penicillin (Sigma-Aldrich; 20 U/ml) and streptomycin (Sigma-Aldrich; 20 g/ml) as well as 2 mM L-glutamine (Sigma-Aldrich) until they reach 80% confluency. Before use, the cells were standardised to a final cell concentration of 1 x 10^5 cells/ml and subsequently seeded into wells of a sterile, disposable 96-well flat-bottom microtitre plate (Greiner Bio-One, Germany).

Drugs. Copper acyl salicylate (a kind donation by Dr Pravin Kendrekar from Health Sciences Department, Central University of Technology), fluconazole (Sigma-Aldrich) and amphotericin B (Sigma-Aldrich) were obtained as standard powders. Copper acyl salicylate was dissolved in absolute ethanol (Merck, South Africa), fluconazole in distilled water while dimethyl sulfoxide (Merck) was used for amphotericin B. These compounds were further diluted using RPMI 1640 media to reach desired final concentrations in the wells of the microtiter plate. Thus, at the end the concentrations of drug diluents, in which stock solutions were prepared, never exceeded 1%. For comparative purposes, a

concentration gradient of 0.01 mM (8.44 µg/ml), 0.1 mM (84.4 µg/ml) and 1 mM (844 µg/ml), similarly to that we used for aspirin (8), was used in the current study.

Susceptibility assay. The *in vitro* susceptibility assay was done according to EUCAST guidelines (13). In brief, wells of sterile 96-well flat-bottom microtitre plates were seeded with 100 µl of standardised cryptococcal cells. The cells were immediately treated with 100 µl of the test drug (CAS, fluconazole or amphotericin B) at twice the desired final concentration as stated above. Non-treated cells were included as control. The plates were incubated at 37°C for 48 h before reading the optical density (OD) of the wells using a spectrophotometer (Biochrom EZ Read 800 Research, United Kingdom) at 562 nm. At the end, the percentage growth reduction was calculated as follows: (OD reading of treated cells/OD reading of non-treated cells) x 100%. Concentrations that led to 50% or more reduction in growth were used in the checkerboard assays. In anticipation of the checkerboard assay, the respective concentrations of amphotericin B and fluconazole that also led to a 50% or more reduction in growth were 1 µg/ml and 8 µg/ml. These figures were based on those we reported for aspirin (8). The current study and the above-mentioned aspirin study were done on the same strains, in the same laboratory and at the same period.

Checkerboard assay. For the assay, cells from LMPE 046 (most sensitive to CAS), LMPE 052 (most resistant to CAS), LMPE 109 (*C. gattii* reference strain) and LMPE 150 (*C. neoformans* reference strain) were used. Standardised cryptococcal cells (in 100 µl of RPMI 1640 media) were seeded into wells of sterile microtitre plate and immediately

138 treated with CAS paired with amphotericin B (50 µl : 50 µl) or CAS paired with
139 fluconazole (50 µl : 50 µl). The plate was incubated at 37°C for 48 h. At the end of the
140 incubation period, OD readings were taken, and subsequently the fractional inhibitory
141 concentration (FIC) index (FICI) was calculated. Fractional inhibitory concentration index
142 (that is, the sum of the FICs [ΣFIC]) was defined as $FIC_A + FIC_B$, where FIC_A is the MIC
143 of drug A in combination/MIC of drug A alone and FIC_B is the MIC of drug B in
144 combination/MIC of drug B alone (8). Fractional inhibitory concentration index values
145 were determined to establish if there was synergism (≤ 0.5), no interaction (> 0.5 - 4) or
146 antagonism (> 4).
147
148 The effect of CAS (at the determined MIC) on the ultrastructure of cryptococcal
149 cells, its mode of action and macrophage interaction studies were examined using the
150 one strain that showed the greatest sensitivity.
151
152 **Effect of CAS on cellular ultrastructure.** To perform scanning electron microscopy
153 (SEM), 48-h old cells (prepared as detailed in the *in vitro* susceptibility assay) i.e. non-
154 treated cells and CAS-treated cells (844 µg/ml) were considered. These chosen cells
155 were prepared for SEM as previously described by van Wyk and Wingfield (14). In brief,
156 cells were chemically fixed with sodium-phosphate-buffered 3% glutardialdehyde (Merck)
157 and sodium-phosphate-buffered 3% osmium tetroxide (Merck) following dehydration in a
158 graded ethanol (Merck) series. Then, the cells were dried (Bio-Rad Microscience
159 Division, England), mounted on stubs, and coated with gold using an SEM coating
160 system (Bio-Rad Microscience Division) (14). Cells were examined using a Shimadzu

Superscan SSX 550 scanning electron microscope (Japan). To determine the diameter of cells, a 100 cells per each experimental condition (randomly selected from different locations acquired from different stubs) were measured.

To complement the above experiment, the effect of CAS on causing cells to shed their capsule (glucuronoxylomannan; GXM) was determined. In brief, the cells (representing treated and non-treated cells) were prepared as detailed in the *in vitro* susceptibility assay and grown over a 48-h period. However, to map the change in the amount of the shed GXM, the supernatant was aspirated (at different time points i.e. 0 h, 12 h, 24 h and 48 h) from respective wells. The supernatant was then transferred to wells of an ELISA (IMMY, United States) microtiter plate specific for GXM quantification. The plate was treated according to guidelines provided by IMMY. The optical density was measured at 450nm using a spectrophotometer.

Effect of CAS on reactive oxygen species (ROS) and phospho-p-38 MAPK. These assays were done as previously detailed by Ogundeji et al (8). For both assays, cells were prepared as detailed in the *in vitro* susceptibility assay. At the end of the 48 h incubation period, the plate was gently agitated to re-suspend the cells. For ROS analysis, 90 µl of media containing cells (separately collected from non-treated cells wells and CAS-treated cells wells) was aspirated and transferred to designated wells on a sterile, black 96-well flat-bottom microtitre plate (Greiner Bio-One). These cells were reacted 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, USA) microplate reader. For phospho-p-38 MAPK analysis, 100 µl of media containing cells (separately collected from non-treated cells wells and CAS-treated cells wells) was aspirated and transferred to sterile, designated 1.5 mL eppendorf tubes (Merck). Following this, the lysate was harvested from the cells according to a protocol and materials provided in the phospho-p-38 MAPK ELISA kit (Sigma-Aldrich). The lysate was then transferred to a sterile microtitre plate specific for phospho-p-38 MAPK assay. The lysate plate was then treated according to the manufacturer's protocol. The plate was read at 450 nm using a Biochrom EZ spectrophotometer.

The cytotoxic effect of CAS on murine macrophages. This assay was done as previously detailed by Ogundeji et al (8). Standardised macrophages (1 x 10^5 cells/ml) were re-suspended in fresh media following overnight seeding at 37°C in a 5% CO_2 incubator (Thermo-Scientific). The cells were immediately treated with 100 µl of CAS (1:1 v/v) to reach a final drug concentration of 844 µg/ml (MIC), 4220 µg/ml (5x the MIC) and 8440 µg/ml (10x the MIC). The non-treated macrophages were included as control. The plate was incubated at 37°C in a 5% CO_2 incubator for 48 h.

