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The role of oxidative and nitrosative bursts caused by azoles and amphotericin B against the fungal pathogen *Cryptococcus gattii*

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Objectives: Although the most accepted mechanisms of action of amphotericin B and azoles are related to ergosterol, it is possible that these drugs have other effects on the fungal cell. In the present study, the role of endogenous reactive oxygen species (ROS) and peroxynitrite produced by azoles and amphotericin B in the fungus *Cryptococcus gattii* were examined.

Methods: We studied distinct parameters to evaluate the effect of oxidative and nitrosative stresses induced by these drugs in *C. gattii* cells: lipid peroxidation, ergosterol content, ROS and peroxynitrite production, enzymatic activity of the antioxidant system and the *in vitro* interaction of antifungal drugs with a peroxidase inhibitor, a superoxide dismutase inhibitor and a peroxynitrite scavenger.

Results: The data demonstrated that itraconazole led to ROS formation and lipid peroxidation in *C. gattii* cells in the early stages of the treatment; this did not occur with fluconazole. This phenomenon strongly increased the activities of enzymes of the antioxidant system. These results were confirmed by synergism observed between the catalase inhibitor and itraconazole. Amphotericin B caused lipid peroxidation in *C. gattii* cells through a greatly enhanced production of oxidative and nitrosative radicals with increased peroxidase activity. These data were confirmed by the synergism between the catalase/superoxide dismutase inhibitors and amphotericin B. In addition, the effect of this antifungal was antagonized by the peroxynitrite scavenger.

Conclusions: Oxidative and nitrosative bursts play an important role in the antifungal activity of itraconazole and amphotericin B against *C. gattii*.

Keywords: reactive oxygen species, peroxynitrite, antifungal drugs, cryptococcosis

Introduction

Cryptococcus gattii and *Cryptococcus neoformans* are the main aetiological agents of the human and animal fungal disease cryptococcosis.[1] The infection is usually acquired by inhalation of environmental basidiospores or desiccated yeasts.[2] Cryptococcal disease in humans involves pulmonary and cutaneous sites, but the most severe manifestation is the CNS involvement with meningoencephalitis.[1] Despite the similarities of the clinical syndromes in cryptococcosis, immunocompromised patients are more frequently infected by *C. neoformans*, while *C. gattii* has emerged as an important cause of infection in immunocompetent individuals.[3,4] It is well established that the infection caused by *C. gattii* has a less favourable response to antifungal therapy and a relatively worse prognosis compared with the infection caused by *C. neoformans*.[5,6] Usually, clinical manifestations of the *C. gattii* infection include severe or prolonged cough, pneumonia, shortness of breath, sputum production, sinusitis, muscle soreness, blurred or double vision, seizures, headache, sharp chest pain, fever, weight loss, skin lesions, change in mental status and a general feeling of discomfort.[7,8]

A small number of antimycotic drugs with different modes of action and target spectra are available to treat these diseases, with polyenes and azoles being the most used against cryptococcosis.[1] The polyenes, represented by amphotericin B, bind to ergosterol, the major sterol in the fungal cell membrane, and form pores, thereby causing membrane damage. The azoles (e.g. fluconazole and itraconazole) affect sterol biosynthesis by inhibiting sterol 14-α-demethylase, which results in ergosterol depletion and accumulation of toxic methylated sterols.[9] Interestingly, resistance to antifungal drugs has been demonstrated among clinical strains of *Cryptococcus* spp.[10] and the

investigation of this phenomenon considering the drugs amphotericin B and azoles against *C. gattii* is very important.

Although the above-mentioned mechanisms of action are the most accepted for amphotericin B and azoles, several reports suggested that these antifungals have other effects on the fungal cell. In particular, it has been suggested that amphotericin B can be auto-oxidized with the subsequent production of oxygen free radicals.[11] Other researchers suggested that amphotericin B rapidly induces a strong oxidative burst in *C. neoformans*.[12] On the other hand, the importance of the enhanced production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) on the antifungal activity and its influence on the pathogen's antioxidant defence system remains unclear. Regarding the azoles, some studies described that the activity of miconazole against *Candida albicans* may be related to ROS production.[13,14] However, these researchers did not focus on the role of ROS production in the antifungal effect of fluconazole and itraconazole (the main azoles used in cryptococcosis treatment). Furthermore, recent studies demonstrated that *C. gattii* possesses an arsenal of ROS scavengers,[15] suggesting a cytoprotective mechanism against endogenous and exogenous ROS.

Indeed, there is no study focused on the importance of RNS in the microbial susceptibility to the antifungals. Therefore, we evaluated distinct parameters involved in the oxidative and nitrosative bursts generated by fluconazole, itraconazole and amphotericin B against *C. gattii*.

Materials and methods

C. gattii strains and study design

We tested three clinical strains of *C. gattii* (L135/03, L27/01 and 23/10993), all from the culture collection of the Laboratório de Micologia of Universidade Federal de Minas Gerais, Brazil, and one reference strain (ATCC 32068) from the culture collection of the University of Georgia, Atlanta, GA, USA. The isolates were maintained on slants of Sabouraud dextrose agar (SDA; Difco Laboratories, Detroit, MI, USA) at 4°C. Prior to each test, the strains were subcultured on SDA for 48 h at 35°C. The following assays were performed with all the strains: antifungal drug susceptibility testing, time–kill curves and *in vitro* interaction of fluconazole, itraconazole, amphotericin B and hydrogen peroxide with 3-amino-1,2,4-triazole (3-AT), diethyldithiocarbamate (DETC) and 5,10,15,20-tetrakis (4′-sulfphonatophenyl) porphyrinato iron III (FETPPS). The reference strain ATCC 32068 was chosen for determination of the ergosterol content, lipid peroxidation, enzyme activities and the amount or ROS and RNS.

