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MICROBIOLOGY

Recent developments on the anti-*Candida* effect of amphotericin B combined with a second drug - a mini-review

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Abstract: Invasive *Candida* infections threaten human health due to the increasing incidence of resistance to the currently available antifungal agents. Amphotericin B (AMB) is the gold standard therapy to treat these infections. Nevertheless, the use of such substance in the clinic is aggravated by its toxicity. Since AMB binds to membrane sterols, it forms pores on human plasma membranes, mainly in kidney cells, leading to nephrotoxicity. The combination of this drug to a second substance could allow for the use of smaller concentrations of AMB, consequently lowering the probability of adverse effects. This mini-review summarizes information regarding an array of substances that enhance AMB antifungal activity. It may be noticed that several of these compounds target plasma membrane. Interestingly, substances approved for human use also presented combinatory anti-*Candida* activity with AMB. These data reinforce the potential of associating AMB to another drug as a promising therapeutical alternative to treat *Candida* infections. Further studies, regarding mechanism of action, pharmacokinetics and toxicity parameters must be conducted to confirm the role of these substances as adjuvant agents in candidiasis therapy.

Key words: Amphotericin B, antifungal activity, *Candida* spp., checkerboard, synergism, yeast.

INTRODUCTION

Candida spp. is a major fungal pathogen that affects human beings. Three main classes of antifungal agents are currently used to treat *Candida.* infections - azoles, polyenes, and echinocandins -, however these compounds may not be effective against all strains, due to the development of resistance by the fungus (Kotey et al. 2021). Because of the small number of substances available to treat *Candida* spp. infections and the increasing incidence of resistance to them, high mortality rates of invasive *Candida* infections can be observed, mainly on immunocompromised individuals. Annualy, more than 400,000 invasive *Candida* spp. infections occur, with a mortality rate of 46 - 75% (Brown et al. 2012). These alarming numbers show that it is imperative the development of new effective treatments against candidiasis.

Amphotericin B (AMB) is a polyene drug widely employed to treat candidiasis, especially when the infection becomes invasive, and visceral leishmaniasis. It is well accepted that this substance binds ergosterol located in the cell membrane, causing the formation of pores and hence the leakage of ions, both in fungi and *Leishmania* spp. Nonetheless, the mechanism of action of AMB may be more complex. According to Chattopadhyay & Jafurulla (2011), the antileishmanial activity of AMB is consequence of its binding to protozoan ergosterol and macrophage cholesterol, avoiding the entry

of *Leishmania* spp. into the macrophages. Anderson et al. (2014) proposed that instead of forming an ion channel, AMB originates an extramembranous structure that extracts ergosterol from plasma membrane, therefore killing the microorganisms. Using a confocal fluorescence lifetime imaging microscopy of giant unilamellar vesicles, Grudzinski et al. (2016) did not detect these extramembranous structures, but observed that membrane sterols may allow AMB to adopt a vertical orientation within the lipid phase of the membrane, originating an aggregate that promotes osmotic imbalance. In *C. albicans*, Grela et al. (2019) showed that AMB binds to the cell membrane of budding yeasts, impairs the formation of cell walls, and penetrate into the cytoplasm. Recently, Dong et al. (2021) observed by stimulated Raman scattering imaging that AMB molecules are indeed organized vertically in the plasma membrane, parallel to phospholipid acyl chains, corroborating to the ion channel model. Besides affecting membrane permeability, AMB may present a second mechanism of action, related to its ability to induce the production of reactive oxygen species (ROS) within the fungus, damaging essential structures such as the membrane itself, DNA, and mitochondria (Mesa-Arango et al. 2014).

Resistance to AMB has already been described, but its incidence is low when compared to azoles and echinocandins. The main disadvantage of administering AMB is its nephrotoxicity, a consequence of the binding of this compound to human cells membrane.

Combinatory approaches have been extensively used in clinical practice to manage infectious diseases. For instance, sulfamethoxazole and trimethoprim, two drugs that inhibit folic acid biosynthesis pathway, are commonly used in association to treat bacterial infections (John 2020). A strategy that could be approached to diminish AMB toxicity would be administering smaller doses of this drug combined to a second substance. Attempts of combining AMB with antifungal drugs currently used in clinical practice have been made, but the results obtained against *Candida* spp. were not satisfactory. Scheid et al. (2012) evaluated the combinatory effect of itraconazole or voriconazole with terbinafine or AMB against *C. dubliniensis*. Treating the yeasts simultaneously with AMB and azole drugs led to a high degree of indifference and antagonism, precluding the use of these substances combined (Scheid et al. 2012). Combining AMB to fluconazole, itraconazole, voriconazole, posaconazole, caspofungin, or flucytosine also presented mainly indifferent interactions against *C. glabrata* (Khalifa et al. 2021). Interestingly, Rosato et al. (2012) observed that AMB was synergic with anidulafungin against *C. albicans*, *C. parapsilosis*, *C. guilliermondii*, *C. krusei*, *C. glabrata*, and *C. tropicalis*. Considering the low number of antifungal drugs available to treat candidiasis and the limited efficacy of combining them to AMB, a feasible strategy to overcome *Candida* spp. infections could be associating AMB to non-antifungal agents. Then, the aim of this mini-review is to provide an overview of the recent developments regarding the anticandidal activity of AMB combined to compounds that are not currently used as antifungal drugs.