For determine the CAS effect on the metabolic activity, 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). Following that, the plate was incubated in the 5% CO_2

incubator. The OD of the wells was measured at 492 nm after 3 h of initiating the tetrazolium reaction, using a Biochrom spectrophotometer.

Moreover, the therapeutic index (TI) of CAS was calculated by dividing the LD_{50} by the obtained MIC (i.e. LD_{50} / $MIC_{844\ \mu g/ml}$) to determine if the range is narrow or wide (15).

The phagocytic function of macrophages i.e. capability to internalise cryptococcal cells in the presence or absence of CAS, was measured using a phagocytosis stain, pHrodo™ Green Zymosan A BioParticles (Life Technologies, USA). In brief, 1 µl of this stain was added to 999 µl of standardised cryptococcal cells in RPMI 1640 medium and incubated at 37°C for 1 h while slowly agitating. After 1 h, cryptococcal cells were washed twice with PBS, spun down and re-suspended in 1000 µl of fresh media that contained twice the desired final concentration of CAS (844 µg/ml). A co-culture was prepared by adding 100 µl of the stained cryptococcal cells to seeded macrophages (1:1 v/v) at an effector-to-target ratio of 1:1. 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.

At the end, the immunological response of macrophages to cryptococcal cells in the presence or absence of CAS was also measured. Here, cryptococcal cells were suspended in fresh RPMI 1640 media that contained twice the desired final concentration of CAS (844 µg/ml) or just in media (devoid of CAS). A co-culture was prepared by adding 100 µl of the cryptococcal cells to seeded macrophages (1:1 v/v) at an effector-to-

target ratio of 1:1. The plate was incubated at 37°C in a 5% CO_2 incubator for 6 h. After the incubation period, the supernatants were collected and kept for cytokine ELISA assays i.e. interferon-gamma (INF-*y*) and interleukin-6 (IL-6). Following this, 100 µl of the supernatant (separately obtained from co-cultures with or without CAS) were transferred to wells on a sterile microtitre plate specific for INF-*y* or IL-6 (BioLegend, USA). The supernatants were then treated according to the respective manufacturer's protocol. The plates were read at 450 nm using 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.

RESULTS AND DISCUSSION

Copper acyl salicylate has anti-fungal activity and acts in synergy with conventional drugs. Table 1 summarises the effect of CAS on the growth of the ten-tested clinical and two reference *Cryptococcus* strains. From the table, it was observed that as the concentration of CAS increased, there was 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, CAS effected 50% or more growth reduction when compared to the drug-free control. Interestingly, we previously reported that aspirin

(at the same drug concentration (1 mM)), also effected 50% or more growth reduction (8). However, when directly comparing the anti-microbial activity of CAS against that of aspirin on all ten strains - CAS was noted to be more effective in reducing growth (data not shown). This determination is important as it confirms what is expected of a metal complex – that is, it should display strong biological activities when compared to its individual parental compounds (10, 11).

In addition to negatively affecting growth, drug treatment also influenced the ultrastructural appearance of the cells as well as their size (Fig. 1). When compared to treated cells (3.91 µm ± 0.06 in cell diameter), the non-treated cells (4.14 µm ± 0.05 in cell diameter) were observed to be bigger in size ($p < 0.05$). Furthermore, the non-treated cells appeared to be covered with an extracellular matrix, which could possibly be the capsule. On the other hand, drug treatment seemed to reduce this covering, which may leave cells "naked" and vulnerable to adverse external conditions i.e. the effects of other drugs when administering combined therapy or even, macrophage action. To ascertain if cells may have lost their capsules in the presence of CAS, the amount of shed GXM in the growth medium was measured (Fig. 2). Here, it was noted that treated cells significantly ($p < 0.01$) shed their GXM than non-treated cells.

Table 2 summarises the combined effect of CAS and amphotericin B as well as CAS and fluconazole on strain LMPE 046 (most sensitive to CAS), LMPE 052 (most resistant to CAS), LMPE 109 (*C. gattii* reference strain) and LMPE 150 (*C. neoformans* reference strain). When analysing the data, it was pleasing to note that certain drug

combinations (shown in colour-coded cells) led to a synergistic outcome that also translated into a corresponding growth reduction of 50% or more. Importantly here, the concerned concentration of a particular drug (CAS, amphotericin B or fluconazole) when used in combined therapy were lower than the concerned concentration of that particular drug when used alone. The significance of the latter is that when CAS is used in combined therapy, it should: 1) minimise the unintended risks associated with using amphotericin B or, 2) enhance the effectiveness of fluconazole, which is known to be fungistatic.

Copper acyl salicylate subjected cells to oxidative stress. Figure 3A shows the effect of CAS on ROS production. The CAS-treated cells were shown to accumulate significant amounts of ROS ($p < 0.01$) when compared to non-treated cells. In addition to being produced as part of normal oxygen metabolism of respiring cells (16), any impairment in the shuttling of electrons and generation of membrane potential can also lead to ROS. This impairment can, among other things, be induced by exposure of cells to certain drugs as it was the case with aspirin (8). In the latter study, it was shown that aspirin exposure lead to loss of membrane potential. It is our assumption that like aspirin, CAS could have led to loss of membrane potential hence the observed ROS accumulation. Given the undesired effects of ROS on cellular macromolecules (17), cells can under conditions of stress signal an adaptive response (18, 19). To investigate the latter, we sought to determine if ROS may serve as a stimulant that may activate a stress response pathway by assaying for the phosphorylation of the p-38 MAPK. Figure 3B shows a significant increase ($p < 0.01$) in the levels of phosphorylation of p-38 MAPK among

CAS-treated cells compared to levels recorded for non-treated cells. Based on Figure 3 (A and B) results, it is reasonable to conclude that CAS seems to have subjected cells to oxidative stress as noted from the elevated levels of p-38 MAPK and excessive accumulation of ROS. It is therefore our argument that ROS may have targeted the cell walls as seen in Figure 1 and compromised their integrity and importantly, this loss in integrity may have led to growth reduction as noted in Table 1.

Copper acyl salicylate improves the functioning of murine macrophages. When considering the effects of CAS on macrophages, it was noted that CAS (at 844 µg/ml) led to a 6% reduction in metabolic activity (Fig. 4) of treated macrophages when compared to non-treated macrophages. This finding (6%) further imply that far more than the 844 µg/ml (1 mM) of CAS is required to be added to the cultivation media of macrophages in order to effect a lethal dosage wherein 50 % of metabolic activity would be negatively affected. However, a 50% reduction was achieved at a concentration that is ten times (i.e. 8440 µg/ml) the determined MIC (Fig. 4). When calculating the TI, it was determined that the therapeutic range was wide enough as 10x the concentration of the MIC was required to effect a 50% lethal dosage. In their review article, Tamargo et al. (15) reported that generally a drug with a TI value of 10 or more is considered a safe drug. Thus, our findings are in line with what is generally accepted in literature.

