IDENTIFICATION OF OFF-PATENT DRUGS THAT SHOW SYNERGISM WITH AMPHOTERICIN B OR THAT PRESENT ANTIFUNGAL ACTION AGAINST CRYPTOCOCCUS NEOFORMANS AND CANDIDA SPP

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Identification of off-patent drugs that show synergism with Amphotericin B or that present antifungal action against *Cryptococcus neoformans* and *Candida* spp.

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Running title: Off-patent drugs that enhance Amphotericin B activity

Abstract

Amphotericin B (AmB) is the antifungal with the strongest fungicidal activity, but its use has several limitations, mainly associated to its toxicity. Although some lipidic and liposomal formulations that present reduced toxicity are available, their price limits their application in developing countries. Flucytosine (FC) has shown synergistic effect with AmB for treatment of some fungal infections, such as cryptococcosis, but again, its price is a limitation for its use in many regions. In the present work, we aimed to identify new drugs that having a minor effect on *C. neoformans,* reduced its growth in the presence of sub-inhibitory concentrations of AmB. In the initial screening, we found fourteen drugs that had this pattern. Later, checkerboard assays of selected compounds such as erythromycin, riluzole, nortriptyline, chenodiol, nisoldipine, promazine, chlorcyclizine, cloperastine and glimepiride, were performed and all of them confirmed for their synergistic effect (FICI<0.5). Additionally, toxicity of these drugs in combination with AmB was tested in mammalian cells and in zebrafish embryos. Harmless compounds, such as the antibiotic erythromycin was found to have synergic activity with AmB, not only against *C. neoformans*, but also against some *Candida* spp., in particular against *C. albicans*. In parallel, we identified drugs that had antifungal activity against *C. neoformans* and found 43 drugs that completely inhibited the growth of this fungus, such as ciclopirox and auranofin. Our results expand our knowledge about antifungal compounds and open new perspectives in the treatment of invasive mycosis based on repurposing of off-patent drugs.

Keywords

Cryptococcus neoformans, Amphotericin B, synergism, drug repurposing.

Introduction

Opportunistic invasive fungal diseases (IFDs) pose a life-threatening problem for the increasing population of immunocompromised patients in our society (1, 2). Their incidence has risen in the last years, and their management has high associated costs, estimated around $30,000-50,000 per patient (3). Despite the availability of antifungal families, treatment of IFDs presents several limitations, such as antifungal cost, off target-toxicity and restricted spectrum of action.

The main antifungal families are azoles, echinocandins, 5-fluoro-cytosine (flucytosine) and polyenes (amphotericin B, AmB). In the case of polyenes, and in particular, AmB, several action mechanisms have been described. Classically, it has been stated that this antifungal binds to ergosterol and forms pores at the cell membrane (4-6). But AmB has other effects on the cells, such as ergosterol sequestration (7, 8) and induction of oxidative damage (9-13), which also contributes to the fungicidal activity of the drug.

AmB is the antifungal that presents the strongest fungicidal activity together with a wider action spectrum (see review in (14)), but its toxicity restricts its use. To overcome this problem, several lipidic and liposomal formulations have been developed. However, their price prevents its frequent use in developing countries. An example are diseases caused by *C. neoformans* which is a basidiomyces that causes disease mainly HIV^+ individuals (15) and it has a high prevalence in developing countries. Treatment for cryptococcosis is based on initial administration of AmB in combination with flucytosine, followed by fluconazole (16). Flucytosine use is also restricted due to its price (17) and limited availability in some regions.

The development of new drugs is an expensive and time-consuming process. However, nowadays, there are other strategies to find new antimicrobial compounds, based on screening of large collection of drugs (18). This approach has been successfully used in the last years to identify drugs that present antifungal activity. For

example, screening of synthetic drug libraries revealed two hydrazides, ([N'-(3-bromo-6-hydroxybenzylidene)-2-methylbenzohydrazide (BHBM) and its derivative 3-bromo-N'-(3-bromo-4-hydroxybenzylidene benzohydrazide) that target the synthesis of fungal sphingolipids and that could be promising drugs for the treatment of cryptococcosis (19, 20). A variation of these screenings is to test the activity of off-patent compounds, which is known as drug repurposing. The repurposing strategy has also been applied to look for active compounds (A.C.) against fungi such as *C. albicans* (21-26)., *C. neoformans* (26-28), the emerging pathogen *C. auris* (26, 29, 30) and multiresistant molds as *Lomentospora prolificans* (26).

Since AmB is the antifungal with the strongest activity, we aimed to identify new drugs or combinations that could potentially enhance its fungicidal effect. For this purpose, we screened the Prestwick Chemical library for compounds that increased the activity of AmB. This library has been used in screenings to find drugs with antifungal activity against *C. neoformans* and *C. albicans* (25, 28). It has even been used to identify synergistic drugs with amphotericin B and caspofungin against *C. albicans* biofilms (31). Once the A.C. were identified, their synergic effect with AmB was tested through checkerboard assay. The synergic combinations (A.C. + AmB) were tested for toxicity through an in vitro test with mammalian cells and in in vivo test with zebrafish embryos. In parallel, other drugs that have full activity against *C. neoformans* were presented, some of them with known antifungal activity, but others still not characterized.

Results

Identification of the optimal sub-inhibitory AmB concentration against *C. neoformans*

We first selected the minimal concentration of AmB that caused a modest inhibitory effect on *C. neoformans*. As shown in figure 1, AmB at 0.03 µg/mL caused 20-30% decrease of *C. neoformans* growth. Higher concentrations (0.06 and 0.12 µg/mL)

caused a more variable effect, depending on the day and plates, ranging the growth inhibition between 20-80%. In all cases, almost full inhibition of growth was found at concentrations ≥ 0.25 µg/mL. In consequence, we selected the concentration of 0.03 µg/mL as the minimum sub-inhibitory concentration of AmB to be used for our initial screening.

Screening for AmB synergistic compounds

We next performed the screening to identify compounds that had a moderate effect on growth in *C. neoformans*, but that had an inhibitory effect when combined with AmB 0.03 µg/mL. For this purpose, we prepared two plates as described in M&M: one with the compounds alone (100 µM), and another one in which they were mixed with AmB (0.03 µg/mL). In each plate we included wells without any drug as growth controls. After inoculation of the plates with H99 strain and incubation for 48 h, we identified 14 potential drugs that enhanced the activity of AmB (Table 1). These A.C. were atracurium besylate, erythromycin, riluzole hydrochloride, nortriptyline hydrochloride, chenodiol, thioguanosine, diflorasone diacetate, oxiconazole nitrate, glimepiride, nisoldipine, promazine hydrochloride, chlorcyclizine hydrochloride, demeclocycline hydrochloride and cloperastine hydrochloride. The combination of these drugs with sub-inhibitory AmB concentrations induced more than 80% of fungal growth inhibition, while the compounds alone did not have any significant effect on *C. neoformans* growth (Table 1).