MATERIALS AND METHODS

Methods performed to assess the interaction between two drugs

Several assays are available to evaluate the interaction between two drugs, as described below. Initially, *in vitro* assays are performed to observe whether the compounds interact with each other. A deep knowledge of these assays is essential to correctly interpret the

data obtained from the experiments. Incubation time, concentrations of each drug, and type of antifungal activity (fungistatic / fungicidal) must be considered in order to accurately describe the combinatory effect of two compounds.

Among the methodologies that can be carried out in order to evaluate the combined antifungal activity of two substances *in vitro*, checkerboard stands out as the most commonly performed (Bidaud et al. 2021). This assay is conducted in 96-well microplates and consists in a two-dimensional microbroth dilution method. Each column and row of the microplate present a two-fold serial dilution of drugs A and B, respectively. Therefore, each well consists of a combination of different concentrations of drugs A and B. Also, the antifungal effect of each drug alone must be assessed. Then, one column and one row are designated to present only drugs A and B, respectively. After incubation at a specific period, the absorbance of the wells is obtained on a microplate reader, cell growth is measured (Figure 1), and the concentrations that present antifungal activity are determined, such as MIC (minimum inhibitory concentration, i.e., the lowest concentration that completely inhibit cell growth) or IC_{50} (the concentration that inhibits 50% of cell growth when compared to untreated control). The fractional inhibitory concentration index (FICI) model is often used to evaluate drug interaction. FICI is defined as the sum of the fractional inhibitory concentration (FIC) of each drug, while FIC is the ratio MIC combined / MIC alone. There are different interpretations for FICI values. Usually, synergistic (one drug enhances the activity of another), indifferent (drugs do not enhance each other activity), and antagonistic (one drug decreases the activity of another) interactions are defined by FICI ≤0.5, >0.5 - 4.0, and > 4.0, respectively (Odds 2003). There are some studies that consider an extra type of interaction, namely "additive", when FICI value is between 0.5 and 1.0 (Trifan et al. 2021). The combinatory antifungal activity may also be determined by adding a specific concentration of drug A to different concentrations of drug B, generally obtained by serial dilution. FICI values can be obtained, but the number of combinations between the drugs is very limited (Fukuda et al. 2019). The concentration of drug B that presents synergism with drug A is designated as the combined MIC of this substance.

Another common method performed to evaluate the interaction between two drugs is

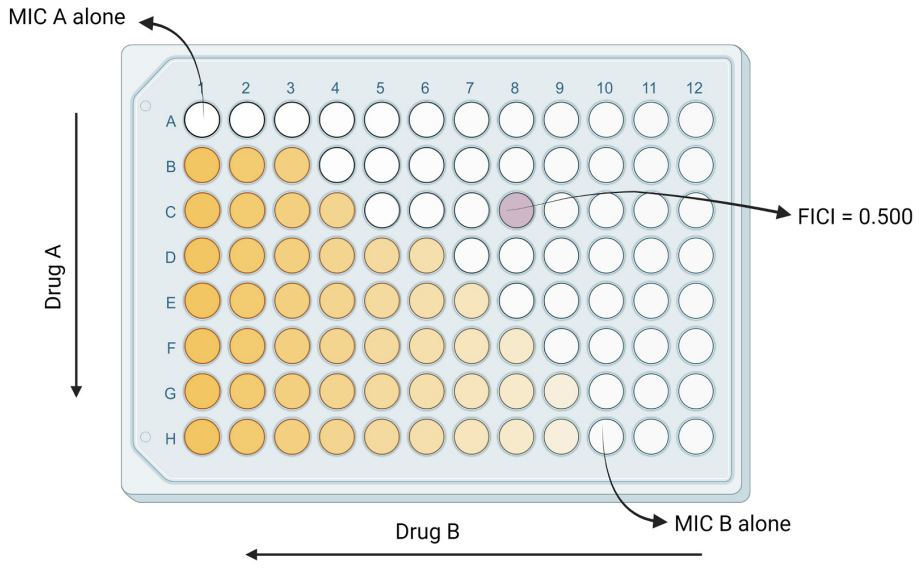

Figure 1. Checkerboard assay scheme. Drug A is vertically diluted from well A to well G within the columns, while Drug B is horizontally diluted from well 12 to well 2 within the rows. White and yellow wells represent no visible cell growth and visible cell growth, respectively. The purple well indicates the combined concentration of drugs A and B in which the FICI value is 0.500, the usual breakpoint used to classify a drug interaction as synergic. Created with BioRender.com.