We also determined if CAS would chemically sensitise macrophages, and thus enhance their phagocytic capability. The data shown in Figure 5 revealed that the presence of CAS significantly enhanced the efficiency of macrophages to internalise

cryptococcal cells by 65%, compared to the non-treated cells. Furthermore, non-CAS-treated macrophages produced significantly less INF-*y* levels when compared to CAS-treated macrophages ($p < 0.05$) (Fig. 6A) and similarly non-CAS-treated macrophages produced significantly less IL-6 levels when compared to CAS-treated macrophages ($p < 0.05$) (Fig. 6B). When taken together Figures 5 and 6 results suggest that CAS may have improved the functioning of macrophages as these immune cells were able to have more cytokines (Fig. 6), which may have aided them to better recognise (perhaps due to the loss of the extracellular covering as seen in Figure 1) and to internalise more cryptococcal cells as seen Figure 5.

In conclusion, the manifestation of disseminated cryptococcal infections in HIV-infected individuals is a serious problem more so for people living in Sub-Saharan Africa. And, undesired issues around the current anti-fungal drugs (amphotericin B and its side effects and fluconazole being fungistatic) only worsens the matter. Toward this end, we have proposed a number of alternative drugs that may be considered to manage cryptococcal infections (8, 20). The current paper looked specifically at the effects of a metal complex, CAS, and is informed by our initial work on aspirin. It was interesting to note that CAS was more effective than aspirin in controlling the growth of cryptococcal cells. This is a quality that is to be expected of a metal complex and our findings are in line with the observations of Sorenson and co-workers with respect to the quality of aspirinate metal complexes. Though CAS may be more attractive than aspirin, it is not known at this point if it will also lead to less gastrointestinal toxicity. Therefore, studies are now required to examine this aspect.

For our *in vitro* susceptibility assays we followed the EUCAST guidelines, which is significant as the work can be reproduced elsewhere and provides insight into the effectiveness of the test drug used. Moreover, the results give an indication of whether the drug may lower the microbial burden in laboratory animals. We are at the moment seeking ethical approval to test the latter. We were able to establish that like aspirin, CAS may have inhibited the growth of the cells by disrupting membrane function. Membranes are crucial for non-fermenting microbes like *Cryptococcus*, which are entirely dependent on the oxygen metabolism to derive energy to support their growth. Because of the latter, it will be interesting to see if laboratory animals (which like humans, also respire) will not be adversely affected by CAS treatment. At this moment, we have only established (*in vitro*) that a mammalian cell line was not negatively affected by CAS rather, exposure to CAS was beneficial.

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TRANSPARENCY DECLARATION

The authors have no conflicts of interest to declare.

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473 **TABLE 1.** The effect of CAS on growth of *C. neoformans* and *C. gattii*.

Species detail		Non-treated cells	Drug response CAS-treated cells		
			8.44 µg/ml	84.4 µg/ml	844 µg/ml
Strain name	**Strain number**	**OD $_{562\ nm}$**	**%GR**	**%GR**	**%GR**
C. neoformans (H99)	LMPE 150	0.458 (0.016)	13% (0.025)	34 (0.028)	78% (0.054)
C. neoformans	LMPE 028	0.439 (0.012)	12% (0.030)	32 (0.044)	77% (0.031)
C. neoformans	LMPE 030	0.446 (0.007)	13% (0.012)	34 (0.007)	76% (0.015)
C. neoformans	LMPE 043	0.449 (0.013)	12% (0.047)	31 (0.005)	77% (0.023)
C. neoformans	LMPE 046	0.469 (0.009)	15% (0.026)	38 (0.052)	80% (0.017)
C. neoformans	LMPE 047	0.460 (0.019)	14% (0.017)	35 (0.051)	78% (0.015)
C. gattii (R265)	LMPE 109	0.456 (0.023)	12% (0.033)	32 (0.019)	77% (0.049)
C. gattii	LMPE 045	0.462 (0.016)	12% (0.037)	30 (0.029)	75% (0.029)
C. gattii	LMPE 048	0.464 (0.016)	13% (0.025)	32 (0.022)	76% (0.004)
C. gattii	LMPE 052	0.474 (0.007)	11% (0.014)	29 (0.014)	74% (0.014)
C. gattii	LMPE 054	0.451 (0.003)	10% (0.005)	31 (0.008)	74% (0.014)
C. gattii	LMPE 070	0.458 (0.003)	11% (0.013)	30 (0.022)	75% (0.099)

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

476 **TABLE 2.** The combined effect of CAS and amphotericin B and CAS and fluconazole on selected *Cryptococcus* strains.

LMPE 046		Fractional inhibitory concentration (FIC) index					Percentage growth reduction (%GR)				
	CAS (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
	105.6	1.65	0.69	0.40	0.37	0.29	31	44	61	73	79
	211	0.93	0.52	0.38	0.33	0.21	42	48	65	75	87
	422	0.61	0.42	0.29	0.27	0.18	49	66	73	78	92
	844 (MIC)	0.42	0.34	0.23	0.20	0.12	60	72	76	85	95
	1688	0.29	0.22	0.17	0.11	0.08	79	82	86	94	98
	CAS (µg/ml)	Fluconazole (µg/ml)					Fluconazole (µg/ml)				
		2	4	8 (MIC)	16	32	2	4	8 (MIC)	16	32
	105.6	2.06	0.65	0.43	0.40	0.34	26	41	58	67	76
	211	1.89	0.56	0.41	0.35	0.31	38	49	63	74	80
	422	0.64	0.47	0.37	0.29	0.26	45	63	72	78	85
	844 (MIC)	0.43	0.39	0.28	0.24	0.19	58	71	80	84	91
	1688	0.30	0.23	0.20	0.16	0.11	73	80	86	88	97
LMPE 150											
	CAS (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
	105.6	1.60	0.67	0.39	0.35	0.24	32	45	63	75	80
	211	0.89	0.55	0.35	0.32	0.19	44	49	66	77	88