Checkerboard assay

To confirm the synergistic activity, we selected nine compounds to perform checkerboard assays, based on different criteria, such as their administration route, and degree of synergistic effect. This assay allows for the calculation of the fractional inhibitory concentration index (FICI, see material and methods). The main results were obtained at concentrations between 0.1 and 0.025 mM. For each combination, we

146 selected the FICI for both 50 and 75% *C. neoformans* growth inhibition. The calculated
147 FICI in all combinations was $\leq$ 0.5, except for nortriptyline hydrochloride, that provided
148 a FICI > 0.5 only when 75% growth inhibition was examined (table 2). These results
149 confirmed the synergistic activity of the selected A.C. + AmB combinations.
150
151 Erythromycin showed a synergistic effect with AmB at concentrations between 0.1 mM
152 (73 µg/mL) and 0.025 mM (18.25 µg/mL). Furthermore, it partially enhanced the
153 antifungal activity even at low AmB concentrations of 0.008 µg/mL (Figure 2A). Riluzole
154 hydrochloride was also synergic at 0.1 mM (27 µg/mL) and 0.05 mM (13.5 µg/mL) in
155 combination with 0.03-0.06 µg/mL AmB (Figure 2B). Nortriptyline hydrochloride at
156 0.1 mM (30 µg/mL), besides in a minor scale, also potentiated AmB activity at 0.03
157 µg/mL and 0.015 µg/mL, inhibiting around 50% and 30% of the *C. neoformans* growth
158 respectively, being also able to increase AmB activity at 0.015 µg/mL (Figure 2C). For
159 chenodiol, the better synergistic activity was observed using 0.1 mM (40 µg/mL),
160 0.05 mM (20 µg/mL) and 0.025 mM (10 µg/mL) combined with 0.015-0.06 µg/mL of
161 AmB (Figure 2D). Nisoldipine induced a drastic decreased in fungal growth even at
162 0.025 mM (9.75 µg/mL) and concentrations as low as 0.008 µg/mL of AmB (Figure 2E).
163 For promazine hydrochloride, chlorcyclizine hydrochloride and cloperastine, where
164 0.1 mM corresponded to 32 µg/mL, 33 µg/mL and 36 µg/mL respectively, the
165 synergistic effect was found with concentrations around 0.1-0.025 mM, which
166 enhanced the antifungal activity of AmB at concentrations even as low as 0.008 µg/mL
167 (Figure 2F, 2G and 2H). Finally, in the presence of 0.03 µg/mL AmB, glimepiride
168 reduced fungal growth 60-70% at concentrations of 0.1 (49 µg/mL), 0.05 (24.5 µg/mL)
169 and 0.025 mM (12.25 µg/mL) (Figure 2I).
170
171 **Cytotoxicity Assay in mammalian cells**
172 We next investigated whether the combinations of the identified A.C. with AmB had
173 deleterious effects on mammalian cells. As shown in Figure 3, erythromycin (A),

chenodiol (B) and glimepiride (C), alone at 0.1 mM or in combination with AmB at 0.03 µg/mL, presented low cytotoxicity, with ~80% cell viability after 24 h. Nisoldipine (Figure 3D) presented about 80% cell viability when used alone, but reduced to 70% cell viability in combination with AmB. Clorcyclizine hydrochloride, riluzole hydrochloride and nortriptyline hydrochloride, induced a higher toxicity at 0.1 mM (around 50% death), although addition of AmB did not increased the inherent toxicity with the A.C. alone (Figure 3E, F and G). Promazine hydrochloride alone showed cell death around 50% and increased to 60% in combination with AmB (Figure 3H). Finally, cloperastine (Figure 3I) was the compound that showed the highest toxicity at the concentration tested (around 70% of mortality, Figure 3I).

Toxicity to zebrafish embryos

Physic-Chemical characteristics of the test solution fell into the range of the valid criteria according to OECD 236 [33].

Table 3 shows the lethality data of the drug solutions (single or in combinations with AmB) for the zebrafish embryos (n=16/group). Single compounds such as AmB, erythromycin and glimepiride did not produce mortality for the time of exposure (0-96 hpf). Other compounds such as nisolpidine, chlorcyclizine, chloperastine, promazine or riluzole exhibited relatively rapid zebrafish embryo mortality (during the first 24-48 hpf), while nortriptyline induced mortality at a later developmental stage (between 48-96 hpf). When the mixtures of A.C. with AmB were tested, we found that the combinations AmB + erythromycin as well as the AmB + glimepiride produced no mortality. The addition of AmB to chlorcyclizine, cloperastine and promazine boosted early mortality (24 hpf) in comparison with the single compound data, indicating increased toxicity of the mixture. Negative control group (dilution water) and negative solvent group (0.4% DMSO in egg water) did not exhibit mortality, while the positive control group showed mortality higher than 30%, in agreement with the OECD 236 guideline (32).

No obvious malformations were noticed in the zebrafish embryos exposed to either erythromycin, glimepiride or nortriptyline alone or in combination with AmB when they were observed through the stereomicroscope. Similarly, these three A.C. or their combination with AmB did not induce significant changes on embryo hatching (Supplemental table 1) as they hatched at a similar rate as the control group and within the physiological hatching period (usually between 48-60 hpf).

Erythromycin enhances the activity of AmB against *Candida* spp.

Among all the compounds, we decided to focus on erythromycin because it did not increase the toxicity in cell lines nor zebrafish models, and it is a well-known macrolide antibiotic used to fight bacterial infections. We tested the effect against *C. albicans, C. glabrata, C. auris, C. parapsilosis, C. tropicalis* and *C. krusei*.

The calculated FICI for all species, except for *C. krusei*, was < 0.5 (Table 4) confirming that erythromycin also enhanced the AmB activity against most *Candida* species. For *C. albicans*, the synergistic effect was stronger with erythromycin concentrations ranging from 0.1-0.025 mM, which enhanced the activity of AmB at concentrations ranging from 0.03-0.12 µg/mL (Figure 4A). When AmB (0.06 µg/mL) was combined with erythromycin (0.1 or 0.05 mM), there was a growth inhibition above 90% for *C. albicans*. Similar results were found for *C. glabrata*. For this species, erythromycin concentrations ranging 0.1-0.025 mM combined with AmB (0.03-0.12 µg/mL) induced a statistically significant decrease in fungal growth (Figure 4B). While AmB alone in concentrations of 0.03 and 0.06 µg/mL inhibited only around 7% and 35% respectively, addition of erythromycin (0.1, 0.05 and 0.025 mM) increased the inhibition to around 95%.

The synergic effect against *C. auris* was also tested, as this yeast is an important emergent pathogen that is resistant to different antifungals (33). As demonstrated for other species, the combination of AmB in concentrations ranging 0.06-0.25 µg/mL with erythromycin ranging 0.1-0.025 mM showed a synergistic effect against *C. auris*

(Figure 4C). AmB at 0.12 µg/mL only inhibited around 30% the growth of *C. auris*; however in combination with 0.1, 0.05 and 0.025 mM of erythromycin, the inhibition increased to around 90%.

For *C. parapsilosis* and *C. tropicalis*, the synergistic effect was moderate (Figure 4D and E). Finally, for *C. krusei*, the combination between both drugs did not show any synergistic effect (Figure 4F).

Screening of compounds with antifungal activity against *C. neoformans*

The experimental approach described above allowed the identification of drugs that inhibited the growth of *C. neoformans* in the control plates that did not contained sub-inhibitory concentrations of AmB. Although previous reports have already identified compounds with anticryptococcal activity using the same library, we decided to analyze our results to check if we could describe new compounds. Results from the new screening showed 43 A.C. that had inhibitory activity against *C. neoformans* (Supplemental table 2). The majority of these compounds act in the central nervous system, followed by drugs belonging to cardiovascular therapeutic class and infectiology. To confirm the results of this screening, five A.C. were selected to be tested by sensitivity assays as described by EUCAST-AFST E.DEF 7.3 protocol using the range of concentration from 0.4 to 0.0003 mM using four *C. neoformans* and four *C. gattii* strains. Some of the compounds showed low MICs such as ciclopirox ethanolamine (around 0.5 µg/mL) while others, such as triflupromazine had higher MIC values (64 µg/mL). Hycanthone had very low activity against *C. neoformans*, with MIC values for both species above 64 µg/mL (Table 5).

Discussion

The design of new antifungal treatments is a challenge in the field of medical mycology. In the last ten years, there has been only one new antifungal licensed

(isavuconazole). The changing epidemiology of fungal infections and their high incidence requires the identification and development of new drugs with antifungal activity. Since the commercialization of new molecules is a time-consuming process and very expensive, repurposing of off-patent drugs offers a feasible alternative to design new therapeutic approaches.