Antifungal drug susceptibility testing

The MICs of fluconazole (Sigma-Aldrich, St Louis, MO, USA), itraconazole (Sigma-Aldrich), amphotericin B (Sigma-Aldrich) and hydrogen peroxide (Sigma-Aldrich) were determined by the antifungal microdilution susceptibility standard test proposed in the CLSI M27-A3 method.[16] The inoculum was prepared in sterile saline and the transmittance of the suspensions was adjusted to 75%–77% (530 nm), followed by further dilution in RPMI-1640 buffered with MOPS (Sigma-Aldrich) medium to achieve 1.0×10^3–5.0×10^3 cfu/mL. Fluconazole was dissolved in sterile distilled water, while itraconazole and amphotericin B were dissolved in 100% DMSO at 1000 mg/mL. These stock solutions and hydrogen peroxide were further diluted in RPMI-1640 (Sigma-Aldrich) to yield twice the final strength required for the test. The final concentrations ranged from 0.125 to 64 mg/L for fluconazole, 0.03 to 16 mg/L for itraconazole and amphotericin B, and 53.1×10^2 to 27.2×10^4 mg/L for hydrogen peroxide.[16]

A 100 μL volume of the inoculum suspension was transferred to sterile flat-bottomed 96-well plates containing 100 μL of each of the antifungals or RPMI-1640 (growth control). The plates were incubated at 35°C for 72 h. The MICs of fluconazole and itraconazole were determined visually as 80% growth inhibition, while for amphotericin B and hydrogen peroxide, the reading was performed as 100% growth inhibition when compared with the control. The results were confirmed by adding the salt MTT (Sigma-Aldrich) (5.0 mg/mL) to determine the reduction in the metabolic cell activity. Briefly, the plates were incubated at 35°C for 3 h and DMSO was added before spectrophotometric reading at 490 nm. The MIC endpoint for interpreting the results was 80% of reduction in metabolic activity for azoles and 100% of reduction in metabolic activity for amphotericin B and hydrogen peroxide compared with the control (no drug). The isolate *Candida parapsilosis* ATCC 22019 was used as a quality control.[16] All the tests were performed in duplicate for each strain.

Time–kill curves

An assay was performed to evaluate the time–kill kinetics of the drugs against *C. gattii* strains. For fluconazole, itraconazole, amphotericin B and hydrogen peroxide, the concentration tested was equivalent to the MIC or 2× MIC for each strain, as described previously.[17] The inoculum was prepared in sterile saline and the transmittance of the suspensions was adjusted to 75% (530 nm), followed by further dilution in RPMI-1640 medium to achieve 1.0×10^3–5.0×10^3 cfu/mL. A 100 μL aliquot of *C. gattii* inoculum was taken from the microtitre plates containing the MIC and 2× MIC of each antifungal agent at different intervals until 72 h and plated on SDA. The plates were incubated at 35°C for 72 h prior to colony counting. The results were expressed as the percentage of growth compared with that of the growth control for each time analysed. The data represent the means of three independent experiments in duplicate assays.

Lipid peroxidation assay

The strain *C. gattii* ATCC 32068 was cultured (35°C for 72 h) on SDA and ∼20.0 mg of the fungal cell mass was transferred to polypropylene tubes, followed by treatment with 8× MIC of the test drugs for 1 or 24 h at 35°C. A growth control was also performed. After the incubation, the tubes were centrifuged (Jouan, model BR4i) at 1643 **g** for 5 min at 4°C and the supernatant was removed. The cells were washed with sterile distilled water and the net wet weight pellet was determined.

The products of lipid peroxidation were measured as thiobarbituric acid-reactive substances (TBARSs).[18] The pellet was frozen and homogenized in 1000 μL ice-cold 1.1% phosphoric acid. The homogenate (400 μL) was mixed with 400 μL of 1% thiobarbituric acid (Sigma–Aldrich) prepared in 50 mM NaOH containing 0.1 mM butylated hydroxytoluene and 200 μL of 7% phosphoric acid (all the solutions were kept on ice during manipulation). Subsequently, samples (pH 1.5) were heated for 60 min at 98°C and 1500 μL of butanol was added. The mixture was mixed vigorously using a vortex and centrifuged for 5 min at 2000 **g**. The organic layer was transferred and the absorbance at 532 nm was measured (Thermo Scientific Multiskan Spectrum, Thermo Fisher Scientific). The thiobarbituric acid solution was replaced with 3 mM HCl in the blank controls. The TBARS values were expressed using the extinction coefficient of 156 mM^{-1} cm^{-1}. The results represent the means of three independent experiments.

Ergosterol quantification

The cells were treated as outlined in the preceding Lipid peroxidation assay section. The ergosterol quantification in the fungal cell membrane was performed as described previously.[17] For the extraction of lipids, 3000 μL of an ethanolic solution of 25% potassium hydroxide was added to each cell mass and agitated for 1 min. The tubes were incubated in a water bath at 85°C for 1 h and further cooled at room temperature. A mixture of 1 mL of sterile water and 3000 μL of *n*-heptane (Sigma-Aldrich) was added, followed by agitation in a vortex for 3 min. The supernatant was removed and the reading was performed using a spectrophotometer at 282 and 230 nm. A calibration curve with standard ergosterol (Sigma-Aldrich) was constructed and used to calculate the amount of ergosterol. The results were expressed as the percentage of ergosterol in comparison with the growth control and represent the means of three independent experiments.

Measurement of ROS and peroxynitrite production

Endogenous amounts of ROS and peroxynitrite were measured by fluorometric assay with specific probes.[13,19] 1.0×10^5 cells/mL of *C. gattii* ATCC 32068 were treated with the MIC of fluconazole, itraconazole, amphotericin B or hydrogen peroxide in RPMI-1640 without phenol red (Sigma-Aldrich) (data not shown) and incubated with 10 μM 2′,7′-dichlorofluorescein diacetate (Invitrogen, Life Technologies, Carlsbad, CA, USA) for ROS quantification or 20 μM dihydrorhodamine 123 (Invitrogen) for peroxynitrite quantification. The fluorescence was measured with a fluorometer (Synergy 2 SL Luminescence Microplate Reader; Biotek) using excitation and emission wavelengths of 500 nm. The kinetics of ROS and peroxynitrite production were performed at 0, 0.5, 1 and 24 h. The data are expressed as arbitrary units of fluorescence ± SEM. These tests were performed in triplicate.