the time-kill assay (Bidaud et al. 2021). In this experiment, two drugs (alone and combined) are incubated with the cells in a tube. Then, serial dilutions of these systems are carried out, and cells are spread onto the surface of a solid medium. After incubation during predetermined periods, colony forming units (CFU) are counted. If the treatment with the combined drugs decreases or increases cell concentration by ≥ $2\log_{10}$ CFU/ml (in comparison to the drugs alone), the interaction is classified as synergistic or antagonistic, respectively. Thus, time-kill assay measures the fungicidal effect of drugs combination through time. A disadvantage of this method is that testing several concentrations is laborious and, usually, only one concentration of each compound is tested (Figure 2). In order to overcome the time-consumption of this assay, an end-point measurement can be performed. Cells are incubated with the drugs alone and combined, and after a specific period CFU counting is done. In this case, the antifungal effect of the drugs is not monitored over time.

RESULTS AND DISCUSSION

Amphotericin B associated to natural products

Natural products have been used throughout history as therapeutical agents to treat a wide array of diseases. There are indications that 60.000 years BC Neanderthals might have used flowers for healing purposes (Lietava 1992). Nowadays, half of the drugs prescribed in industrialized countries present substances directly or indirectly obtained by natural products (Palmeira-de-Oliveira et al. 2013). Due to the extensive production of secondary metabolites, plants stand out as invaluable sources of bioactive compounds, such as the well-known molecules salicylic acid (used to obtain the anti-inflammatory drug acetylsalicylic acid), the analgesic morphine, and the cardiotonic digoxin (Rishton 2008). The high biodiversity of plants

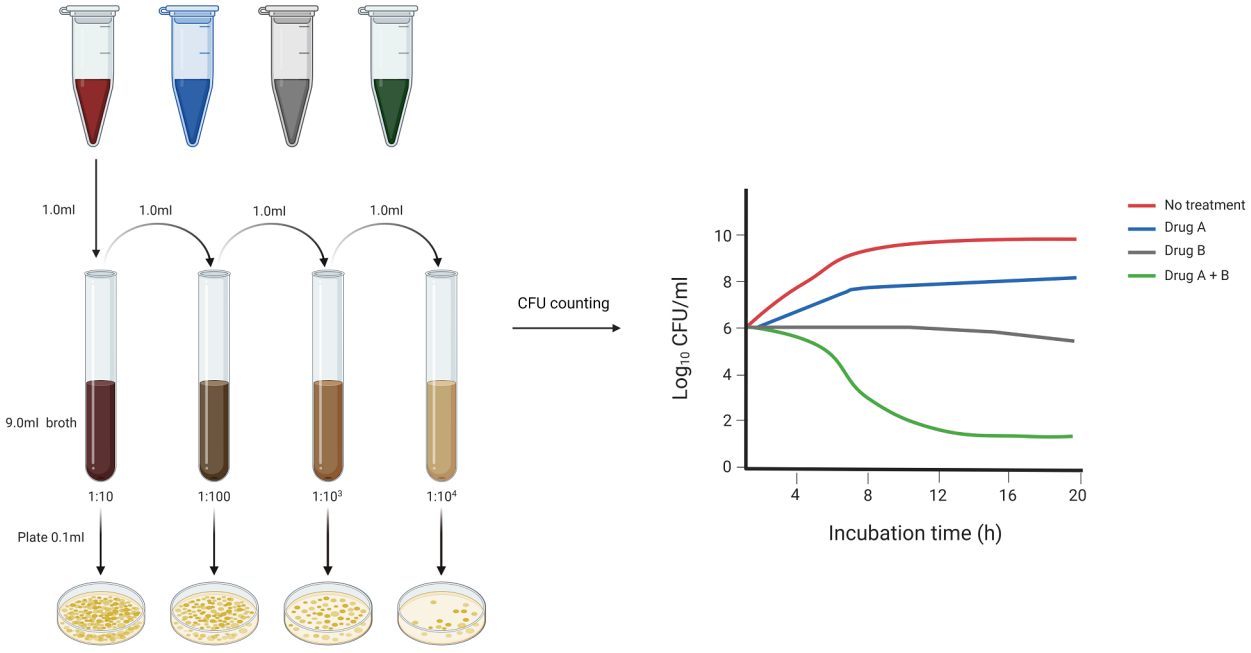

Figure 2. Time-kill assay scheme. Cells are incubated with the drugs, alone and combined, for predetermined periods, and then are spread onto solid medium to allow colony forming unit (CFU) counting. The result is usually expressed as a graphic that describes cellular concentration over time. Created with BioRender.com.

and their ability of producing several distincts molecules increases the possibility of finding substances capable of inhibiting fungal growth or enhancing the antifungal activity of another drug.