AmB shows the strongest antifungal activity among antifungals, but its administration is associated with toxicity. For this reason, in the present work, we attempted to identify new drugs that could enhance the activity of AmB at lower therapeutic doses that could reduce the toxicity at the same time. In a preliminary screening, we identified several compounds that could produce the desired effect. Furthermore, the synergism was confirmed by checkerboard assays. We also investigated the toxicity of the compounds in two different models: a mammalian cell line, and the zebrafish embryo model as a promising tool in toxicology. We showed that some of the compounds identified in this study as synergistic with AmB presented toxicity in these models at the concentrations used (such as chenodiol, chlorcyclizine, promazine and riluzole). However, this did not limit their possible interest as synergistic compounds with AmB, since these compounds are already used in clinic for different purposes, and we only discarded compounds that showed increased toxicity when combined with AmB. This was the case of chlorcyclizine and cloperastine, which produced a slight increase in toxicity in the zebrafish model.

Glimepiride showed synergism with AmB and presented low toxicity in the in vitro and in vivo systems. This compound is used to treat diabetes mellitus type 2 by stimulating the release of insulin. However, it can cause side effects, (headache, nausea and dizziness) due to endocrine unbalance. In our toxicity assays we did not find any lethal toxicity of the combination of the drugs, but the fact that both AmB and glimepiride have side effects in humans, indicate that this might not be the most suitable therapeutic option to treat cryptococcosis.

In the present study, one of the most promising compounds showing synergic activity with AmB was the erythromycin. Either alone or in combination with AmB, Erythromycin did not exert lethal toxicity in the in vitro or in vivo test models. In addition, no adverse effects on embryo development or hatching were evident after the exposure to these drugs. Erythromycin is a macrolide antibiotic that inhibits protein synthesis in bacteria through binding to the ribosomal complex (34, 35), which could be a related mechanism for fungi. The mechanism by which this antibiotic enhanced the activity of AmB has not been studied yet. Nevertheless, it is known that AmB creates pores at the fungal membrane that increase permeability. In this way, AmB might promote influx of erythromycin into fungal cells, which could present an inhibitory effect for the fungal ribosomal complexes. In *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, erythromycin inhibits mitochondrial protein synthesis by causing the separation of the tRNA from the mitochondrial ribosomes (36-38). Interestingly, AmB induces oxidative damage through a mechanism that involves the mitochondria (10-13), and inhibition of complex I conferred resistance to this antifungal (9). In consequence, it is reasonable to suggest that both drugs could synergistically increase mitochondrial damage.

Fungal infections often affect patients who are multi-treated, and it is common that they receive antibiotics for a long period. For this reason, the fact that erythromycin enhances the antifungal effect of AmB might offer a feasible combination in clinic that eliminates fungal burden. On the other hand, antibiotics can also decrease the bacterial population and in turn, favor fungal replication; therefore future studies using in vivo models are warranted. Another issue to consider is that due to their lipophilic properties and relatively high molecular weight, macrolides do not efficiently cross the blood-brain barrier (BBB) (39, 40), so their use during cryptococcal meningitis might be limited. However, in conditions of meningeal inflammation and disruption of the integrity of the BBB, their penetration into the CNS might increase, so further investigations will be required to fully evaluate the role of erythromycin and AmB combination during

cryptococcal meningitis. On the other hand, erythromycin shows a good tissue distribution, so its use during candidemia and candidiasis might be a feasible strategy to improve the effectivity of AmB.

Chenodiol, which is used to dissolve gallstones (41-43), also showed synergy with AmB. This compound is a bile acid that acts as a detergent and dissolves lipid particles. Besides it reduces cholesterol biosynthesis (44). Although fungi do not contain cholesterol in their membranes, it is possible that chenodiol reduces the main sterol of fungal membranes, ergosterol, either by direct binding and sequestration or by inhibition of its synthesis, which would increase the susceptibility to AmB.

Three compounds showed 3-4 times more antifungal activity when combined with AmB: riluzole hydrochloride, nortriptyline hydrochloride and promazine hydrochloride. However, they showed some toxicity in vitro and in vivo, either alone or in combination with AmB. These medicines act on the central nervous system (CNS). Interestingly, other anti-psychotic drugs, such as quetiapine, and olanzapine and the antidepressant sertraline, have already demonstrated activity against *C. neoformans* with MIC values of 0.5 mg/ml for quetiapine and 0.25 mg/ml for olanzapine. Although we have not studied the mechanisms of action of the described compounds, there are already reports that antipsychotic drugs could act on the cellular membrane of eukaryotes increasing their permeability (45-47). In the case of sertraline, it shows potent in vitro anticryptococcal activity by blocking protein synthesis (48, 49). For other purposes, riluzole could inhibit primary cancer cell proliferation, probably by inducing the arrest of cells in the G2/M phase (50). In addition, these authors described that riluzole hidrochloride also induced the production of ROS and apoptosis in liver cancer cells by inhibiting glutamate release.

Nisoldipine inhibited around 25% the growth of *C. neoformans*, and when combined with AmB, the inhibitory effect increased to around 95%. However, there was a statistically significant increase in the cell toxicity when combined with AmB. Nisoldipine acts as a calcium channel blocker, and it has been previously reported to

inhibit growth of some *Cryptococcus, Candida* spp, *S. cerevisiae* and *A. fumigatus* (51). Other study demonstrated that another calcium channel blocker, Nifedipine showed a synergistic effect with itraconazole being also active against itraconazole-resistant strains of *A. fumigatus* (52). Liu *et al.* combined four calcium channel blockers, amlodipine, nifedipine, benidipine and flunarizine with fluconazole and found synergism even against *C. albicans* fluconazole-resistant strains (53). These results suggest that inhibiting calcium channels might be another strategy to augment the efficacy of AmB.

In general, we found a good agreement between the toxicity data found in cell lines and in the zebrafish embryo model, although some differences were also detected, being some combinations and compounds more toxic in the zebrafish embryo model. These differences may be primarily due to the complexity of the zebrafish embryo system in which drugs usually show different toxicokinetics and undergo metabolism to some extent. In addition, zebrafish embryos have developed some of the main organs, such as heart, liver and nervous system by 72 hpf, which can be potential offtargets for the drugs. Therefore, the zebrafish model is a complementary model to the cell system for screening toxicity of drugs.

In our experimental approach, we always carried out a control plate with the compounds of the Prestwick Library without AmB, so it was unavoidable to identify compounds that inhibited the growth of *C. neoformans.* We found 43 drugs that inhibited the growth of *C. neoformans*. From these compounds we selected 11 to confirm the antifungal activity against different *C. neoformans* and *C. gattii* strains. The anti-cryptococcal activity was confirmed for 10 compounds (Supplemental table 2 and Table 5). Previous studies have also identified compounds among the Prestwick Chemical library with fungicidal effect against *C. neoformans*. Butts *et al* using the Prestwick library, described 31 compounds active against *C. neoformans* through adenylate kinase release approach (28). When compared with those results, we found

12 compounds in common, thioridazine, perhexiline maleate, chlorprothixene, fluspirilen, trifluoperazine, methiothepin maleate, prochlorperazine dimalate, suloctidil, thonzonium bromide, clomiphene, tamoxifen and thiethylperazine dimalate. Suloctidil was one of the compounds with lowest MIC value in both studies. This drug was also active against *C. albicans* in planktonic and biofilm cells in vitro and was also able to reduce fungal burden in vivo (28, 54). Recently, Truong *et al.* used the repurposing strategy to identify A.C. against *C. deuterogatti*. The authors identified 54 active drugs using the Enzo drug library that contains 640 off-patent drugs. Among them, they suggested the use of flubendazole, an anthelmintic drug, as a new anti-cryptococcal drug due to its activity against all pathogenic *Cryptococcus* spp, including isolates resistant to fluconazole. They also identified that the calcium channel blockers nifedipine, felodipine and nisoldipine are active against all pathogenic *Cryptococcus* spp (51).