	422	0.72	0.40	0.29	0.26	0.15	48	66	75	80	91
	844 (MIC)	0.47	0.31	0.22	0.20	0.11	61	74	79	84	94
	1688	0.31	0.26	0.19	0.14	0.07	77	80	88	91	96
	CAS (µg/ml)	**Fluconazole (µg/ml)**					**Fluconazole (µg/ml)**				
		2	**4**	**8 (MIC)**	**16**	**32**	**2**	**4**	**8 (MIC)**	**16**	**32**
	105.6	2.17	0.84	0.42	0.38	0.32	24	38	56	69	73
	211	1.98	0.62	0.39	0.34	0.28	35	46	61	73	77
	422	0.81	0.44	0.34	0.26	0.23	42	61	70	76	81
	844 (MIC)	0.46	0.41	0.28	0.23	0.20	57	70	78	80	86
	1688	0.39	0.30	0.23	0.19	0.15	71	82	86	85	90
LMPE 052											
	CAS (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**
	105.6	1.71	0.66	0.41	0.35	0.27	28	41	60	71	77
	211	0.99	0.54	0.36	0.31	0.20	40	47	66	74	86
	422	0.75	0.41	0.28	0.25	0.16	47	64	72	79	90
	844 (MIC)	0.44	0.32	0.22	0.21	0.12	61	70	78	83	93
	1688	0.27	0.25	0.19	0.15	0.09	78	83	87	90	96
	CAS (µg/ml)	**Fluconazole (µg/ml)**					**Fluconazole (µg/ml)**				
		2	**4**	**8 (MIC)**	**16**	**32**	**2**	**4**	**8 (MIC)**	**16**	**32**
	105.6	2.12	0.71	0.45	0.39	0.35	22	39	55	65	75

	211	1.94	0.59	0.40	0.32	0.30	36	47	60	71	79
	422	0.78	0.46	0.38	0.28	0.26	41	60	69	77	82
	844 (MIC)	0.48	0.40	0.32	0.25	0.21	56	68	75	81	89
	1688	0.41	0.28	0.24	0.18	0.13	70	78	83	87	94
LMPE 109											
	CAS (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**
	105.6	1.62	0.75	0.42	0.33	0.25	30	43	62	73	78
	211	0.95	0.53	0.39	0.30	0.20	38	48	67	76	85
	422	0.68	0.43	0.31	0.25	0.16	45	65	71	81	90
	844 (MIC)	0.44	0.30	0.26	0.20	0.13	62	73	77	86	94
	1688	0.26	0.23	0.19	0.15	0.09	76	81	85	92	97
	CAS (µg/ml)	**Fluconazole (µg/ml)**					**Fluconazole (µg/ml)**				
		2	**4**	**8 (MIC)**	**16**	**32**	**2**	**4**	**8 (MIC)**	**16**	**32**
	105.6	2.03	0.72	0.40	0.37	0.31	25	40	60	68	75
	211	1.94	0.60	0.38	0.33	0.28	36	47	64	70	81
	422	0.59	0.45	0.34	0.28	0.24	40	62	69	78	86
	844 (MIC)	0.46	0.37	0.26	0.22	0.17	56	70	75	82	90
	1688	0.32	0.25	0.21	0.19	0.14	72	79	83	86	95

477 Colour-coded cells represent the define MIC for each respective drug and the combine effect of the respective MIC on percentage growth
478 reduction and FICI.

479

FIGURE LEGENDS

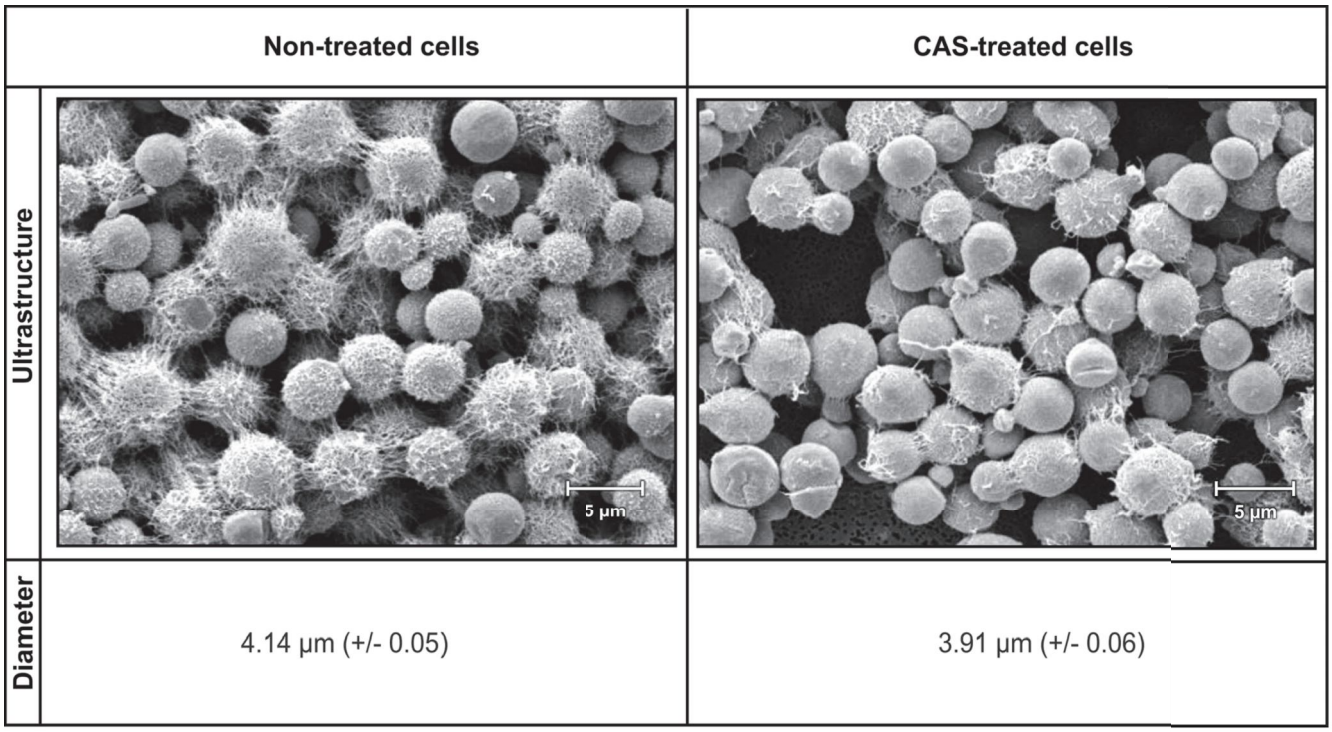
FIG. 1. Scanning electron micrographs showing the effect of CAS on the ultrastructure and the size of cells. CAS-treated cells showed more extracellular matrix (this may be the capsule) on their cell wall surfaces compared to non-treated cells. The treated cells were significantly smaller ($p < 0.05$) in cell diameter (CAS-treated cells = 3.91 µm +/- 0.06) when compared to that of non-treated cells (4.14 µm +/- 0.05).

FIG. 2. The ELISA quantitative results showing the levels of shed GXM by non-treated cells ad treated cells. The results indicate that cells significantly ($p < 0.01$) shed their capsules in the presence of CAS than in its absence.

FIG. 3. Reactive oxygen species (ROS) assay results showing the effect of CAS on treated cells compared to non-treated cells (A). Treatment of cells with CAS led to a significant increase ($p < 0.01$) in ROS accumulation compared to non-treated cells. MAPK p-38 assay results showing the effect of CAS on p-38 phosphorylation levels (B). The p-38 phosphorylation levels of CAS-treated cells were significantly higher ($p < 0.01$) compared to that of non-treated cells.