In order to survive in an environment that presents a limited amount of nutrients, bacteria and fungi may produce substances to kill each other (Kong et al. 2010). The first observation of this phenomena, known as antibiosis, was made by Fleming in 1929, which allowed the discovery of penicillin. Then, several antimicrobial agents have been extracted from microorganisms, such as bacitracin, streptomycin, polymyxins, and amphotericin B itself. Therefore, besides plants, microorganisms appear as attractive sources of antifungal or adjuvant agents to be used against fungal infections.

Below, this review describes substances obtained from plants or microorganisms that are able to enhance the antifungal activity of amphotericin B against *Candida* spp.

Amphotericin B associated to substances obtained from plants

Using a panel of 76 *C. albicans* strains, Zhu et al. (2021) observed that artemisinin, an antimalarial drug obtained from *Artemisia annua*, did not inhibit fungal growth alone. On the other hand, this compound at 25 µg/ml presented synergism against all the tested strains when combined to AMB. Interestingly, this combined activity was also seen in a murine oropharyngeal candidiasis model. Authors observed that artemisinin upregulated the expression of genes related to ergosterol biosynthesis, namely *ERG1*, *ERG3*, *ERG9*, and *ERG11*, therefore increasing the fungal ergosterol level and the binding of AMB to the plasma membrane (Zhu et al. 2021) (Table I).

Yamada et al. (2021) observed that the viability of *C. albicans* was significantly reduced after a 3-hour incubation with 50 µM benzyl isothiocyanate (BITC), a natural substance found in *Alliaria petiolata*. Further, this study verified that combining 0.15 µM (0.139 µg/ml) AMB to 40 µM BITC promoted vacuolar membrane disruption, leading to cell death possibly due to the release of hydrolytic enzymes (Yamada et al. 2021).

Silva et al. (2020) assessed the anti-*Candida* activity of the monoterpene enantiomers R-(+)-β-citronellol and S-(-)-β-citronellol, and observed MIC values of 32 - 256 µg/ml against *C. albicans* and *C. tropicalis* strains. Both compounds

Table I. Natural products obtained from plants with combined anti-*Candida* activity with amphotericin B, their active concentrations (alone and combined), the active concentration of amphotericin B combined to these substances and method to assess the combinatory effect.

Substance	Antifungal activity alone	Combinatory activity	Amphotericin B concentration	Method	Reference
Artemisinin	-	25 µg/ml	0.125 – 1 µg/ml	Checkerboard	Zhu et al. 2021
Benzyl isothiocyanate	50 µM	40 µM	0.15 µM	CFU counting	Yamada et al. 2021
Carvacrol	63 – 250 µg/ml	0.125 – 8 µg/ml	0.25 – 2 µg/ml	Checkerboard	Shaban et al. 2020
Citronellol	64 – 512 µg/ml	NI	NI	Checkerboard	Silva et al. 2020
Eugenol	625 µg/ml	156 µg/ml	0.05 µg/ml	Checkerboard, Time-kill assay	Khan et al. 2019
Hydroxychavicol	120 – 240 µg/ml	2 – 30 µg/ml	0.5 – 2 µg/ml	Checkerboard	Himratul-Aznita et al. 2016
Isoquercitrin	2.5 µg/ml	0.31 µg/ml	0.16 µg/ml	Checkerboard	Kim et al. 2019
Vanillin	125 µg/ml	NI	NI	Checkerboard	Saibabu et al. 2020

CFU – colony forming unit; NI – not informed.

presented synergism with AMB against the growth of two *C. albicans* strains, while indifference was observed when the combinations were tested against *C. tropicalis*. Considering that in the presence of exogenous ergosterol MIC values of the enantiomers increased 64 - 128 times, it may be hypothesized that the compounds bind to the plasma membrane ergosterol and increase its fluidity, enhancing AMB antifungal activity (Silva et al. 2020). More studies need to be conducted to clarify why synergism was not observed against *C. tropicalis*, and whether the combination display antifungal activity against other *Candida* species.

The phenolic compounds hydroxychavicol and isoquercitrin showed combined activity with AMB against *Candida* spp. Himratul-Aznita et al. (2016) observed that 2 - 8 µg/ml hydroxychavicol presented synergism with AMB against *C. albicans*, *C. parapsilosis*, *C. dubliniensis*, and *C. lusitaniae*, but the mechanism of the combinatory activity is unclear (Himratul-Aznita et al. 2016). Kim et al. (2019) observed synergism between isoquercitrin and AMB against a *C. albicans* strain. Combining the compounds promoted intracellular ROS accumulation, increased mitochondrial ROS production, and decreased superoxide dismutase activity. The consequent oxidative stress led to lipid peroxidation and membrane disruption, therefore causing cell death (Kim et al. 2019).