In the present work we have also identified other compounds that were not previously reported by other authors using the same chemical library. There are several explanations for this discrepancy, but differences in the experimental conditions, such as the concentration used, or the detection method are key points. Another major difference is the screening strategy as we were interested in compounds with effects on fungal growth (fungicides and/or fungistatic). Nevertheless, previous reports identified drugs with fungicidal activity but using an assay based on adenylate kinase release from the cells. Expanding the screening for compounds with both fungicidal and fungistatic activity might have highlighted more compounds as some of the antifungals used in clinic are fungistatic, or even can behave as fungistatic or fungicidal depending on the species.

The compound that showed lowest MICs against *C. neoformans* and *C. gattii* was ciclopirox ethanolamine (0.5 µg/mL). Ciclopirox ethanolamine is an antifungal agent used topically to treat a variety of fungal infections mainly dermatophytosis, seborrheic dermatitis and cutaneous candidiasis (55). It acts through the chelation of polyvalent

398 metal cations, such as Fe^{3+} and Al^{3+}. Its activity was tested *in vitro* against distinct
399 fungal species (56, 57), but its use for the treatment of systemic mycosis has not been
400 reported yet.
401 Auranofin also showed activity against *C. neoformans* with a MIC value of 4 µg/mL.
402 This compound is used for treatment of rheumatoid arthritis. Our results are in
403 agreement with previous findings that reported that this drug was active against some
404 fungal species (23, 58). Although the inhibitory mechanism is not known, it has been
405 suggested that auranofin exerts its action through the production of reactive-oxygen
406 mediated cell death.
407 Tamoxifen has also been described its antifungal activity by different groups (59-63). It
408 is an anticancer drug that acts as an anti-estrogen in the mammary tissue. The
409 mechanism of action still is not fully known, but it is known that this drug can bind to
410 calmodulin (63). This protein is required for growth at high temperature in
411 *C. neoformans* (64).
412 Triclabendazole is another compound also presenting activity against *C. neoformans*
413 and *C. gattii* (65) with MICs around 4-8 µg/mL. It is an anthelminthic drug that binds to
414 the β-tubulin molecule, causing an ultrastructural disruption by impairing the
415 maintenance of the integrity of the surface membrane (66, 67). Other antifungals such
416 as griseofulvin, carbendazim and thiabendazole bind to tubulin and thus preventing
417 microtubule polymerization. Also, there are a great number of patents of distinct
418 molecules that have the tubulin as an alternative target with antifungal activity against a
419 great diversity of pathogenic fungi (68).
420
421 In conclusion, drug repurposing showed to be an efficient tool to find potential drugs
422 that could enhance the AmB activity and helped to find new active drugs against
423 several pathogenic yeasts. In this sense, the combination of erythromycin or
424 glimepiride with AmB reduced the antifungal effective concentration while showing no
425 adverse effects in the toxicity assays, which included the in vitro test with mammalian

cell system and the in vivo toxicity test with zebrafish embryos. The synergism between AmB and erythromycin could be a new promising drug combination to improve the current antifungal therapy against several invasive fungal diseases. Finally, we also identified drugs with anti-*Cryptococcus* activity, such as ciclopirox or auranofin, which should be further characterized to be considered as potential treatments for cryptococcal disease.

Material and methods

Strains and growth conditions

Cryptococcus neoformans H99 strain (69) was used for the initial screening. *Cryptococcus neoformans* strains KN99, CL0741 and 24067 and *C. gattii* strains NIH34, CBS10514, CBS10865 and CL4999 were also used. The following *Candida* spp. strains were also used; *C. albicans* SC5314 (70), *C. glabrata* (CL-9555), *C. tropicalis* (CL-10621) *C. krusei* (ATCC 6258), *C. parapsilosis* (ATCC 22019) and *C. auris* (CL-10013). The yeast strains were incubated in Sabouraud medium (Oxoid, liquid or solid containing 1.5% agar) at 30 °C.

Antifungals and Chemical library

We used the Prestwick Chemical Library® to identify drugs that potentially enhance the activity of AmB. This library contains 1,280 off-patent drugs approved by the Food and Drug Administration (FDA), European Medicines Agency (EMA) and other agencies. The library was provided in 96-wells format, each containing 100 µl of each compound at a concentration of 10 mM in DMSO (dimethyl sulfoxide). In addition, we also used Amphotericin B (Sigma-Aldrich).

Antifungal susceptibility testing

453 The minimal inhibitory concentration (MIC) to AmB to *C. neoformans* H99 strain was
454 tested according to the antifungal susceptibility testing (AFST) following EUCAST-
455 AFST E.DEF 7.3 protocol (71). Briefly, *C. neoformans* cells were inoculated in
456 Sabouraud agar plates for 48 h, and a suspension of 1-5x10^5 cells/mL was prepared in
457 distilled H_20. Antifungal susceptibility plates were prepared with a range of AmB
458 concentrations from 16 to 0.03 µg/mL (1/2 dilutions) in RPMI containing 2% glucose
459 and buffered at pH 7.0 with 165 mM MOPS. One hundred microliters (µl) of the
460 *C. neoformans* suspensions were added to the antifungal susceptibility plates
461 (prepared as 2x stocks) and incubated at 35 °C without shaking for 48 h. After
462 incubation, the OD was measured at 530 nm and the MIC was calculated. MIC was
463 defined as the antifungal concentration that produced a 90% inhibition of growth
464 compared to the control well without antifungal. The strains *Candida parapsilosis*
465 (ATCC 22019) and *C. krusei* (ATCC 6258) were used as quality control isolates in the
466 AFST experiments. This experiment was performed three times (triplicate).
467 Susceptibility of fungal strains to selected compounds from the Prestwick Chemical
468 Library was evaluated as described above (EUCAST-AFST E.DEF 7.3 protocol).
469 Concentration range for all the tested compounds was from 64 to 0.12 µg/mL.
470
471 **Screening of drugs from the Prestwick Chemical Library which enhance the**
472 **activity of Amphotericin B on antifungal activity**
473 The use of the Prestwick Chemical library in our conditions allowed the identification of
474 compounds that showed synergism with Amphotericin B, but also of off-patent drugs
475 that had antifungal activity against *C. neoformans*. The original stocks of the 1,280
476 compounds present in the Prestwick Chemical Library were diluted (1:10) to obtain a
477 concentration of 1 mM in intermediate plates with 2.2x RPMI medium supplemented
478 with glucose. From these intermediate plates, 20 µL of each well were again diluted
479 (1:5) in new plates prepared with 80 µL of 0.06 µg/mL AmB in 2x RPMI medium
480 supplemented with 4% glucose per well. Parallel plates without AmB were carried out

as control to test the activity of the compounds alone. These plates were then inoculated with 100 µL of a *C. neoformans* suspension (1-5x10^5 cells/mL) to achieve a final concentration of 100 µM and 0.03 µg/mL of the A.C. and AmB (sub inhibitory for *C. neoformans*), respectively. In the final plates, columns 1 and 12 did not contain any compound, so they were used to include the following controls: 1) sterility controls, 2) AmB alone at sub inhibitory (0.03 µg/mL) and in inhibitory (0.25 µg/mL) concentrations and 3) a growth control with 2x RPMI medium supplemented with 2% glucose and 1% DMSO.

After 48 h of incubation at 35 ºC, readings at 530 nm were performed. We scored as negative those drugs that did not have any effect on *C. neoformans* by themselves (<40% inhibition), and positive A.C. those that in combination with AmB 0.03 µg/mL induced more than 80% decrease of growth.