Enzymatic activity of the antioxidant system

Prior to the tests, a cell-free extract from *C. gattii* ATCC 32068 treated with the antifungals was prepared according to the method described by Khan *et al.*,[20] with minor modifications. Untreated cells were used as control. Cells (20.0 mg wet weight) were treated with 8× MIC of the drugs. Aiming the evaluation of the oxidative burst on the action of the antifungals, these drugs were combined with the catalase-specific inhibitor 3-AT (Merck, Rahway, NJ, USA) at 10 mM,[21] the superoxide dismutase (SOD) 1 inhibitor DETC (Merck) at 1 mM[22] and the peroxynitrite scavenger FETPPS (Merck) at 12.5 μM[23] for 24 h at 35°C. A growth control was also performed. These cells were centrifuged and suspended in 2 mL of grinding medium (250 mM sucrose/10 mM Tris-HCl, pH 7.5/1 mM PMSF) and 100 mg of glass beads (0.45 mm). The suspension was disrupted mechanically in a vortex mixer for five cycles of 270 s each of 10000 vibrations/min. The homogenate was collected and centrifuged at 1000 **g** for 5 min at 4°C to remove unbroken cells and glass beads and further centrifuged at 15000 **g** for 40 min at 4°C. The supernatant was used as a cell-free extract. Soluble protein was determined using the biuret test using a standard curve of BSA.[24]

SOD activity

The SOD activity was measured by the inhibition of pyrogallol autoxidation as described previously,[20,25] with modifications. The control was performed as 100 μL of grinding medium plus 100 μL pyrogallol (20 mM; Sigma-Aldrich) diluted in PBS (pH 8.5). Both solutions were freshly prepared at the time of the assay. In the test samples, 100 μL of cell-free extract was added to pyrogallol and the inhibition of autoxidation was monitored every 30 s for 3 min at a wavelength of 420 nm. The units of SOD were considered as pyrogallol autoxidation per 200 μL, calculated as follows:

$$\text{Units of SOD/mL of sample} = [(A - B)/A \times 50 \times 100] \times 0.6 \text{ (dilution factor)}$$

where A is the difference in absorbance per 1 min in the control and B is the difference in absorbance per 1 min in the test samples. The results are expressed in units/mg of protein and represent the means of three independent experiments.

Peroxidase (PER) activity

The PER activity was determined according to the methodology proposed previously[19] and measured in a system containing hydrogen peroxide and guaiacol as substrate. The absorbance changes at $\lambda = 470$ nm were monitored using a molar extinction coefficient value of 26.61 M^{-1} cm^{-1} for the product tetraguaiacol formed by the enzymatic reaction. Briefly, 100 μL of the cell-free extract was incubated for 13 min in HEPES solution (25 mM, pH 6.8) containing 10 mM hydrogen peroxide and 4 mM guaiacol (Sigma-Aldrich). The change in absorbance caused by the formation of tetraguaiacol at 470 nm was monitored every 30 s for 3 min of reaction. The least count of the absorbance measurement was 0.001 and one enzyme unit is the amount of enzyme that produces 1 nmol/min of product. The results are expressed in nmol/min/mg of protein and represent the means of three independent experiments.

In vitro interaction of fluconazole, itraconazole, amphotericin B and hydrogen peroxide with 3-AT, DETC and FETPPS

Fluconazole, itraconazole, amphotericin B and hydrogen peroxide were also tested in combination with the catalase-specific inhibitor 3-AT, the SOD1 inhibitor DETC and the peroxynitrite scavenger FETPPS. A chequerboard microdilution method,[26] which provides a matrix of all possible drug combinations in the required concentration range, was used to test the susceptibility of the four *C. gattii* strains to the drugs. The concentrations ranged from 0.25 to 64 mg/L for fluconazole, 0.03 to 0.5 mg/L for itraconazole, 0.06 to 1 mg/L for amphotericin B and 2.1×10^3 to 34×10^3 mg/L for hydrogen peroxide. Furthermore, the concentrations ranged from 5 to 40 mM for 3-AT, 0.5 to 4 mM for DETC and 6.25 to 50 μM for FETPPS. These ranges were used based on the MIC found in the antifungal drug susceptibility testing. The inoculum was prepared as described in the Antifungal drug susceptibility testing section. A volume of 100 μL of the inoculum suspension was transferred to sterile flat-bottomed 96-well plates containing 50 μL of each of the antifungal drugs and 50 μL of each inhibitor/scavenger or RPMI-1640 (growth control). The plates were incubated at 35°C for 72 h. The cellular metabolic activity was determined using the MTT salt.

Interaction between the drugs was quantitatively evaluated by determining the fractional inhibitory concentration index (FICI)[27] and also by the construction of interaction curves. The formula for calculating the FICI was FICI = (MIC of antifungal in combination/MIC of antifungal alone) + (MIC of 3-AT or DETC or FETPPS in combination/MIC of 3-AT or DETC or FETPPS alone). The FICI was calculated for all the possible combinations of different concentrations. The interaction between these drugs was classified as synergism if FICI ≤0.5, indifferent if FICI >0.5 to ≤4.0 and antagonism if FICI >4.0. This assay was tested in duplicate and was repeated twice.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). A one-way ANOVA

using a Kruskal–Wallis non-parametric test was used to statistically compare the differences among groups. Individual comparison between groups was done using the Bonferroni post test. A 95% CI was considered in all experiments.

Results

Antifungal drug susceptibility testing and time–kill curves

In the antifungal susceptibility testing, the MIC range values for fluconazole and itraconazole were 4.0–32.0 and 0.125–0.5 mg/L, respectively. For amphotericin B and hydrogen peroxide, the MIC range values were 0.12–0.25 mg/L and 8.5×10^3 mg/L, respectively (Table 1). To evaluate the kinetics of the action of the antifungal agents tested, the time–kill curve assay was performed. Fluconazole and itraconazole provided fungistatic curves, with reduction of ∼80% of growth after 36 and 12 h of incubation at the MIC and 2× MIC, respectively. On the other hand, amphotericin B and hydrogen peroxide at the MIC and 2× MIC provided similar curves, with complete reduction of growth after 24 h (Figure 1).

Lipid peroxidation is important for the effect of the antifungal agents

Our results showed high levels ($P<0.05$) of TBARSs after 1 h of treatment with itraconazole, amphotericin B and hydrogen peroxide, indicating damage of the lipids compared with the growth control (Figure 2a). It should be noted that lipid peroxidation only remained higher after 24 h when amphotericin B and hydrogen peroxide were tested ($P<0.05$) (Figure 2a). Fluconazole did not influence the levels of TBARSs. Interestingly, the ergosterol levels were reduced for all drugs tested after 1 h of treatment ($P<0.05$) in comparison with the growth control. After 24 h, these levels remained lower ($P<0.05$) for fluconazole and

Table 1. MICs of antifungal drugs, mean FICIs and interactions between antifungal drugs with 3-AT, DETC and FETPPS against the four strains of *C. gattii*

Drug	MIC range alone (mg/L)	Mean FICI+3-AT/interaction	Mean FICI+DETC/interaction	Mean FICI+FETPPS/interaction
Fluconazole	4–32	1.0/indifference	1.0/indifference	2.0/indifference
Itraconazole	0.12–0.5	0.5/synergism	0.6/indifference	2.0/indifference
Amphotericin B	0.12–0.25	0.5/synergism	0.5/synergism	4.5/antagonism
Hydrogen peroxide	8.5×10^3	0.5/synergism	0.5/synergism	5/antagonism