The phenolic aldehyde vanillin displayed antifungal activity alone against *C. albicans*, with MIC of 125 µg/ml. At 62.5 µg/ml, the compound decreased ergosterol levels by approximately 70%. Combining vanillin with AMB presented a remarkable synergistic interaction, with FICI value of 0.1. Vanillin was able to inhibit CaCdr2p, an efflux pump related to fluconazole resistance. Then, a synergistic effect when combined to this antifungal agent was also observed (Saibabu et al. 2020). Nonetheless, the mechanism involved with the interaction between vanillin and AMB needs to be clarified.

The terpenes carvacrol and eugenol also possess anti-*Candida* activity when combined to AMB. Carvacrol at 125 µg/ml presented antifungal activity alone against 25 strains of *C. auris*. At 4 µg/ml, carvacrol lowered AMB MIC of sensitive strains from 0.5 µg/ml to 0.25 µg/ml. Moreover, at 8 µg/ml, carvacrol led to a 4-fold decrease of resistant strains AMB MIC (Shaban et al. 2020). Eugenol presented weaker antifungal activity, with MIC value of 625 µg/ml against *C. albicans*. Combining 156.3 µg/ml eugenol to 2 µg/ml AMB completely inhibited cell growth. The synergism between the substances was also observed by the time-kill assay. The findings of this study indicate that eugenol blocks Ca^{2+} channel, impairing its influx and hence promoting ROS production, therefore enhancing AMB antifungal activity and leading the yeast to apoptosis (Khan et al. 2019).

Amphotericin B associated to substances obtained from microorganisms

Teixeira-Santos et al. (2016) observed that colistin, an antibacterial drug produced by *Bacillus colistinus*, does not display anti-*Candida* activity alone, but decreases AMB MIC value by 4 - 8 times. Colistin may enhance AMB activity due to the formation of a stable complex with this antifungal, which may lead to pore formation and membrane disruption (Teixeira-Santos et al. 2016) (Table II).

Rossi et al. (2020) observed that 0.025 - 0.1 mM erythromycin, an antibacterial drug produced by *Saccharopolyspora erythraea*, enhanced AMB activity against *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. auris*, *C. tropicalis*, and *C. krusei*. Moreover, the combination erythromycin + AMB did not show toxicity *in vitro* (against RAW264.7 macrophages) and *in vivo* (against zebrafish embryos) (Rossi et al. 2020). The *in vitro* efficacy

Table II. Natural products obtained from microorganisms with combined anti-*Candida* activity with amphotericin B, their active concentrations (alone and combined), the active concentration of amphotericin B combined to these substances and method to assess the combinatory effect.

Substance	Antifungal activity alone	Combinatory activity	Amphotericin B concentration	Method	Reference
Colistin	-	3 µg/ml	0.06 – 2 µg/ml	Combined MIC measurement	Teixeira-Santos et al. 2016
Erythromycin	-	0.025 – 0.1 mM	0.03 – 0.5 µg/ml	Checkerboard	Rossi et al. 2020
Myriocin	0.5 µg/ml	0.25 µg/ml	0.008 – 0.031 µg/ml	Checkerboard	Yang et al. 2021
Nectriatide	-	8 – 32 µg/ml	0.031 – 0.25 µg/ml	Combined MIC measurement	Fukuda et al. 2019
Phialotides	-	0.25 – 4 µg/ml	0.016 – 0.5 µg/ml	Combined MIC measurement	Yagi et al. 2020
Simpotentin	-	2 – 64 µg/ml	0.031 – 0.5 µg/ml	Combined MIC measurement	Ohtawa et al. 2019, Uchida et al. 2019

and *in vivo* safety of this combination point out to the potential of repurposed drugs to be used to treat *Candida* spp. infections.

Yang et al. (2021) observed that myriocin, an amino fatty acid produced by certain fungi, such as *Myriococcum albomyces*, inhibited the growth of 20 *Candida* strains, including *C. albicans*, *C. tropicalis*, *C. krusei*, *C. glabrata*, and *C. parapsilosis*, with MIC values ranging from 0.125 to 4 µg/ml. In the presence of ergosterol or phytosphingosine, myriocin MIC values against a *C. albicans* strain increased from 0.5 µg/ml to 8 µg/ml and >64 µg/ml, respectively. Using the fluorescent probe propidium iodide, it was observed that myriocin promotes membrane damage. The combination myriocin + AMB presented an additive interaction against *C. albicans* growth, which may be explained by the action of myriocin on plasma membrane. Although no synergism was observed, it is worth mentioning that combining both substances reduced AMB MIC against one of the tested strains by 128 times (Yang et al. 2021).