In the screening described above, one of the control plates contained only the compounds from the Prestwick Library. For this reason, it was unavoidable to also identify compounds that caused almost full inhibition of *C. neoformans* growth (more than 80% of growth inhibition). The inhibition of these compounds was further tested in microdilution plates using EUCAST protocol, using a concentration range of 64-0.12 µg/mL in 96-wells plates, prepared as described above. The inoculum was prepared as described above and the results were obtained after 48 h of incubation at 35 ºC through spectrophotometer readings at 530 nm.

Checkerboard assay

To confirm the data obtained in the screening for drugs that enhance the activity of AmB, checkerboard assays were performed expanding the concentration range for the A.C. from 0.4 to 0.0003 mM and for AmB from 0.5 to 0.008 µg/mL.

For this purpose, each compound or AmB solutions where prepared at 50x in DMSO. So, the solutions were diluted in RPMI (1:50) plates to ensure that in the assay, DMSO concentration did not exceeded 1%. Finally, 50 µL from each solution concentration

were mixed in the final assay plate. The *C. neoformans* inoculum was prepared as described above in the Antifungal Susceptibility Testing section following the EUCAST-AFST E.DEF 7.3 protocol. The OD at 530 nm was measured after 48 h of incubation at 35 ºC and the Fractional Inhibitory Concentration Index (FICI) was calculated according to the equation: ΣFIC = FIC (compounds) + FIC (AmB), where the FIC is the ratio of the MIC of the combination with the MIC alone.

The combination of AmB with the A.C. was considered synergic when the FICI ≤ 0.5, indifferent when FICI > 0.5 and ≤ 4, and antagonist when FICI > 4 (72, 73).

Toxicity assays

In vitro cytotoxicity assay

In vitro cytotoxic activity was evaluated using the macrophage cell line RAW 264.7. Macrophages (10^5 cells/well) were exposed to 0.1 mM of each compound alone or combined with 0.03 µg/mL AmB, and incubated at 37 ºC, 5% CO_2 for 24 h. Cytotoxicity was obtained by measuring the release of LDH to the medium using the Cytotox 96® non-radioactive cytotoxicity assay kit (Promega) following the manufacturer's recommendations. Untreated cells (viable cells), dead cells (with lysis solution) and wells with only medium were used to determine the background LDH activity and served as controls. Results were represented as percentage of mortality in comparison to the wells in which full death was induced with the lysis buffer provided in the kit.

In vivo toxicity assessment with zebrafish embryos

Fish husbandry and embryo collection

Zebrafish (*Danio rerio*) progenitors were wild type, obtained from a local pet store and maintained at standard laboratory conditions of 26 °C on a 14:10 dark/light photoperiod in a recirculation system for at least 2 months, before using them as progenitors. The water was prepared according to UNE-EN ISO 7346-3:1998. Fishes were fed twice a

536 day with a commercial diet (Zeigler®), and every two days the diet was complemented
537 with living *Daphnia magna*.
538 One day before the experiment, parent animals were separated from the rest and
539 caged in tanks (one female and two males) overnight. Spawning was induced when the
540 light was turned on the following morning. Each spawning was examined for mortality,
541 and when lower than 15%, the eggs were accepted for their use in the toxicity tests.
542
543 *Waterborne exposure of zebrafish embryos*
544 The zebrafish eggs from two spawning were washed with dilution water (UNE-EN ISO
545 7346-3:1998 or ISO 15088:2007). Only fertilized eggs, which staged at 2-4 cells were
546 disposed into 96-well plates with 200 µL of the test solution (n= 16 embryos/test
547 solution). Plates were covered and incubated in a calibrated climate chamber at 28.5 ±
548 0.1 ºC with a 14h/10h light/darkness cycle and for 96 h, with fresh medium renovation
549 every 24h.
550
551 Test solutions included the negative control with dilution water, the solvent control with
552 0.4% DMSO, the positive control with 3,4-dichloroaniline (4 µg/mL in dilution water)
553 and the drug solutions. Chenodiol, nisoldipine, chlorcyclizine hydrochloride,
554 cloperastine hydrochloride, erythromycin, glimepiride, promazine hydrochloride, riluzole
555 hydrochloride and nortriptyline hydrochloride were either tested alone at 0.1 mM or
556 combined with 0.03 µg/mL AmB.
557 Dissolved oxygen, pH and conductivity were assessed in freshly prepared test
558 solutions to comply with test solution requirements according to OECD 236
559 recommendations (32). We included the following test solutions; negative control with
560 dilution water, solvent control with 0.4% DMSO, positive control with 3,4-dichloroaniline
561 (4 µg/mL in dilution water).
562
563 *Toxicological assessment*

Zebrafish mortality and development was assessed at 0, 8, 24, 48, 72 and 96 hours post fertilization (hpf) through stereomicroscope observation. Hatching was evaluated by visual inspection of all zebrafish embryos every 3 h from 48 hpf to 60 hpf.

Statistical analysis

To evaluate the significance of the results of the cytotoxicity assay we used ANOVA followed by Tukey test using the GraphPad Prism 6 (GraphPad Software. Inc, San Diego, CA).

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842 **Table 1.** Synergic compounds at 0.1 mM with AmB 0.03 µg/mL against *C. neoformans*

Name	**µg/mL**	**Therapeutic class**	**Target/Action mecanism**	**Compound inhibition (%)**	**Combination inhibition (%)**
Atracurium besylate	124	**Neuromuscular**	Non-depolarizing neuromuscular blocking agent. Antagonizes the neurotransmitter action of acetylcholine	40%	95%
Erythromycin	73	Infectiology/Metabolism	Inhibits bacterial protein synthesis by binding to bacterial 50S ribosomal subunits	15%	90%
Riluzole hydrochloride	27	Central Nervous System	Inhibitory effect on glutamate release, inactivation of voltage-dependent sodium channels and interfere in intracellular events that follow transmitter binding at excitatory amino acid receptors	25%	95%
Nortriptyline hydrochloride	30	Central Nervous System	Inhibits the reuptake of the neurotransmitter serotonin at the neuronal membrane or acts at beta-adrenergic receptors	30%	90%
Chenodiol	40	Gastroenterology	Suppresses hepatic synthesis of both cholesterol and cholic acid, gradually replacing the latter and its metabolite, deoxycholic acid in an expanded bile acid pool	30%	90%
Thioguanosine	31	Metabolism/Oncology	Antineoplastic action. Inhibits the synthesis of DNA and RNA of cells.	40%	85%
Diflorasone diacetate	49	Endocrinology	It's a synthetic glucocorticoid that binds to the glucocorticoid receptor (GR) in the cytoplasm.	30%	90%
Oxiconazole nitrate	49	Infectiology/Metabolism	Inhibits ergosterol biosynthesis, inhibits DNA synthesis and suppresses the intracellular concentrations of ATP.	5%	95%
Nisoldipine	39	Cardiovascular	By deforming the channel, inhibiting ion-control gating mechanisms, and/or interfering with the release of calcium from the sarcoplasmic reticulum. Inhibits the influx of extracellular calcium across the myocardial and vascular smooth muscle cell membranes	25%	95%
Promazine hydrochloride	32	Central Nervous System	Antagonism at dopamine and serotonin type 2 receptors, with greater activity at serotonin 5-HT2 receptors than at dopamine type-2 receptors	20%	80%
Chlorcyclizine hydrochloride	33	Allergology/Central Nervous System	Histamine H1 receptor	15%	100%
Demeclocycline hydrochloride	50	Metabolism	Inhibits the translation by binding to the 30S and 50S ribosomal subunit, impairing protein synthesis.	35%	90%
Cloperastine hydrochloride	36	Respiratory	NOT IDENTIFIED	5%	90%
Glimepiride	49	Endocrinology	Binds to ATP-sensitive potassium channel receptors on the pancreatic cell surface. Reduce potassium conductance causing depolarization of the membrane and stimulate calcium ion influx through voltage-sensitive calcium channels. Induces the secretion of insulin.	5%	80%

