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REVIEW

Antifungal drugs combinations: a patent review 2000-2015

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ABSTRACT

Introduction: Combination therapy has emerged as an approach to improve the efficacy of antifungal drugs. Its main objective is to achieve synergistic interaction with higher antifungal properties and lower toxic effects than each substance alone.

Areas covered: Twenty-four patents disclosed in the period of 2000-2015 were covered in this review. Twenty of them were devoted to pharmacodynamic potentiation, while four were dedicated to pharmacokinetic actions.

Expert opinion: The common characteristic of most patents published in this area is that the main partner is a commercial antifungal drug. In the most innovative combinations the second component was either a modifier of proton homeostasis, an antibody, an inhibitor of the adhesion of epithelial or endothelial cells or a keratinolytic agent that improves the skin penetration. The evaluation of synergism is always made with simple *in vitro* methods, which constitutes a weakness of the disclosed patents, due to the lack of *in vivo* studies, since the *in vitro* tests cannot predict the *in vivo* behavior. Also, it is surprising that none of the patents analyze the toxicity of the new combinations, taking into account that one of the main objectives of the combinations is to reduce the toxicity of the existing antifungal drugs.

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Antifungal; combination; synergism; mycoses

1. Introduction

1.1. Fungal infections

Fungal infections have increased significantly in recent years and this fact is strongly related to the rising number of immunocompromised patients.[1,2] Most prevalent mycoses belong to one of the following categories: superficial, in which the fungus is confined to the outer layers of the skin, and systemic, in which the fungus affects internal organs of the body.[3] Of them, superficial fungal infections are caused by fungi of the genera *Epidermophyton, Microsporum, Trichophyton, Candida*, and *Malassezia*, while systemic fungal infections are mainly produced by *Candida* or *Aspergillus*, *Cryptococcus neoformans*, and by the emerging pathogens of the genera *Trichosporon, Mucor, Rhizopus, Fusarium*, and *Scedosporium*.[4–8]

1.2. Antifungal drugs in clinical use: advantages and drawbacks

Among the different drugs for treating mycoses, polyenes, allylamines, azoles, and lipopeptides are the most commonly used.

The polyenes amphotericin B (AmpB, **1**) and nystatin **2** (Figure 1) act by joining to the ergosterol in the fungal membrane. Of them, **1** in its deoxycholate form is the most commonly used drug to treat invasive mycoses,[9] However, dose-related nephrotoxicity is well documented and limits its widespread use in clinical practice. To overcome this drawback, lipid formulations of **1** such as liposomal AmpB (LAmpB), AmpB lipid complex (ABLC), and AmpB colloid dispersion (ABCD) were further developed; however, renal toxicity still persists at high cumulative doses.[9]

The allylamines, such as terbinafine **3**, naftifine **4**, and butenafine **5** (Figure 1) target the squalene epoxidase enzyme in the ergosterol biosynthesis pathway and are mainly used to treat superficial fungal infections.

Azoles, the largest class of antifungal agents in clinical use, inhibit the cytochrome P-450-dependent enzyme lanosterol-14α-demethylase. Among them, the first developed imidazoles (e.g. ketoconazole **6**, miconazole **7**, elubiol **8**, and clotrimazol **9**; Figure 1) are generally used for superficial fungal infections and the more recently developed triazoles are the most commonly used drugs for invasive fungal infections. The triazoles can be further classified into first-generation (e.g. fluconazole **10** and itraconazole **11**; Figure 1) and second-generation triazoles (e.g. voriconazole **12**, posaconazole **13**, and ravuconazole **14**; Figure 1).[10] Other antifungal drugs in clinical use are griseofulvin **15** (Figure 2), an antifungal agent useful for superficial mycoses that binds to tubulin and inhibits spindle formation during mitosis, and 5-fluorocytosine (5-FC) **16** (Figure 2), that interferes with ribonucleic acid (RNA) biosynthesis and disturbs the building of certain essential proteins.

The echinocandins caspofungin **17**, micafungin **18**, and anidulafungin **19** (Figure 2) are lipopeptides derived from

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Article highlights

- Combination antifungal therapy has emerged as an alternative to monotherapy for patients that fail to respond to standard antifungal treatments.
- Antifungal synergy is achieved with two compounds acting on different fungal targets or with one compound increasing the penetration of an antifungal agent, thus helping to reach the site of action.
- We review here 24 patents disclosed from 2000 to 2015 comprising new antifungal combinations to treat human fungal infections. The common characteristics of the published patents were as follows: (a) at least one partner was a commercial antifungal drug, and (b) the evaluation of synergism was always made with simple methods (mainly disk diffusion and checkerboard).
- The soil fungus metabolite (+)-FKI-0076 **22** (a 2-derivative of methyl 3,5-dimethoxybenzoate) appears as a promising partner for combination with azole antifungal drugs.
- The most innovative patented combinations dealing with the pharmacodynamic enhancement of antifungal effects comprise either a modifier of proton homeostasis or an antibody as the partner of the antifungal drug.
- Patents dealing with pharmacokinetic interactions contain either an inhibitor of the adhesion of epithelial or endothelial cells or a keratinolytic agent as the partner of the antifungal drug.