Fukuda et al. (2019) isolated the cyclotetrapeptide nectriatide from the fungus *Nectriaceae* sp., and also synthesized eight derivatives. These compounds did not present antifungal activity alone against *C. albicans* but, at 8 - 32 µg/ml, decreased AMB MIC from 0.5 µg/ml to 0.031 - 0.25 µg/ml. Using cells from human embryonic kidney (HEK293 cell line), authors observed IC_{50} values of 15 µg/ml and >128 µg/ml for AMB and nectriatide, respectively. Moreover, 32 µg/ml nectriatide did not change AMB IC_{50} against these cells, but decreased AMB MIC from 0.5 µg/ml to 0.031 µg/ml against *C. albicans*, pointing out the selectivity of the combination to this yeast (Fukuda et al. 2019). HEK293 lineage is an interesting model of study to evaluate AMB toxicity, since it consists on kidney-derived cells, and the most relevant adverse effect of AMB is nephrotoxicity.

From the rare fungus *Pseudophialophora* sp., Yagi et al. (2020) isolated eight phialotides that do not impair fungal growth alone, but enhance AMB activity against *C. albicans*. At 4 µg/ml, all the compounds reduced AMB MIC from 0.5 µg/ml to 0.031 µg/ml (Yagi et al. 2020). Further studies must be carried out to assess whether these compounds may be used as AMB potentiators.

Uchida et al. (2019) purified a novel compound, namely simpotentin, from the fungus *Simplicillium minatense*. This substance did not inhibit *C. albicans* growth at the tested concentrations. However, 64 µg/ml simpotentin decreased AMB MIC from 0.5 µg/ml to 0.0625 µg/ml. This compound is structurally related to

rhamnolipids. Therefore, it may enhance AMB activity by interacting with plasma membrane. Interestingly, simpotentin led to an 8-fold decrease of AMB MIC against *C. albicans*, and did not change AMB IC_{50} against HEK293 cells, showing that the compound potentiate the antifungal activity of AMB without increasing its toxicity (Uchida et al. 2019). Ohtawa et al. (2019) synthesized eight simpotentin stereoisomers and observed that all of them were able to diminish AMB MIC against *C. albicans*. Data revealed that the isomer (3R,5S,13S) was the most active, decreasing AMB MIC from 0.5 µg/ml to <0.0313 µg/ml (Ohtawa et al. 2019).

Amphotericin B associated to synthetic substances

The development of the synthetic organic chemistry in the last century allowed the obtention of drugs completely built in laboratories, such as the antifungal agent fluconazole. It is possible to generate a group of derivatives from a core molecule in order to evaluate which compound possesses the greatest pharmacological activity and toxicity profile. A possibility is to evaluate the ability of these derivatives in enhancing the antimicrobial activity of another compound, and compare the results obtained to determine the most active substance. For example, FICI values can be compared between analogue molecules to understand which modifications emerge as the most promising ones. In this context, some studies tested the combinatory effect of synthetic substances and amphotericin B against *Candida* spp, as depicted below.

Sixteen alkylated tetrahydro-β-carboline derivatives were synthesized from L-Tryptophan by Singh et al. (2019) and had their anti-*Candida* activity evaluated. One compound, namely 12c, presented antifungal activity alone, with MIC values ranging from 3.67 to 14.8 µg/ml against six *Candida* species. Moreover, 12c at 0.46 µg/ml presented synergism with AMB against *C. glabrata*, leading to a 11-fold decrease of AMB MIC. Scanning electron microscopy showed that 12c affects fungal cell surface. Further studies need to be conducted to clarify whether this compound targets plasma membrane and/or cell wall (Singh et al. 2020) (Table III).

Patil et al. (2020) synthesized three arginine-based fatty acids named arginolipids, and evaluated their anti-*Candida* activity against 21 strains of different species. Besides being able to inhibit *Candida* growth alone, one of the substances (oleoyl arginine ethyl ester, or OAEE) was synergic with fluconazole and AMB against all strains tested. It was observed that OAEE alone depolarizes and damages plasma membrane, which in turn may favor the antifungal activity of fluconazole and AMB. Moreover, complexation between OAEE and AMB may fluidize plasma membrane, enhancing AMB antifungal activity (Patil et al. 2020).

Lazić et al. (2018) synthesized four bis-guanylhydrazones, and observed MIC values from 2 to 15.62 µg/ml against *C. albicans*, *C. glabrata*, and *C. parapsilosis*. BG3, the most active compound, synergized with AMB, but not with nystatin, another polyene drug. Also, BG3 induced ROS production and depolarized mitochondrial membrane potential at high concentrations. These mechanisms could explain the synergism with AMB, however they were not observed at sub-inhibitory concentrations (Lazić et al. 2018).