Table 2. Fractional Inhibitory Concentration Index for the selected compounds

Compound	FICI	
	50%	**75%**
Erythromycin	0.35	0.43
Riluzole hydrochloride	0.39	0.37
Nortriptyline hydrochloride	0.40	0.62
Chenodiol	0.31	0.37
Nisoldipine	0.40	0.38
Promazine hydrochloride	0.34	0.43
Chlorcyclizine hydrochloride	0.16	0.37
Cloperastine hydrochloride	0.25	0.34
Glimepiride	0.15	0.28

Table 3: Mortality (%) in zebrafish embryos exposed to single drugs (100 µM) or their combination with AmB (0.03 µg/mL)

SINGLE a.c.	24h	48h	72h	96h	Total
AmB	0	0	0	0	0
Chenodiol	0	100	100	100	100
Nisoldipine	100	100	100	100	100
Chlorcyclizine	0	100	100	100	100
Cloperastine	0	100	100	100	100
Erythromycin	0	0	0	0	0
Glimepiride	0	0	0	0	0
Promazine	0	100	100	100	100
Riluzole	100	100	100	100	100
Nortriptyline	0	0	0	100	100
DMSO 0.4%	0	0	0	0	0
Cont –	0	0	0	0	0
Cont +	18.75	43.75	100	100	100

MIXTURES	24h	48h	72h	96h	Total
Chenodiol + AmB	0	100	100	100	100
Nisoldipine + AmB	100	100	100	100	100
Chlorcyclizine + AmB	100	100	100	100	100
Cloperastine + AmB	100	100	100	100	100
Erythromycin + AmB	0	0	0	0	0
Glimepiride + AmB	0	0	0	0	0
Promazine + AmB	56.25	81.25	100	100	100
Riluzole + AmB	100	100	100	100	100
Nortriptyline + AmB	0	0	0	100	100
DMSO 0.4%	0	0	0	0	0
Cont –	0	0	0	0	0
Cont +	6.25	50	100	100	100

Table 4. Fractional Inhibitory Concentration Index for the combination of erythromycin with AmB against *Candida* spp.

***Candida* spp.**	**FICI**	
	50%	**75%**
C. albicans	0.33	0.33
C. glabrata	0.46	0.46
C. auris	0.43	0.37
C. parapsilosis	0.27	0.36
C. tropicalis	0.30	0.38
C. krusei	0.46	0.56

893 Table 5. MICs for the active compounds against *C. neoformans* and *C. gattii* isolates

	MICs (μg/mL)							
	C. neoformans				*C. gattii*			
	H99	KN99	CL0741	24067	NIH34	CBS10514	CBS10865	CL4999
Auranofin	4	2	4	2	2	2	2	2
Ciclopirox ethanolamine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Clofilium tosylate	32	32	32	32	16	16	16	16
Hycanthone	>64	>64	>64	>64	>64	>64	>64	>64
Perphenazine	16	32	32	32	32	16	32	16
Suloctidil	4	4	4	4	4	4	4	4
Tamoxifen	8	8	8	8	8	8	8	8
Thiethylperazine dimalate	32	32	32	32	32	32	32	32
Triclabendazole	8	8	8	8	8	8	8	8
Trifluoperazine dihydrochloride	16	16	16	16	16	16	16	16
Triflupromazine	64	64	32	32	64	32	64	32

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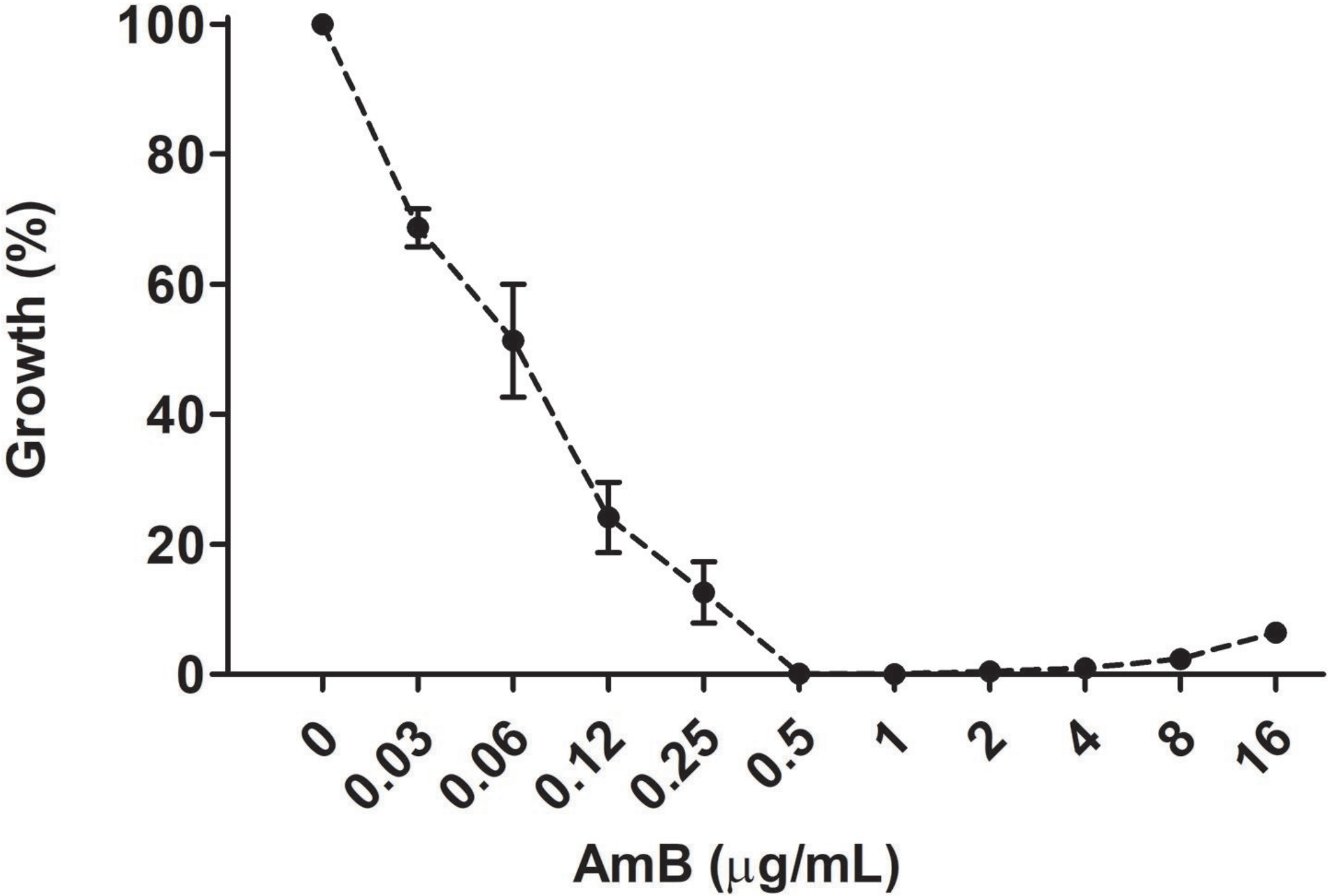
FIGURE LEGENDS