FIG. 4. The effect of CAS on macrophage (MØ) metabolic activity was expressed as percentage (%) change in metabolic activity. At the MIC (844 µg/ml), CAS was non-toxic to macrophages – as it did not yield a 50% reduction in metabolic activity. However, at ten times the MIC (8440 µg/ml) – A 50% reduction in the metabolic activity was achieved.

503 **FIG. 5.** The effect of CAS on macrophage (MØ) phagocytic capability. Addition of CAS
504 significantly enhanced ($p < 0.01$) the phagocytic capability of macrophages compared
505 to that of non-treated macrophages.
506
507 **FIG. 6.** The effect of CAS on macrophage (MØ) immunological response. Addition of
508 CAS lead to significantly higher levels of pro-inflammatory cytokines compared to non-
509 treated macrophages. To the point, (A) shows a 74% increase in the levels of interferon-
510 gamma while (B) shows an 88% increase in the levels of interleukin-6 in the presence of
511 CAS.
512



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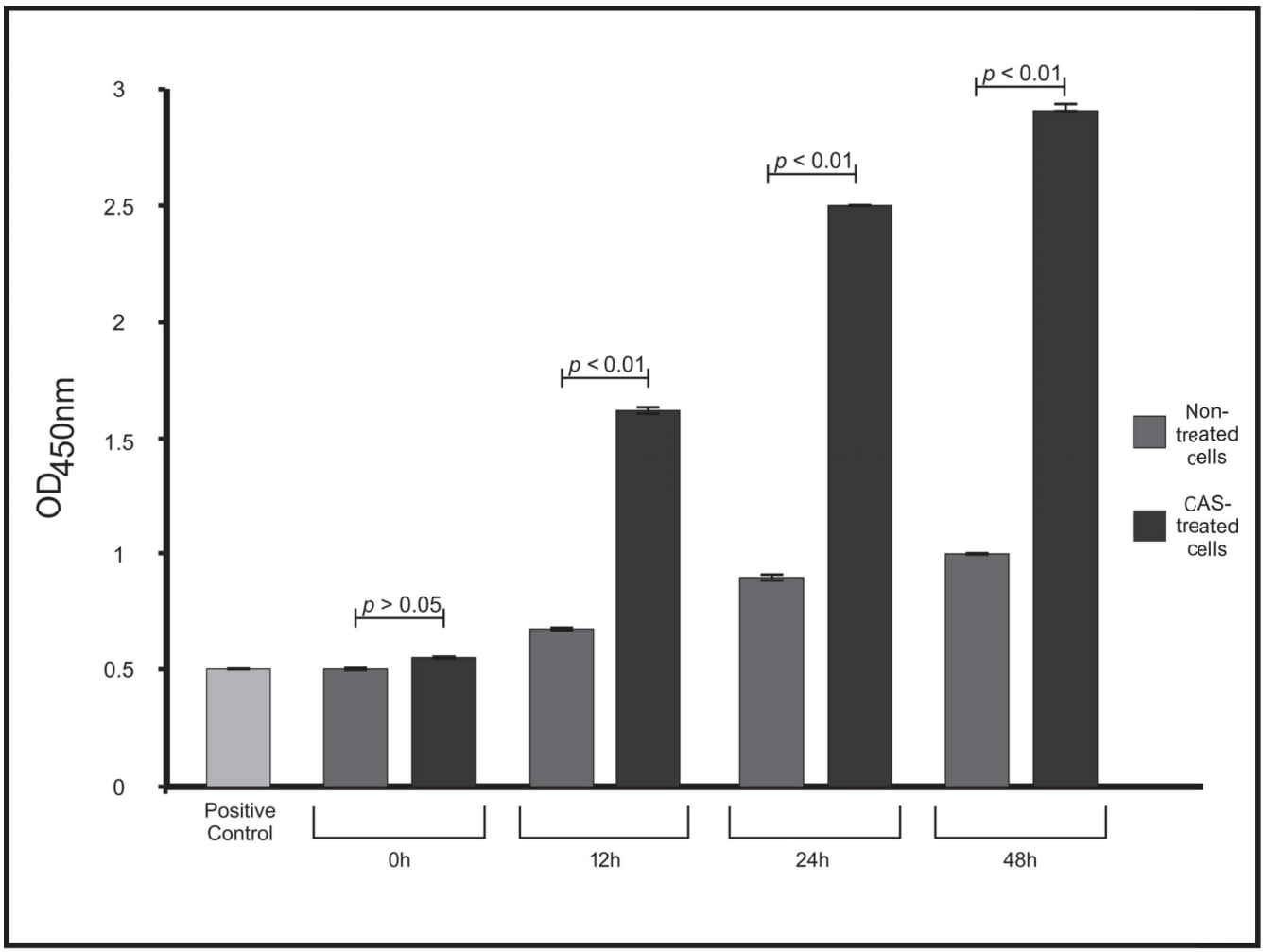

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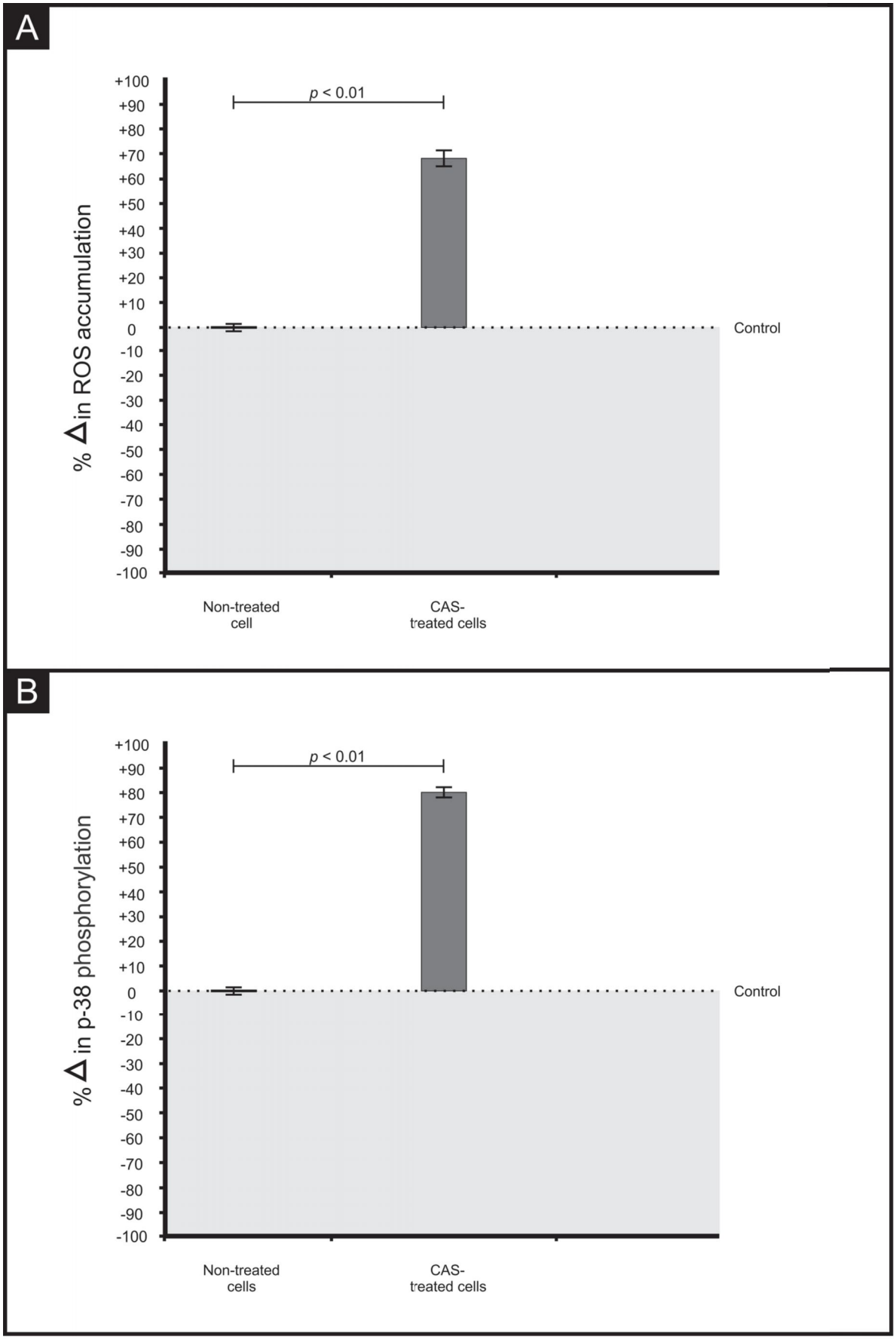