A group of cationic steroidal antimicrobials, namely ceragenins, was synthesized by Bozkurt-Guzel et al. (2018), and the anti-*Candida* activity of five compounds (CSA-8, CSA-13, CSA-44, CSA-131, and CSA-138) was evaluated. All the compounds presented antifungal activity alone, with MIC values ranging from 0.25 µg/ml to 128 µg/ml. Since CSA-8 was the less active compound, it was not tested in combination to AMB. The other four ceragenins presented synergism with AMB

Table III. Synthetic compounds with combined anti-*Candida* activity with amphotericin B, their active concentrations (alone and combined), the active concentration of amphotericin B combined to these substances and method to assess the combinatory effect.

Substance	Antifungal activity alone	Combinatory activity	Amphotericin B concentration	Method	Reference
Alkylated Tetrahydro-β-carboline derivatives	3.67 – 14.8 µg/ml	0.46 µg/ml	0.065 µg/ml	Checkerboard	Singh et al. 2019
Arginolipid	15.25 – 62.5 µg/ml	1.95 – 15.62 µg/ml	0.01 – 1 µg/ml	Checkerboard, Time-kill assay	Patil et al. 2020
Bis-guanylhydrazones	2 – 15.6 µg/ml	NI	NI	Checkerboard	Lazić et al. 2018
Ceragenins	8 – 512 µg/ml	NI	NI	Checkerboard	Bozkurt-Guzel et al. 2018
Fingolimod	-	1.38 µg/ml	0.04 µg/ml	Checkerboard	Wei et al. 2021
Flucytosine analogues	1 – 500 µg/ml	NI	NI	Checkerboard	Wani et al. 2017
Fluphenazine	-	0.78 µg/ml	0.0625 µg/ml	Checkerboard	Lu et al. 2019
Imipramine	40 µg/ml	NI	NI	Checkerboard	Caldara & Marmiroli 2018
LMM6	8 – 32 µg/ml	NI	NI	Checkerboard	Faria et al. 2021
Nortriptyline	50 µg/ml	NI	NI	Checkerboard	Caldara & Marmiroli 2018
Promethazine	64 µg/ml	8 – 16 µg/ml	0.0625 – 0.125 µg/ml	Checkerboard	Brilhante et al. 2018
Schiff base derivatives	9 – 625 µg/ml	NI	NI	Checkerboard	Malik et al. 2018

NI – not informed.

against some of the strains tested. For example, the most active compound (CSA-13) was synergic with AMB against 22 out of the 50 strains tested (Bozkurt-Guzel et al. 2018). More studies must be conducted to evaluate the applicability of ceragenins either as antifungal or as adjuvant agents in candidiasis treatment.

In a study conducted by Lu et al. (2019), the antipsychotic drug fluphenazine did not present anti-*Candida* activity alone at concentrations below 25 µg/ml. However, at 0.78 µg/ml, this substance decreased AMB MIC from 0.25 µg/ml to 0.0625 µg/ml. Treating *C. albicans* with 3.13 µg/ml fluphenazine for 1 hour increased *ERG11* expression, therefore explaining the synergism between this substance and AMB. Interestingly, the combination fluphenazine + AMB downregulated the expression of *ALS3* and *HWP1*, genes that encode surface proteins involved in *C. albicans* virulence. Using a immunocompromised mouse model of *C. albicans* disseminated infection, authors observed that combining fluphenazine and AMB enhanced the survival rate and body weight of the animals, and decreased kidney and brain fungal burden (Lu et al. 2019).

Caldara & Marmiroli (2018) assessed the anti-*Candida* activity of the tricyclic antidepressants doxepin, imipramine, and nortriptyline. The MIC values obtained against *C. albicans* were 200 µg/ml, 40 µg/ml, and 50 µg/ml, respectively. At concentrations that inhibit 10% (MIC_{10}) and 75% (MIC_{75}) of cell growth, imipramine and nortriptyline decreased AMB MIC at 32 - 450 times and 40 - 900 times, respectively, with FICI values ranging from 0.001 to 0.032, pointing out to their potential

use as potentiators of AMB activity (Caldara & Marmiroli 2018).

Faria et al. (2021) evaluated the antifungal activity of the synthetic 1,3,4-oxadiazole derivative 4-[cyclohexyl(ethyl)sulfamoyl]N[5-(4-fluorophenyl)-1,3,4-oxadiazol-2-yl]benzamide, also known as LMM6, and observed MIC values of 8 - 32 µg/ml against *C. albicans*. The combination LMM6 + AMB presented synergistic interaction against *C. albicans*, with FICI values ranging from 0.531 to 0.75 (Faria et al. 2021). It is interesting that results obtained by checkerboard assay may have different interpretations. Results obtained by checkerboard assay may have different interpretations. Based on the FICI values found in this study, interaction would be classified as "additive" or "indifferent" if one consider distinct breakpoints. Therefore, this substance could be discarded even with a FICI of 0.531, that is close to 0.500 (the FICI value that is often used as synergism breakpoint). Thus, assuming strict interpretations based solely on FICI values may not be advised.