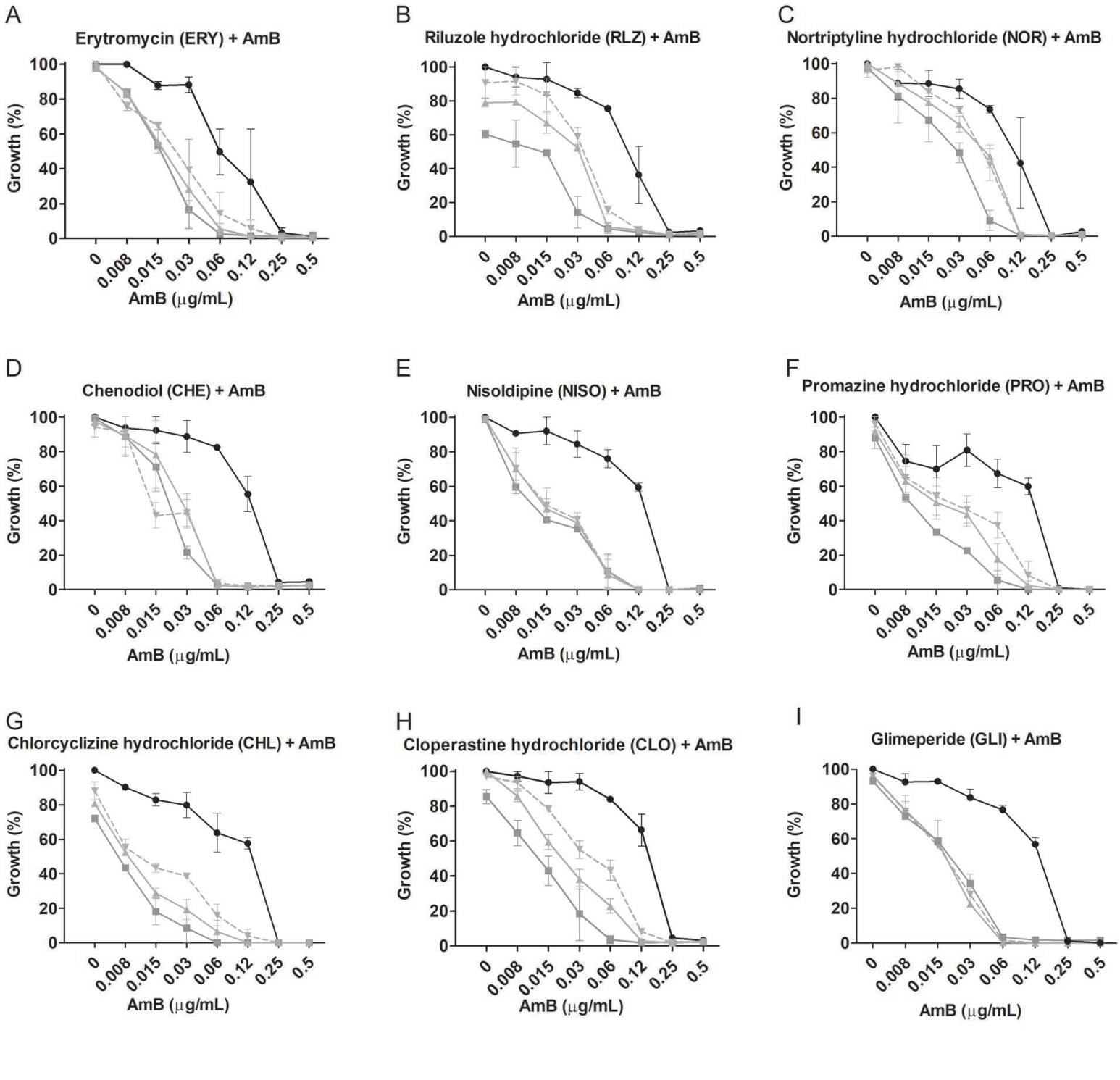
Figure 1. Effect of different AmB concentrations on growth in *C. neoformans*, *C. krusei* and *C. parapsilosis*.

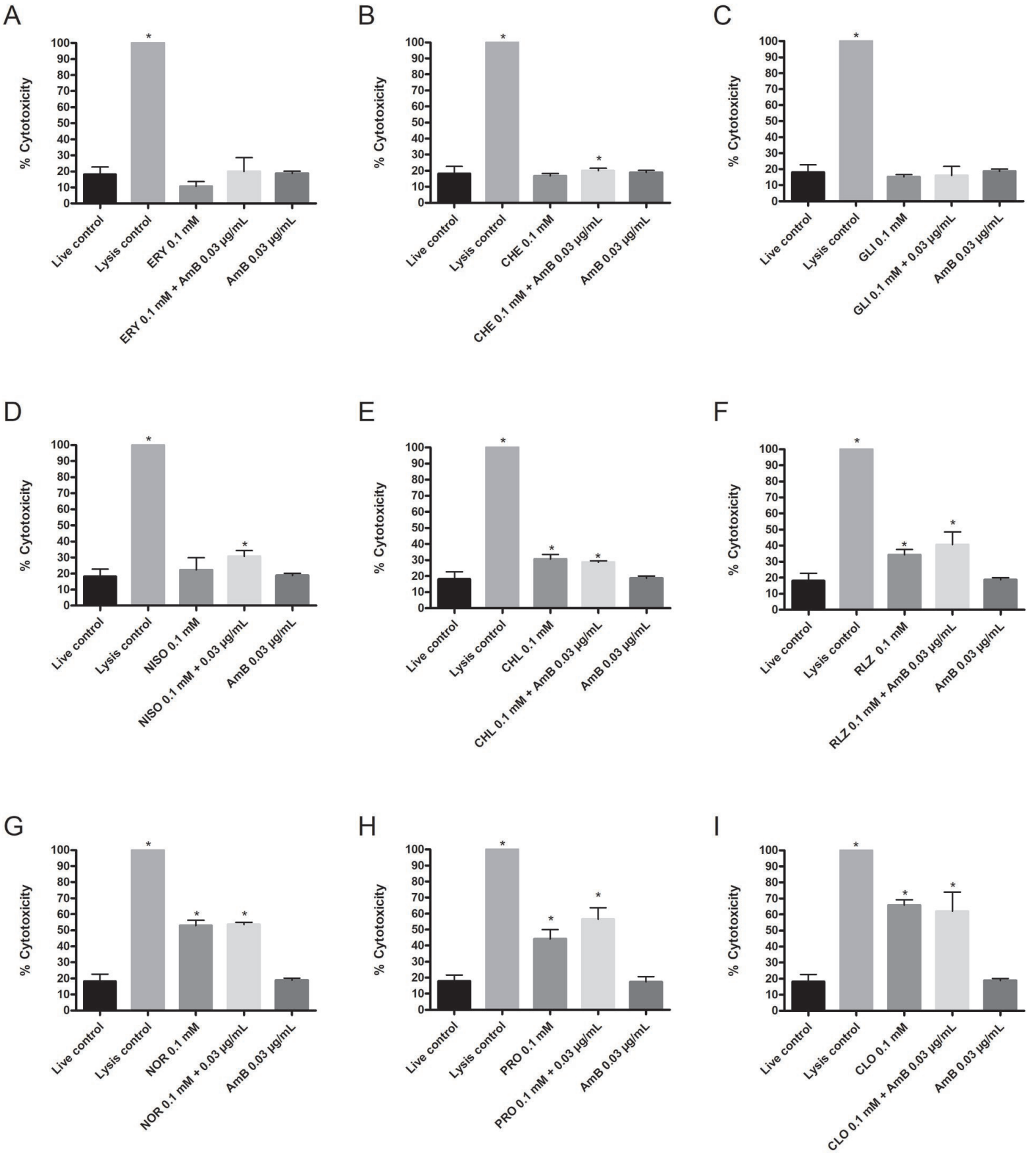
Figure 2. Effect of combination of erythromycin (A), riluzole (B), nortryptiline (C), chenodiol (D), nisoldipine (E), promazine, (F), chlorcyclizine (G), cloperastine (H) and glimepiride (I) with AmB (0.008-0.5 µg/mL) on *C. neoformans* growth (H99 strain). In each case, the graph shows the effect of 0.1 (gray straight line, squares), 0.05 (gray straight line, triangles) and 0.025 mM (dotted gray line, inverted triangle) of each compound. Black straight line shows the growth of *C. neoformans* in the presence of AmB without any A.C. The results were obtained from the checkerboard assays (see M&M for details). The experiment was performed in triplicates, and the average and standard deviation for each point are plotted.