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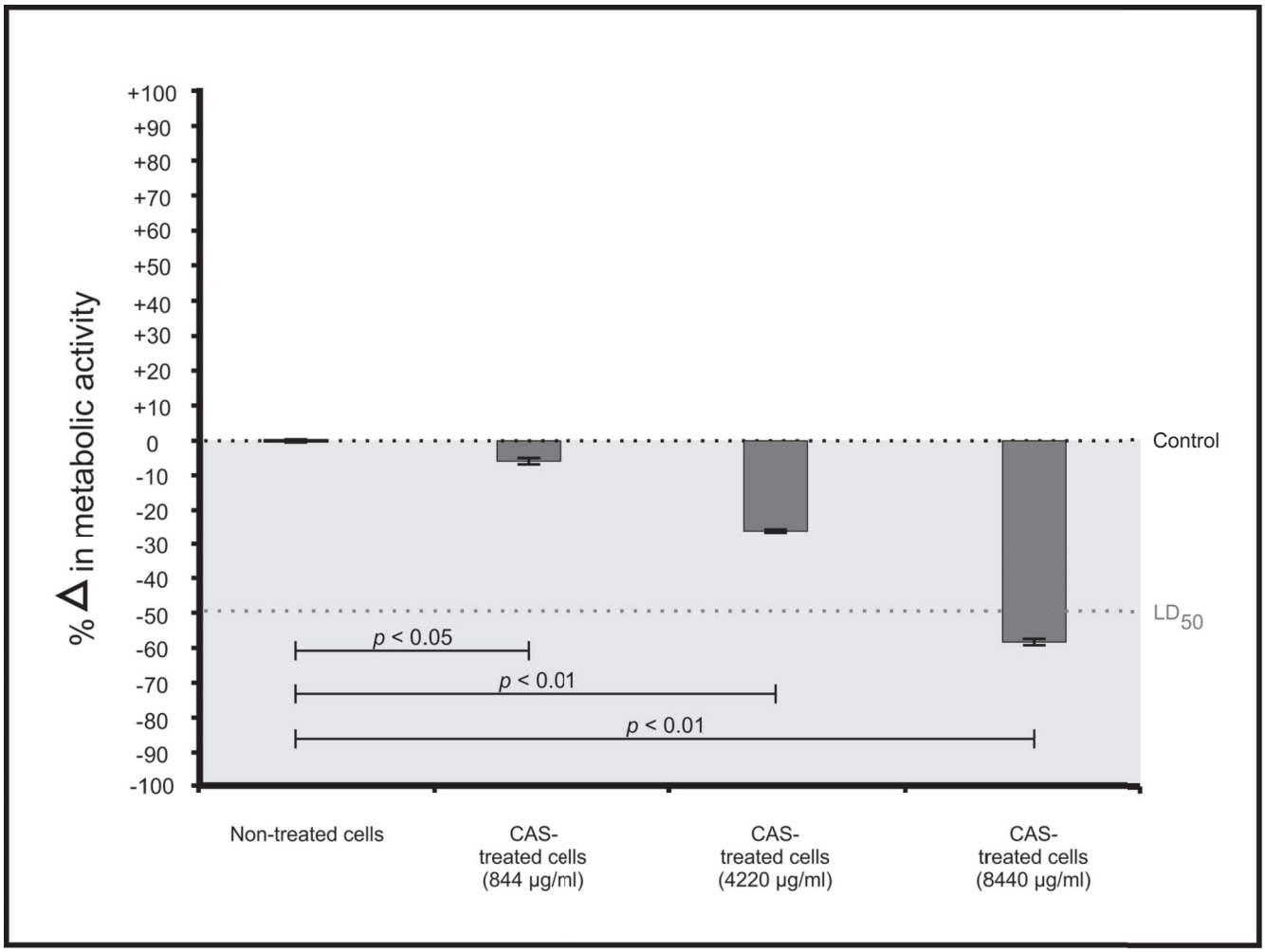

FIG. 4. The effect of CAS on macrophage (MØ) metabolic activity was expressed as percentage (%) change in metabolic activity. At the MIC (844 µg/ml), CAS was non-toxic to macrophages – as it did not yield a 50% reduction in metabolic activity. However, at ten times the MIC (8440 µg/ml) - a 50% reduction in the metabolic activity was achieved.