Wani et al. (2017) synthesized four pyrimidinone/thione analogues of the antifungal drug flucytosine, namely BG1, BG2, BG3, and BG4, and obtained MIC values of 1 - 500 µg/ml against eight *C. albicans* strains. When associated to AMB, BG4 was the most promising substance, since the combination was synergic against 7 out of the 8 strains tested (Wani et al. 2017). BG4 was also the most active analogue when tested alone. These results show that using old antifungal agents as scaffolds to the development of new ones may be a proper strategy to overcome fungal infections.

Wei et al. (2021) assessed the anti-*Candida* effect of fingolimod, a derivative of the natural product myriocin. This study observed that fingolimod does not inhibit *C. albicans* growth alone, but enhances AMB activity. Combining 1.38 µg/ml fingolimod with 0.04 µg/ml AMB presented a synergic interaction against *C. albicans*, with a FICI value of 0.19. This combination also impaired the growth of *C. glabrata* and *C. tropicalis* strains. Since myriocin affects *Candida* plasma membrane, further studies must be conducted to unveil whether fingolimod also targets this structure, in order to clarify the mechanism of synergism with AMB (Wei et al. 2021).

The anti-*Candida* effect of the antihistamine drug promethazine has also been investigated (Brilhante et al. 2018). This substance inhibited *C. tropicalis* growth alone, with MIC value of 64 µg/ml and, at 8 µg/ml, decreased AMB MIC by 4 to 8 times. This study also observed that 64 µg/ml promethazine damaged plasma membrane and depolarized mitochondria. Although these effects justify the antifungal activity of this drug, the mechanism of synergism with AMB remains unclear, since the combinatory activity was achieved with lower concentrations of promethazine.

Malik et al. (2018) synthesized and evaluated the antifungal activity of seven novel Schiff base derivatives against ten *C. albicans* strains. These compounds exhibited MIC values from 6 to 1250 µg/ml. Only 2 out of the 7 substances did not present synergism with AMB against *C. albicans*. Data show that the compounds at MIC/2 and MIC decreased ergosterol content by 10 - 97%. The most active compound, SB5, may impair ergosterol biosynthesis by binding to lanosterol 14α-demethylase (Malik et al. 2018). It must be noticed that decreasing ergosterol amount in plasma membrane diminishes AMB affinity but may increase membrane fluidity, enhancing AMB antifungal activity (Mukhopadhyay et al. 2002). Further studies using lower concentrations of the Schiff base derivatives, alone and combined to AMB should be performed to enlighten the mechanisms involved in their combinatory antifungal activity.

CONCLUSION

The development of resistance against azoles and echinocandins by *Candida* spp. and the adverse effects related to the use of AMB jeopardize antifungal therapy. Then, it is essential that new treatments be created in order to achieve the desired therapeutical success.

Among the compounds described in this study, isoquercitrin, myriocin, alkylated tetrahydro-β-carboline derivatives, and fluphenazine appear as the most promising candidates to be used in combination with AMB, when considered only their active concentrations. Unfortunately, some studies did not report the concentration of the drugs that were synergic with AMB, therefore precluding a more accurate analysis. Moreover, only three studies assessed the toxicity of AMB combined to a second drug. The mechanisms related to the anti-*Candida* activity of these combinations are mostly related to the plasma membrane, which is also the target of AMB. Therefore, toxicity determination is essential to ensure that the combinations are suitable for clinical use. *In vitro* experiments may be employed as screen methodologies to choose the less toxic combinations towards mammalian cells, using viability dyes such as tetrazolium salts (Wijesinghe et al. 2021). Then, *in vivo* assays should be performed to measure the efficacy and toxicity of the combinations in more complex organisms. Finally, the most promising combinations may be submitted to clinical trials, in order to allow their use to treat fungal infections.

It may be highlighted that artemisinin, colistin, erythromycin, fluphenazine, imipramine, nortriptyline, and promethazine are already approved for human use to treat other diseases. Repositioning "old" substances is an important approach to accelerate drug discovery. However, as aforementioned, clinical trials must be performed to assure the suitability of the combinations as antifungal drugs.

In summary, this review shows that combining AMB to a second substance may be a promising strategy to treat patients with candidiasis. Further studies regarding mechanism of action, pharmacokinetics and toxicity parameters must be conducted to confirm the role of these substances as adjuvant agents in candidiasis therapy.

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