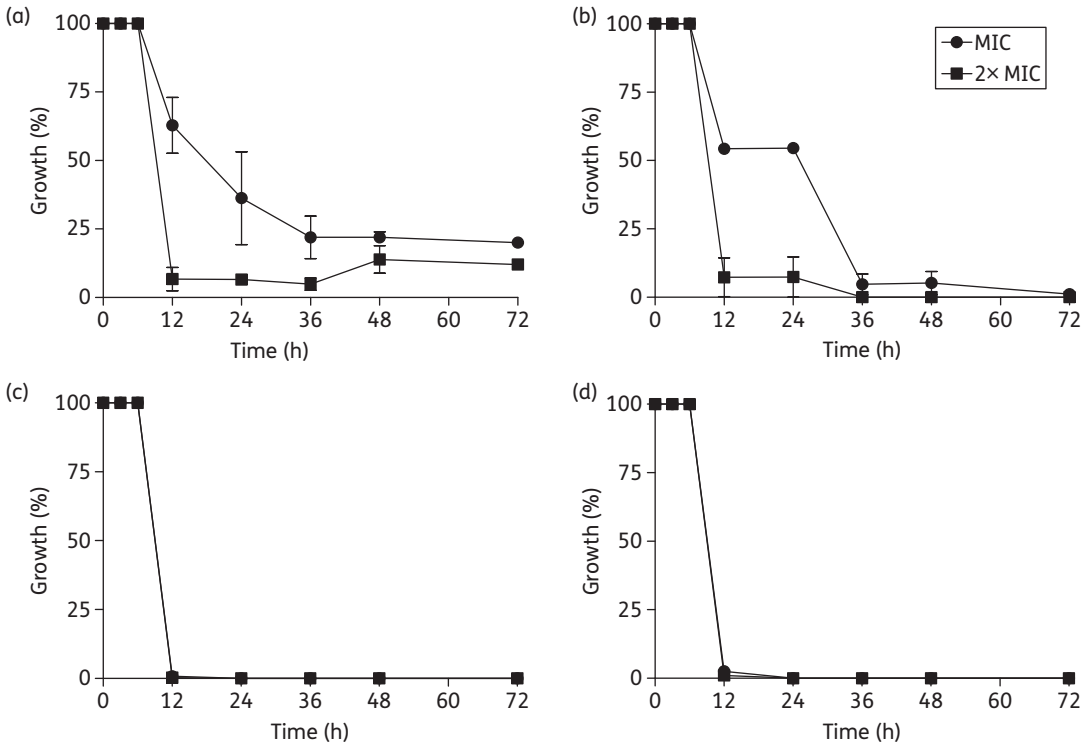

Figure 1. Time–kill curves of the drugs against four *C. gattii* strains. Time–kill curves of fluconazole (a), itraconazole (b), amphotericin B (c) and hydrogen peroxide (d) at MIC and 2× MIC. Results are expressed as the percentage of growth compared with the growth control.

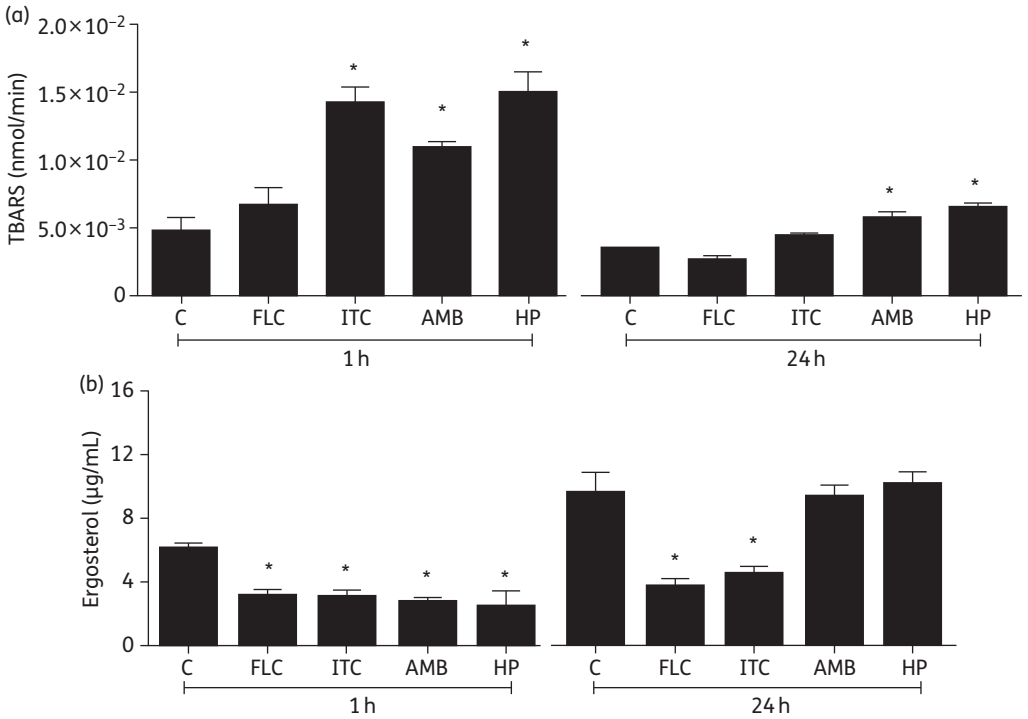

Figure 2. Lipid peroxidation (a) and reduction of the ergosterol content (b) are consequences of the treatment with antifungal drugs. (a) Effect of fluconazole (FLC), itraconazole (ITC), amphotericin B (AMB) and hydrogen peroxide (HP) exposure on lipid peroxidation (TBARSs) in *C. gattii* after 1 and 24 h of treatment. The results are expressed in nmol/min. (b) Ergosterol levels of the strain ATCC 32068 after 1 and 24 h of FLC, ITC, AMB and HP treatment. Results are expressed in μg/mL. Data of these two experiments are represented as the mean ± SEM of two independent experiments in triplicate assays. Statistically significant differences between the treatments and the control are indicated with an asterisk ($P<0.05$). C, control (untreated cells).

itraconazole (Figure 2b). It is well established that lipid peroxidation is mediated by free radicals. Thus, it was hypothesized that this effect may be related to ROS or RNS.