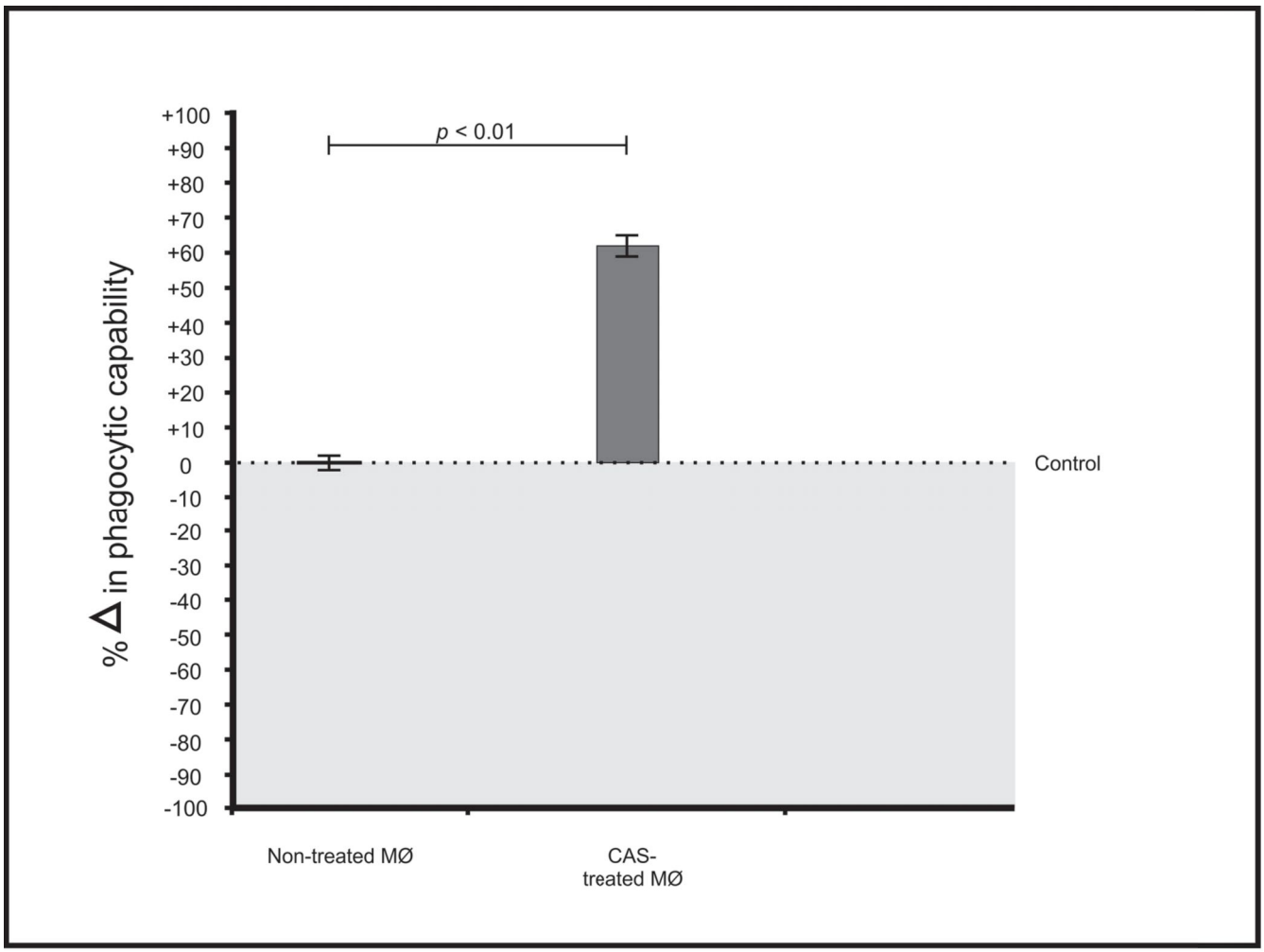

FIG. 5. The effect of CAS on macrophage (MØ) phagocytic capability. Addition of CAS significantly enhanced ($p < 0.01$) the phagocytic capability of macrophages compared to that of non-treated macrophages.

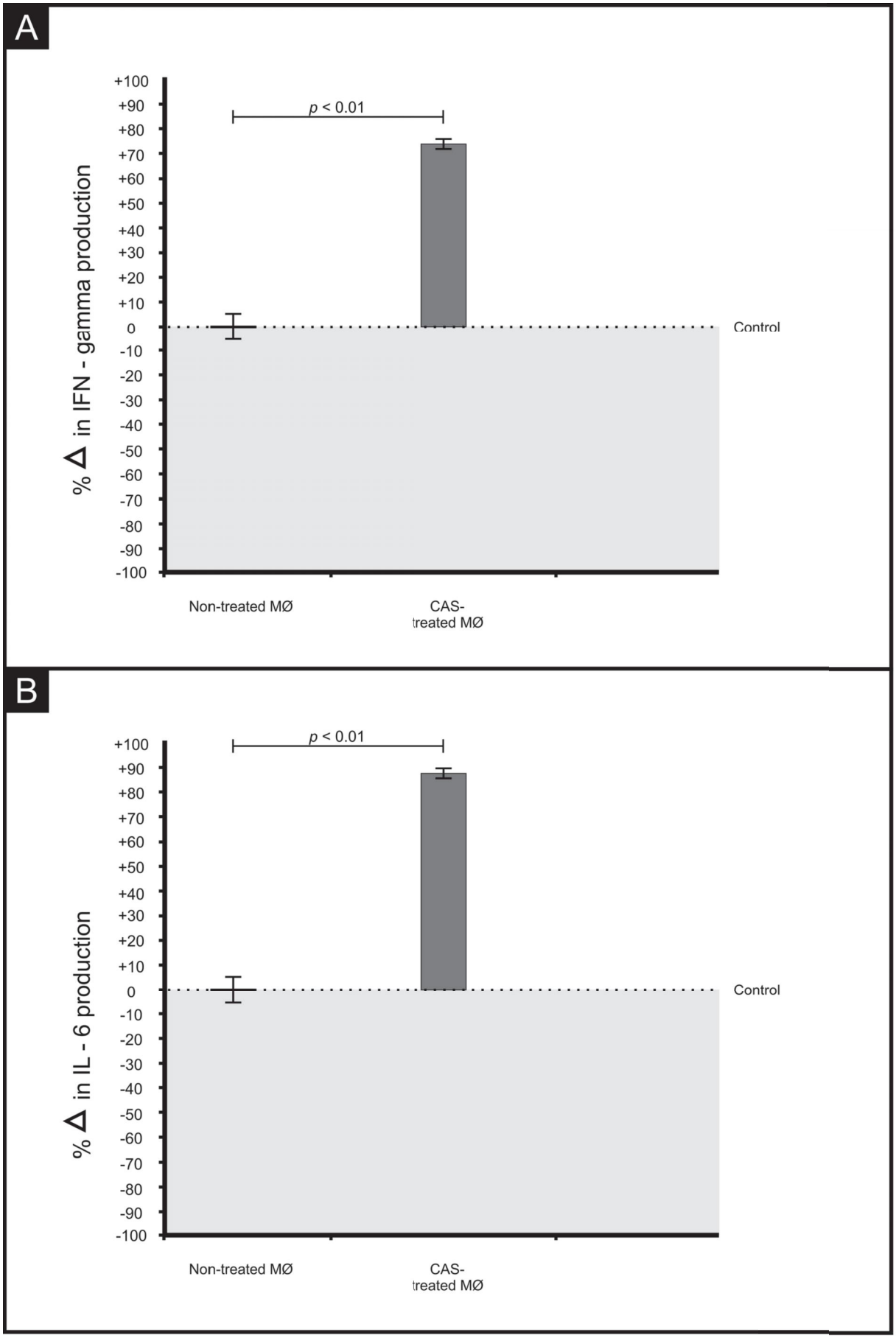

FIG. 6. The effect of CAS on macrophage (MØ) immunological response. Addition of CAS lead to significantly higher levels of pro-inflammatory cytokines compared to non-treated macrophages. To the point, (A) shows a 74% increase in the levels of interferon-gamma while (B) shows an 88% increase in the levels of interleukin-6 in the presence of CAS.