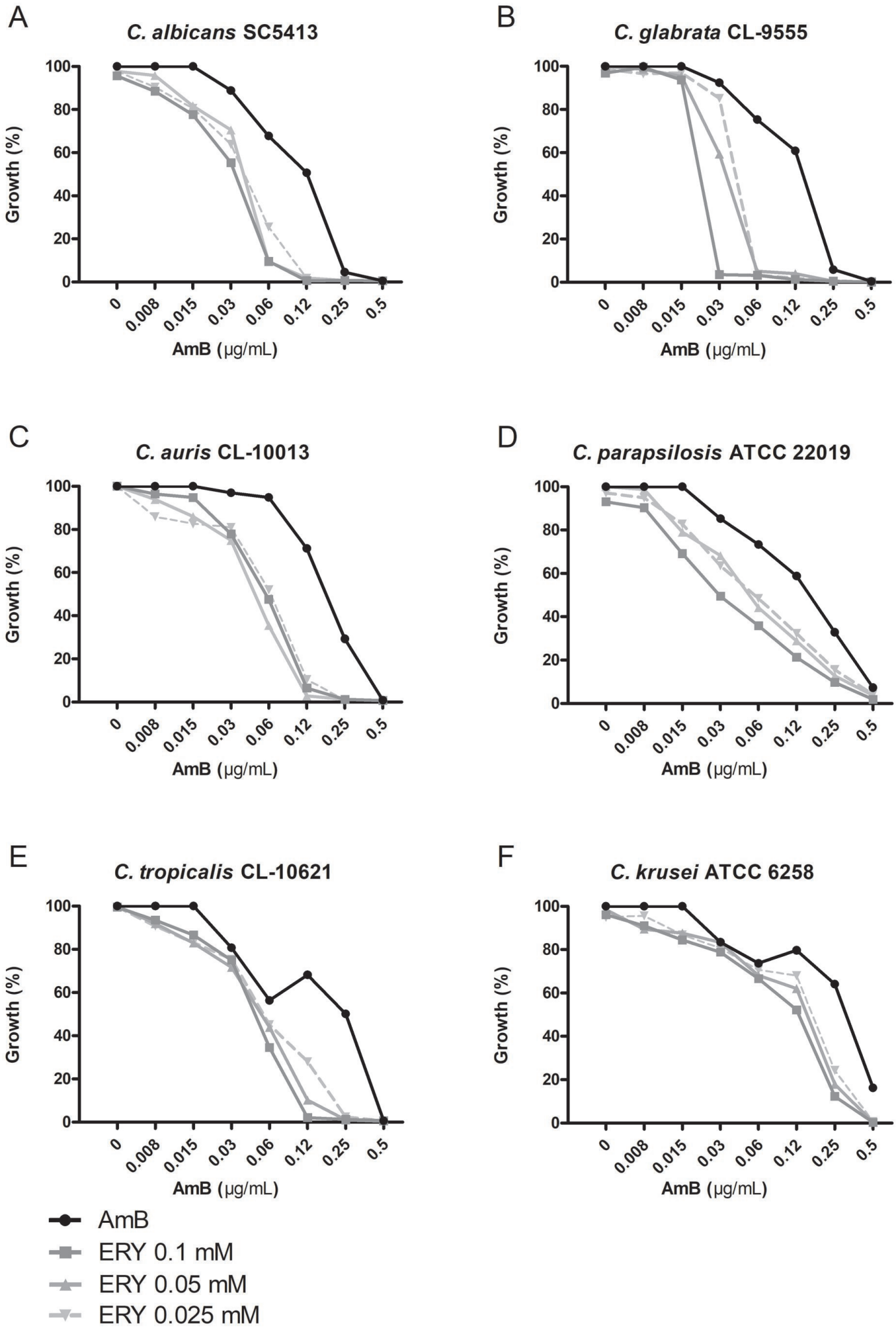
Figure 3. Toxicity of drug combination in RAW264.7 cell line. Toxicity assays in macrophage-like RAW264.7 were performed as described in M&M. For each combination, the graphs show the death percentage of control cells (Live control), lysis control, AmB (0.03 µg/mL), the corresponding A.C. (0.1 mM) and combination of both are shown. The experiment was performed in triplicates in different days, and the bars show the average and standard deviation. Asterisks denote statistical difference between the samples and the live control cells.

Figure 4. Effect of the combination of erythromycin and AmB on *C. albicans* (A), *C. glabrata* (B), *C. auris* (C), *C. parapsilosis* (D), *C. tropicalis* (E) and *C. krusei* (F). In each case, the graph shows the effect of 0.1 (gray straight line, squares), 0.05 (gray straight line, triangles) and 0.025 mM (dotted gray line, inverted triangle) of erythromycin. Data shown in the graphs were obtained from the checkerboard assays (see M&M).


100
80
60
40
20
0
Growth (%)
0
0.03
0.06
0.12
0.25
0.5
1
2
4
8
16
AmB (μg/mL)


A
Erytromycin (ERY) + AmB
B
Riluzole hydrochloride (RLZ) + AmB
C
Nortriptyline hydrochloride (NOR) + AmB
D
Chenodiol (CHE) + AmB
E
Nisoldipine (NISO) + AmB
F
Promazine hydrochloride (PRO) + AmB
G
Chlorcyclizine hydrochloride (CHL) + AmB
H
Cloperastine hydrochloride (CLO) + AmB
I
Glimeperide (GLI) + AmB
Growth (%)
0
20
40
60
80
100
0
0.008
0.015
0.03
0.06
0.12
0.25
0.5
AmB (μg/mL)
AmB
Compound 0.1mM
Compound 0.05mM
Compound 0.025mM


A
% Cytotoxicity
Live control
Lysis control
ERY 0.1 mM
ERY 0.1 mM + AmB 0.03 µg/mL
AmB 0.03 µg/mL
B
% Cytotoxicity
Live control
Lysis control
CHE 0.1 mM
CHE 0.1 mM + AmB 0.03 µg/mL
AmB 0.03 µg/mL
C
% Cytotoxicity
Live control
Lysis control
GLI 0.1 mM
GLI 0.1 mM + 0.03 µg/mL
AmB 0.03 µg/mL
D
% Cytotoxicity
Live control
Lysis control
NISO 0.1 mM
NISO 0.1 mM + 0.03 µg/mL
AmB 0.03 µg/mL
E
% Cytotoxicity
Live control
Lysis control
CHL 0.1 mM
CHL 0.1 mM + AmB 0.03 µg/mL
AmB 0.03 µg/mL
F
% Cytotoxicity
Live control
Lysis control
RLZ 0.1 mM
RLZ 0.1 mM + AmB 0.03 µg/mL
AmB 0.03 µg/mL
G
% Cytotoxicity
Live control
Lysis control
NOR 0.1 mM
NOR 0.1 mM + 0.03 µg/mL
AmB 0.03 µg/mL
H
% Cytotoxicity
Live control
Lysis control
PRO 0.1 mM
PRO 0.1 mM + 0.03 µg/mL
AmB 0.03 µg/mL
I
% Cytotoxicity
Live control
Lysis control
CLO 0.1 mM
CLO 0.1 mM + AmB 0.03 µg/mL
AmB 0.03 µg/mL


A
C. albicans SC5413
B
C. glabrata CL-9555
C
C. auris CL-10013
D
C. parapsilosis ATCC 22019
E
C. tropicalis CL-10621
F
C. krusei ATCC 6258
Growth (%)
AmB (µg/mL)
0
0.008
0.015
0.03
0.06
0.12
0.25
0.5
AmB
ERY 0.1 mM
ERY 0.05 mM
ERY 0.025 mM