Antifungal drugs lead to ROS and peroxynitrite production in C. gattii

Since the treatment of *C. gattii* cells with itraconazole and amphotericin B enhanced the levels of TBARSs (Figure 2), it was hypothesized that ROS/peroxynitrite may be important to the effect of the antifungals. Itraconazole resulted in increasing ROS levels compared with the growth control at the beginning of the treatment ($P<0.05$) (Figure 3a). Treatment with amphotericin B resulted in a significantly enhanced production of ROS throughout the protocol ($P<0.05$) (Figure 3a). There was a tendency to increase the peroxynitrite levels by the cells treated with amphotericin B at times 0 and 0.5 h ($P=0.07$) (Figure 3b). Hydrogen peroxide induced the production of high levels of ROS and peroxynitrite at all times tested ($P<0.05$). Itraconazole and amphotericin B induced the production of free radicals, which probably are responsible for the lipid peroxidation observed (Figure 3c). Furthermore, the ability of *C. gattii* to adapt to these higher levels of ROS and RNS through increasing PER and SOD activities was verified.

Antifungal drugs increased the activities of the antioxidant enzymes PER and SOD

To determine whether treatment with fluconazole, itraconazole, amphotericin B and hydrogen peroxide influences the antioxidant enzymes, the activities of SOD and PER were assessed. The results showed that SOD activity was higher ($P<0.05$) in cells exposed to itraconazole and hydrogen peroxide compared with the growth control (Figure 4a) and PER activity was higher ($P<0.05$) in cells exposed to itraconazole, amphotericin B and hydrogen peroxide (Figure 4b).

To further explore the relationship between antioxidant enzymes and the stress response mediated by the antifungals, SOD activity in the presence of the catalase inhibitor 3-AT and PER activity in the presence of the Cu,Zn-isoenzyme-SOD1 inhibitor DETC (Figure 4a–c) were measured. The PER activity was significantly diminished ($P<0.05$) when fungal cells were treated with itraconazole, amphotericin B and hydrogen peroxide in the presence of DETC (Figure 4b), but the PER inhibition did not alter the SOD activity (Figure 4a). These results suggest a functional role of SOD and a possible connection between these enzymes in regulating responses to these drugs.

The SOD and PER activities were also analysed in the presence of FETPPS, a scavenger of peroxynitrite (Figure 4). It was noted that the activity of PER was reduced in the presence of FETPPS when the yeast cells were treated with itraconazole, amphotericin B and hydrogen peroxide (Figure 4b). FETPPS led to a

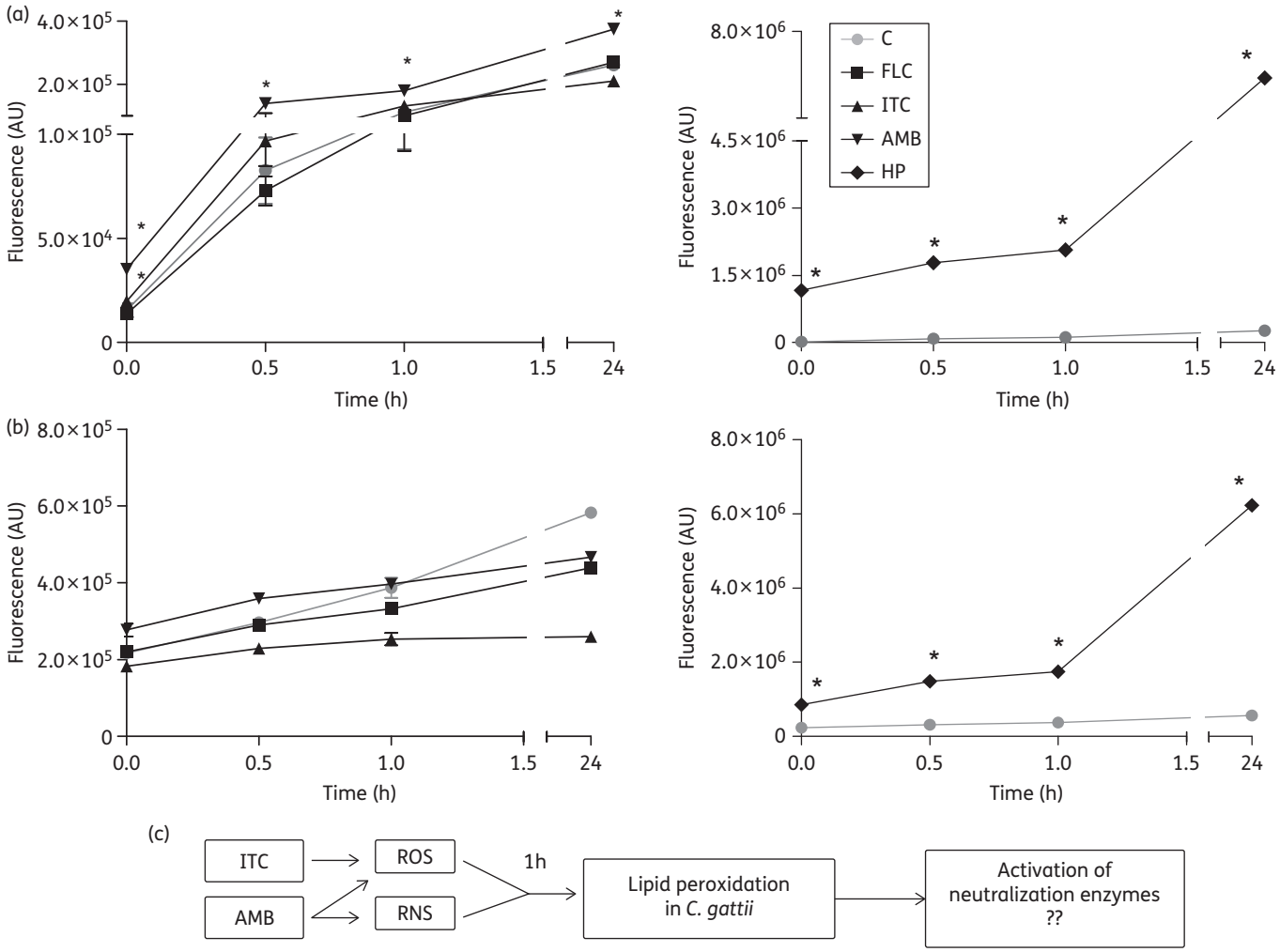

Figure 3. Amounts of ROS (a) and peroxynitrite (b) in azole- and amphotericin B (AMB)-treated *C. gattii* cells within 24 h. Cells were either untreated or treated with the MICs of the antifungals. The results are expressed in arbitrary units (AU) of fluorescence. Each data point represents the mean of three independent samples. Statistically significant differences between the treatments and the control are indicated with an asterisk ($P<0.05$). (c) Proposed mechanism of how itraconazole (ITC) and AMB cause lipid peroxidation through ROS and RNS production and the possibility of this phenomenon resulting in activation of neutralization enzymes. C, control (untreated cells); FLC, fluconazole; HP, hydrogen peroxide.

reduction of SOD activity, regardless of the presence of the antifungals (Figure 4a).