TABLE 1. The effect of CAS on growth of *C. neoformans* and *C. gattii*.

Species detail		Non-treated cells	Drug response		
			CAS-treated cells		
			8.44 µg/ml	84.4 µg/ml	844 µg/ml
Strain name	**Strain number**	**$OD_{562\ nm}$**	**%GR**	**%GR**	**%GR**
C. neoformans (H99)	LMPE 150	0.458 (0.016)	13% (0.025)	34 (0.028)	78% (0.054)
C. neoformans	LMPE 028	0.439 (0.012)	12% (0.030)	32 (0.044)	77% (0.031)
C. neoformans	LMPE 030	0.446 (0.007)	13% (0.012)	34 (0.007)	76% (0.015)
C. neoformans	LMPE 043	0.449 (0.013)	12% (0.047)	31 (0.005)	77% (0.023)
C. neoformans	LMPE 046	0.469 (0.009)	15% (0.026)	38 (0.052)	80% (0.017)
C. neoformans	LMPE 047	0.460 (0.019)	14% (0.017)	35 (0.051)	78% (0.015)
C. gattii (R265)	LMPE 109	0.456 (0.023)	12% (0.033)	32 (0.019)	77% (0.049)
C. gattii	LMPE 045	0.462 (0.016)	12% (0.037)	30 (0.029)	75% (0.029)
C. gattii	LMPE 048	0.464 (0.016)	13% (0.025)	32 (0.022)	76% (0.004)
C. gattii	LMPE 052	0.474 (0.007)	11% (0.014)	29 (0.014)	74% (0.014)
C. gattii	LMPE 054	0.451 (0.003)	10% (0.005)	31 (0.008)	74% (0.014)
C. 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) x 100%]. Values represent the mean values from three biological replicates, and values in parentheses represent standard deviations.

TABLE 2. The combined effect of CAS and amphotericin B and CAS and fluconazole on selected *Cryptococcus* strains.

LMPE 046		Fractional inhibitory concentration (FIC) index					Percentage growth reduction (%GR)				
	CAS (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
	105.6	1.65	0.69	0.40	0.37	0.29	31	44	61	73	79
	211	0.93	0.52	0.38	0.33	0.21	42	48	65	75	87
	422	0.61	0.42	0.29	0.27	0.18	49	66	73	78	92
	844 (MIC)	0.42	0.34	0.23	0.20	0.12	60	72	76	85	95
	1688	0.29	0.22	0.17	0.11	0.08	79	82	86	94	98
	CAS (µg/ml)	Fluconazole (µg/ml)					Fluconazole (µg/ml)				
		2	4	8 (MIC)	16	32	2	4	8 (MIC)	16	32
	105.6	2.06	0.65	0.43	0.40	0.34	26	41	58	67	76
	211	1.89	0.56	0.41	0.35	0.31	38	49	63	74	80
	422	0.64	0.47	0.37	0.29	0.26	45	63	72	78	85
	844 (MIC)	0.43	0.39	0.28	0.24	0.19	58	71	80	84	91
	1688	0.30	0.23	0.20	0.16	0.11	73	80	86	88	97
LMPE 150											
	CAS (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
	105.6	1.60	0.67	0.39	0.35	0.24	32	45	63	75	80
	211	0.89	0.55	0.35	0.32	0.19	44	49	66	77	88

	422	0.72	0.40	0.29	0.26	0.15	48	66	75	80	91
	844 (MIC)	0.47	0.31	0.22	0.20	0.11	61	74	79	84	94
	1688	0.31	0.26	0.19	0.14	0.07	77	80	88	91	96
	CAS (µg/ml)	**Fluconazole (µg/ml)**					**Fluconazole (µg/ml)**				
		2	**4**	**8 (MIC)**	**16**	**32**	**2**	**4**	**8 (MIC)**	**16**	**32**
	105.6	2.17	0.84	0.42	0.38	0.32	24	38	56	69	73
	211	1.98	0.62	0.39	0.34	0.28	35	46	61	73	77
	422	0.81	0.44	0.34	0.26	0.23	42	61	70	76	81
	844 (MIC)	0.46	0.41	0.28	0.23	0.20	57	70	78	80	86
	1688	0.39	0.30	0.23	0.19	0.15	71	82	86	85	90
LMPE 052											
	CAS (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**
	105.6	1.71	0.66	0.41	0.35	0.27	28	41	60	71	77
	211	0.99	0.54	0.36	0.31	0.20	40	47	66	74	86
	422	0.75	0.41	0.28	0.25	0.16	47	64	72	79	90
	844 (MIC)	0.44	0.32	0.22	0.21	0.12	61	70	78	83	93
	1688	0.27	0.25	0.19	0.15	0.09	78	83	87	90	96
	CAS (µg/ml)	**Fluconazole (µg/ml)**					**Fluconazole (µg/ml)**				
		2	**4**	**8 (MIC)**	**16**	**32**	**2**	**4**	**8 (MIC)**	**16**	**32**
	105.6	2.12	0.71	0.45	0.39	0.35	22	39	55	65	75

	211	1.94	0.59	0.40	0.32	0.30	36	47	60	71	79
	422	0.78	0.46	0.38	0.28	0.26	41	60	69	77	82
	844 (MIC)	0.48	0.40	0.32	0.25	0.21	56	68	75	81	89
	1688	0.41	0.28	0.24	0.18	0.13	70	78	83	87	94
LMPE 109											
	CAS (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**
	105.6	1.62	0.75	0.42	0.33	0.25	30	43	62	73	78
	211	0.95	0.53	0.39	0.30	0.20	38	48	67	76	85
	422	0.68	0.43	0.31	0.25	0.16	45	65	71	81	90
	844 (MIC)	0.44	0.30	0.26	0.20	0.13	62	73	77	86	94
	1688	0.26	0.23	0.19	0.15	0.09	76	81	85	92	97
	CAS (μg/ml)	**Fluconazole (μg/ml)**					**Fluconazole (μg/ml)**				
		2	**4**	**8 (MIC)**	**16**	**32**	**2**	**4**	**8 (MIC)**	**16**	**32**
	105.6	2.03	0.72	0.40	0.37	0.31	25	40	60	68	75
	211	1.94	0.60	0.38	0.33	0.28	36	47	64	70	81
	422	0.59	0.45	0.34	0.28	0.24	40	62	69	78	86
	844 (MIC)	0.46	0.37	0.26	0.22	0.17	56	70	75	82	90
	1688	0.32	0.25	0.21	0.19	0.14	72	79	83	86	95

Colour-coded cells represent the define MIC for each respective drug and the combine effect of the respective MIC on percentage growth reduction and FICI.