Inhibition of PER, SOD and peroxynitrite alters the activities of antifungal drugs

To determine the influence of the antioxidant enzymes and peroxynitrite/ROS production on the *C. gattii* tolerance of the antifungal drugs, we tested 3-AT, DETC and FETPPS in combination with azoles, amphotericin B and hydrogen peroxide. A synergistic effect of 3-AT with itraconazole (FICI=0.5), amphotericin B (FICI=0.5) and hydrogen peroxide (FICI=0.5) was observed (Figure 5a and Table 1). A synergist effect of DETC with amphotericin B (FICI=0.5) and hydrogen peroxide (FICI=0.5) was also noted (Figure 5b and Table 1).

An antagonistic effect was observed in the interaction between amphotericin B plus hydrogen peroxide (FICI=4.5) and amphotericin B plus FETPPS (FICI=5) (Figure 5c and Table 1). It is important to note that the curves of the combination between FETPPS and amphotericin B or hydrogen peroxide (Figure 5c) presented the same profile, which is characteristic of an antagonistic interaction.

A schematic association of all the data obtained from the experiments involving the oxidative and nitrosative bursts in the mechanism of action of azoles and amphotericin B is proposed in Figure 6.

Discussion

The *C. gattii* immunoproteome project identified a great part of the antioxidant defence system of this yeast.[28] However, little is known about the activity of antioxidant enzymes in *C. gattii* in the presence of antifungal compounds and its susceptibility to oxidative and nitrosative challenges. In this study, we

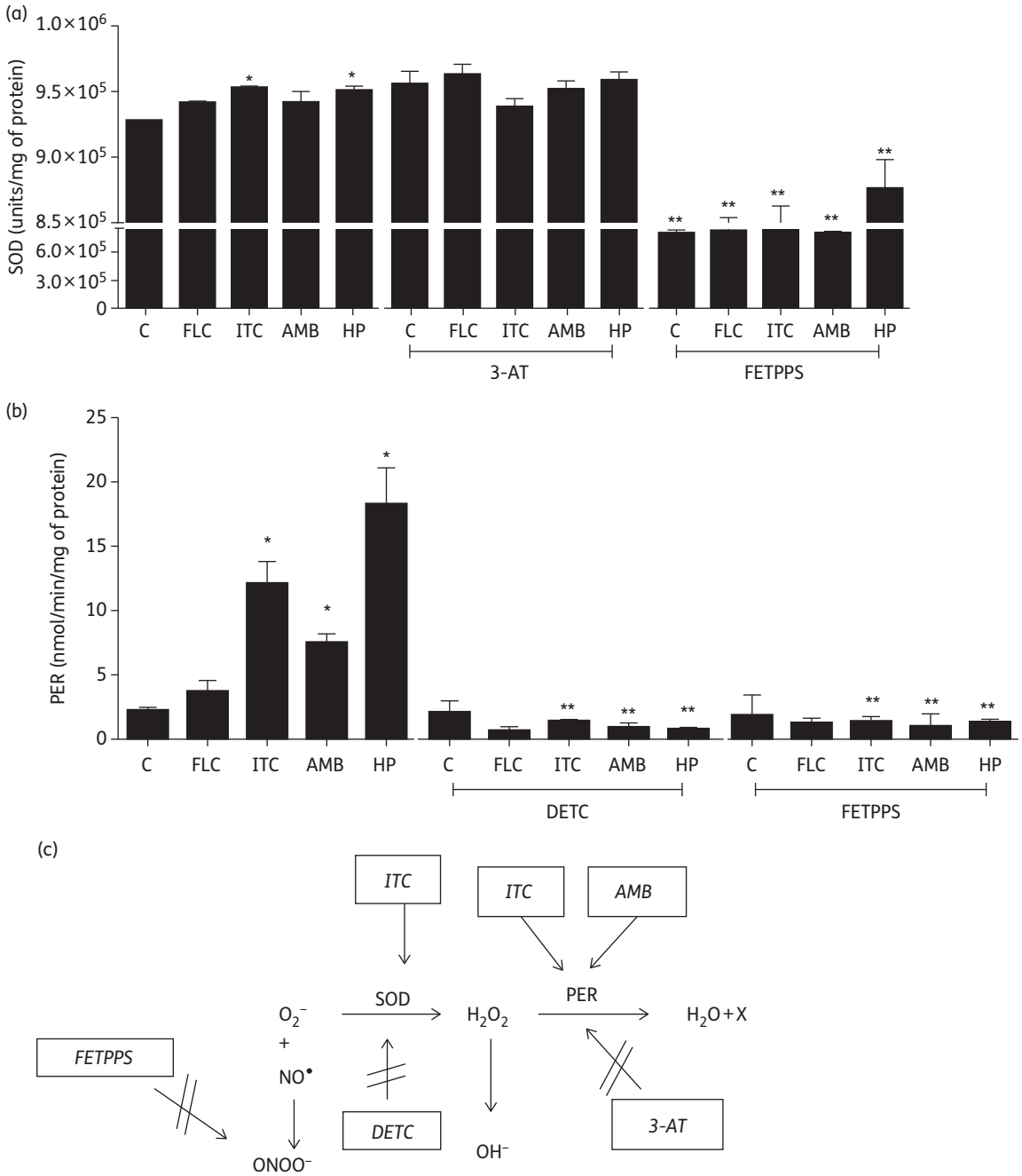

Figure 4. SOD (a) and PER (b) activities of yeast cells after the treatments. (a) Total of SOD specific activity after 24 h of treatment with fluconazole (FLC), amphotericin B (AMB), itraconazole (ITC) or hydrogen peroxide (HP) in the presence or absence of 3-AT or FETPPS. Results are expressed in units/mg of protein. (b) Total of PER specific activity after 24 h of treatment with FLC, AMB, ITC or HP in the presence or absence of DETC or FETPPS. Results are expressed in nmol/min/mg of protein. Data represent the mean ± SEM of two independent experiments in triplicate assays. Statistically significant differences between the antifungal drugs and the control are indicated with one asterisk and statistically significant differences between the presence and absence of inhibitor/scavenger following treatment with the same antifungal are indicated with two asterisks ($P < 0.05$). (c) Schematic representation of the antioxidant defence system and the mechanism of action of 3-AT, DETC and FETPPS. C, control (untreated cells).

have provided evidence that itraconazole and amphotericin B induce the production of free radicals in *C. gattii* cells. Also, the role of ROS and RNS during the action of the antifungals is proposed.

Itraconazole, an orally active broad-spectrum triazole antimycotic, has anti-*Cryptococcus* activity *in vitro* and in animal models of cryptococcal meningitis.[29] High levels of TBARSs were observed after 1 h of *C. gattii* treatment with itraconazole, but

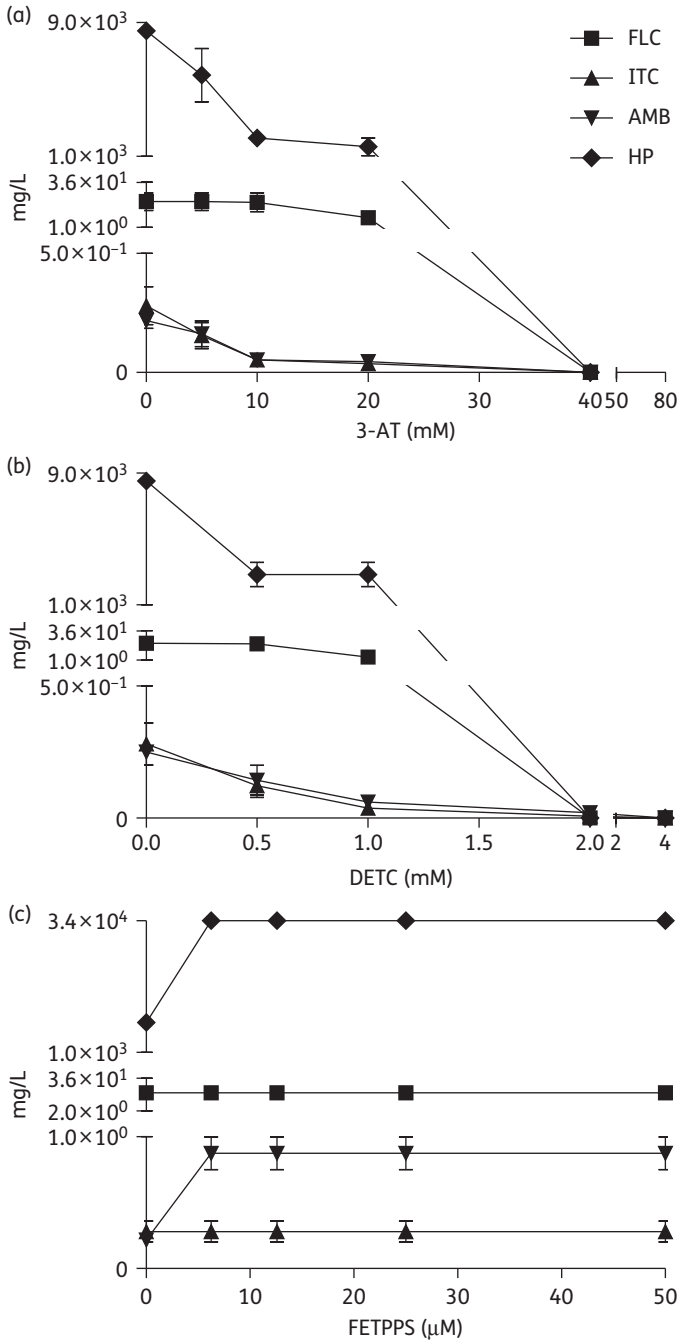

Figure 5. Combination curve of antifungal drugs with 3-AT, DETC and FETPPS against four *C. gattii* strains. Fluconazole (FLC), itraconazole (ITC), amphotericin B (AMB) and hydrogen peroxide (HP) were tested in combination with the catalase-specific inhibitor 3-AT (a), the SOD1 inhibitor DETC (b) and the peroxynitrite scavenger FETPPS (c). The cellular metabolic activity was determined using the MTT dye and the growth was measured by monitoring absorbance at 490 nm.

not with fluconazole. The assays showed that high lipid peroxidation occurred together with a reduction of the ergosterol content after 1 h of treatment with itraconazole. The data indicated that these two events occur simultaneously. Therefore, we decided to measure peroxynitrite and ROS produced by *C. gattii* cells after treatment with itraconazole.

The results show that itraconazole resulted in an increase in the intracellular amount of ROS. Based on these data, it was hypothesized that this drug does not have a unique target in the cell: itraconazole also induces the production of ROS, leading to lipid peroxidation, which corresponds to the oxidative degradation of lipids, in which a free radical chain 'steals' electrons from the lipids (mainly polyunsaturated fatty acids) in cell membranes, resulting in cell damage. This phenomenon would happen in a dynamic way together with inhibiting sterol 14α-demethylase,[9] explaining the fact that the MIC for itraconazole is always lower than for fluconazole for almost all yeasts.[30,31] In addition, fluconazole was not able to induce ROS, RNS and lipid peroxidation.

We observed no significant lipid peroxidation after 24 h of treatment with itraconazole; on the other hand, it was possible to note an increase in PER and SOD activities at this time. Synergism between itraconazole and 3-AT, a catalase inhibitor, was also verified. These results suggest that oxidative stress in *C. gattii* exposed to itraconazole occurs more significantly at the beginning of the treatment and contributes to the antifungal effect. It is probable that the cells which survive to the ROS have increased antioxidant activity. This hypothesis helps to explain the fact that itraconazole is a fungistatic drug.[32]

Amphotericin B is an antifungal agent that presents potent activity against the majority of fungi, including yeast and moulds.[33] It is an oxidizing molecule, which may be related to the oxidative burst.[11] In our experiments, amphotericin B and hydrogen peroxide provided similar time–kill curves, damage to lipids and interaction with 3-AT, DETC and FETPPS: TBARS levels were increased after 1 and 24 h of treatment with these two drugs. Therefore, we also decided to measure peroxynitrite and ROS produced by *C. gattii* cells after treatment with amphotericin B. The results showed that amphotericin B induced high levels of ROS and peroxynitrite immediately after contact with this drug and the oxidative burst continued to be enhanced after 24 h.

Our data reinforce that amphotericin B, like itraconazole, has multiple effects on the cell, as suggested previously.[12,34] We suggest that amphotericin B has two fungicidal mechanisms: oxidative and nitrosative bursts plus increased cellular permeability. In addition, *C. gattii* enhanced the activity of its antioxidant system in response to the oxidative and nitrosative bursts.[35,36]

The assays demonstrated increased PER activity after 24 h of treatment with amphotericin B, besides synergism between amphotericin B and 3-AT and between amphotericin B and DETC. However, this activation of the antioxidant system may not be sufficient to neutralize all the ROS and RNS, which does not allow an efficient cellular adaptation to this stress (Figure 6). It also explains the lower resistance rate to amphotericin B described among clinical strains of *C. gattii* when compared with azoles.[10]

Peroxynitrite is a short-lived species that is a potent inducer of cell death.[37] Although peroxynitrite is not a free radical by chemical nature (as it has no unpaired electron), it is a powerful oxidant exhibiting a wide array of tissue-damaging effects, including lipid peroxidation, inactivation of enzymes and other effects in the cell.[38] The present study demonstrated that the

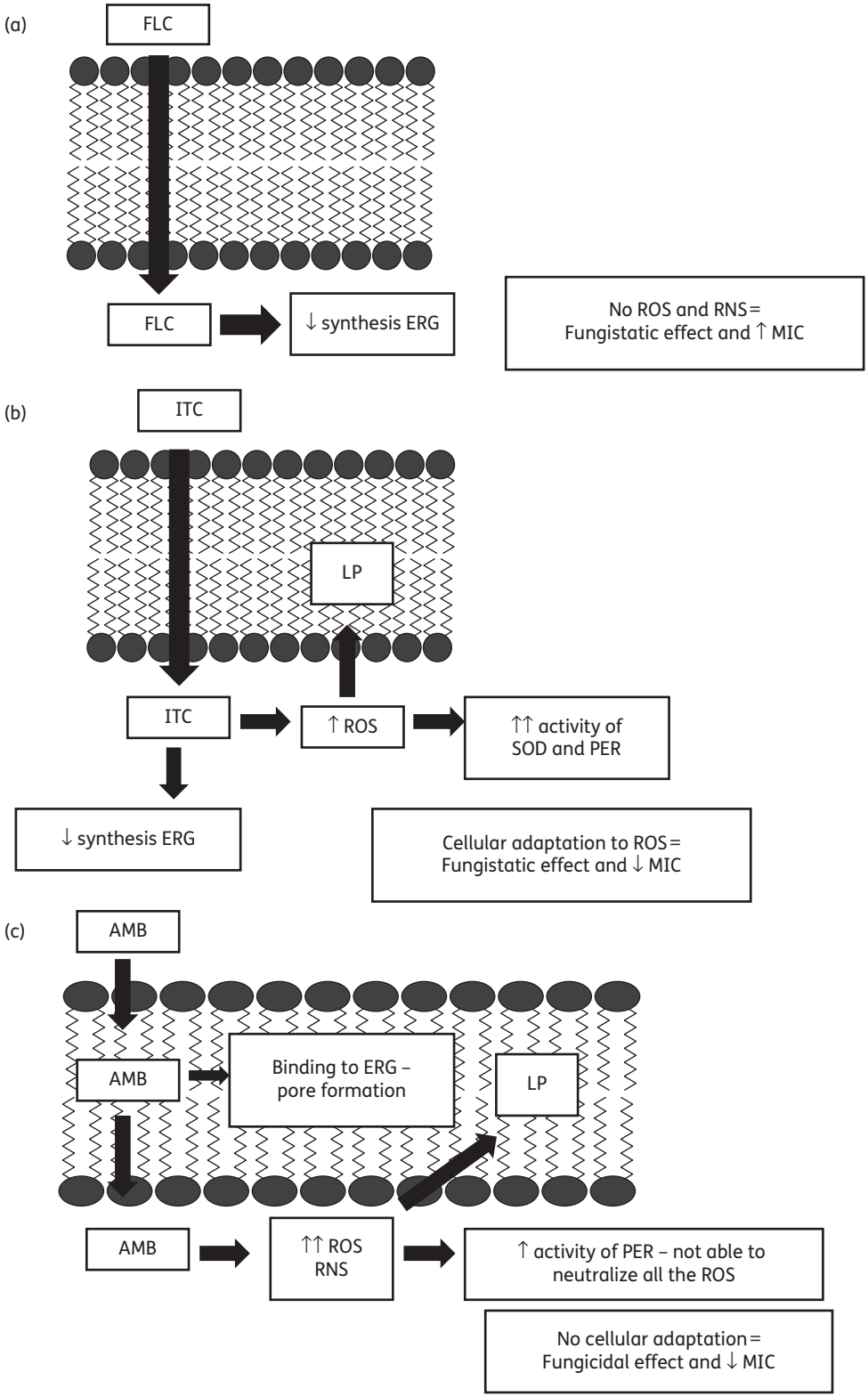

Figure 6. Schematic representation of the role of oxidative and nitrosative bursts caused by itraconazole (ITC) and amphotericin B (AMB) against *C. gattii*. (a) Fluconazole (FLC) does not promote free radical formation and only interferes in the synthesis of ergosterol (ERG), which results in a fungistatic action and higher MIC values. (b) In a dynamic way, ITC reduces the content of ERG, but also induces oxidative stress in *C. gattii* at the beginning of the treatment [leading to lipid peroxidation (LP)], which is sufficient to activate the antioxidant enzymatic system, resulting in a fungistatic effect and lower MIC values. (c) AMB binds to ERG and also induces strong oxidative and nitrosative stress in yeast cells (leading to LP). These two fungicidal actions do not allow for efficient cellular adaptation, resulting in lower MIC values.

addition of FETPPS, a scavenger of peroxynitrite, diminished SOD activity after all treatments tested (included growth control). In this context, it was previously shown that nitric oxide efficiently competes with SOD for superoxide.[39] Overall, peroxynitrite was constantly removed from system by FETPPS, probably shifting the equilibrium to the formation of this species by the reaction between nitric oxide and superoxide.[37]

Moreover, the activity of PER was also reduced when FETPPS was added to the cells treated with itraconazole and amphotericin B. These results may be explained by the reduction of its substrate (hydrogen peroxide), which may be caused by the reduced activity of SOD.

Finally, our data showed an interesting role of oxidative and nitrosative bursts caused by azoles and amphotericin B against the fungal pathogen *C. gattii*. We believe that these findings are important to a better understanding of the mechanism of action of these drugs and the emergence of resistant strains.

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Transparency declarations

None to declare.

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