This box summarizes key points contained in the article.

natural fungal compounds that act by specific and noncompetitive inhibition of (1,3)β-D-glucan synthase, enzymatic complex that is essential for the biosynthesis of glucan polymers by the fungal cell wall.[11–13]

In spite of the several antifungal agents in clinical use, there is no drug that meets all the desirable requirements t since all have notable drawbacks. For example, in addition to its nephrotoxicity, **1** has two main drawbacks: it must be used intravenously in its conventional form and it is very expensive in its new lipidic forms. Regarding the azoles, they are currently used as first-line antifungal drugs [14] because of their excellent oral bioavailability, their stable parenteral formulations, and their low toxicity. However, resistance to azoles has emerged in clinical isolates from immunocompromised patients, probably due to the fact that they are fungistatic rather than fungicide and need long periods of time to eradicate the fungal infections.[15] In addition, azoles can produce adverse effects [16] and since they are inhibitors of the cytochrome P-450 family CY3A4,[17] they cause adverse interactions with immunosuppressants, chemotherapeutics drugs, benzodiazepines, tricyclic antidepressants macrolides, and selective serotonin reuptake inhibitors. Therefore, new approaches for treating these infections are highly needed.

1.3. Combination therapy

Combination therapy has emerged as an approach to improve the efficacy of antimicrobial therapy for difficult-to-treat infections.[18] The combination of two or more antifungals [19,20] or of an antifungal drug with a non-antifungal compound, has been tested on an empirical basis, with the aim of coping with treatment failures.[21]

The main advantages of an antifungal combination are to achieve a synergistic antifungal effect with lower quantities of each of the components, also lowering the toxic side effects and the appearance of fungal resistance.[22–24] However, they can also produce lower effects than the expected (antagonism) or an effect that is the sum of the actions of each drug when used alone (additivism or indifference).[25,26]

The pharmacodynamic interactions of drugs (study of biochemical and physiological effects and their mechanisms of action at an organ as well as at a cellular level) were traditionally the focus of studies of antimicrobial combinations, but pharmacokinetic interactions, affecting the amount, rate, and *ratio* of drug concentrations achieved at the site of infection, are also of great importance for the effect of a drug.[27]

1.3.1. Assessment of the type of interaction

There are many *in vitro* as well as *in vivo* methods to assess the type of interaction of a combination. However, it is worth to take into account that the *in vitro* tests do not predict the *in vivo* behavior.[28]

Regarding the *in vitro* tests, it is quite common that they give contradictory results. With the same set of data, a method can arrive to the conclusion that a mixture is synergistic while the other can lead to antagonistic results.[28,29] In addition, the type of interactions do not always coincide when different fungal species of the same genera, or diverse strains of the same species, are tested.[28,30]

Another important issue is that a combination of two compounds may act synergistically within one dose range while also showing antagonism within another.[31]

From the above considerations, it is clear that the interaction effects between two agents depend on many factors that must be taken into account for the design of the study. It is also important to consider that synergism is not the same concept of enhancement or potentiation. According to Chou, [32] synergism (or antagonism) needs two active drugs (it is 'mutual'), whereas enhancement or potentiation is 'one-sided' (one of the drugs has no effect). So, to determine if this enhancement constitutes synergy, a well-designed method should be used. The most utilized and also the simplest designs to detect *in vitro* interactions between two substances are the following: the qualitative disk diffusion method, the checkerboard design, the construction of isoboles, and the time-kill studies. However, several more sophisticated designs that give more reliable information have been developed. Most of them define an index that is useful to interpret the obtained results and to define the type of interaction.[33]

Since only simple *in vitro* assays are used in the patents covered by this review, only such tests were selected to be described below.

1.3.1.1. Disk diffusion assay. The disk diffusion assay can be performed following two designs: (a) two disks are placed at a distance of 20 mm from each other (center to center) and after a suitable incubation time, an inhibition halo is formed between both disks if a synergistic effect is present [34]; (b) two sterile paper disks are embedded, each with one of the drugs alone and a third disk is impregnated with a sample containing a mixture of both drugs. The diameter of the inhibition halo around each disk is measured after an incubation time according to the fungal growth (Figure 3).[35]

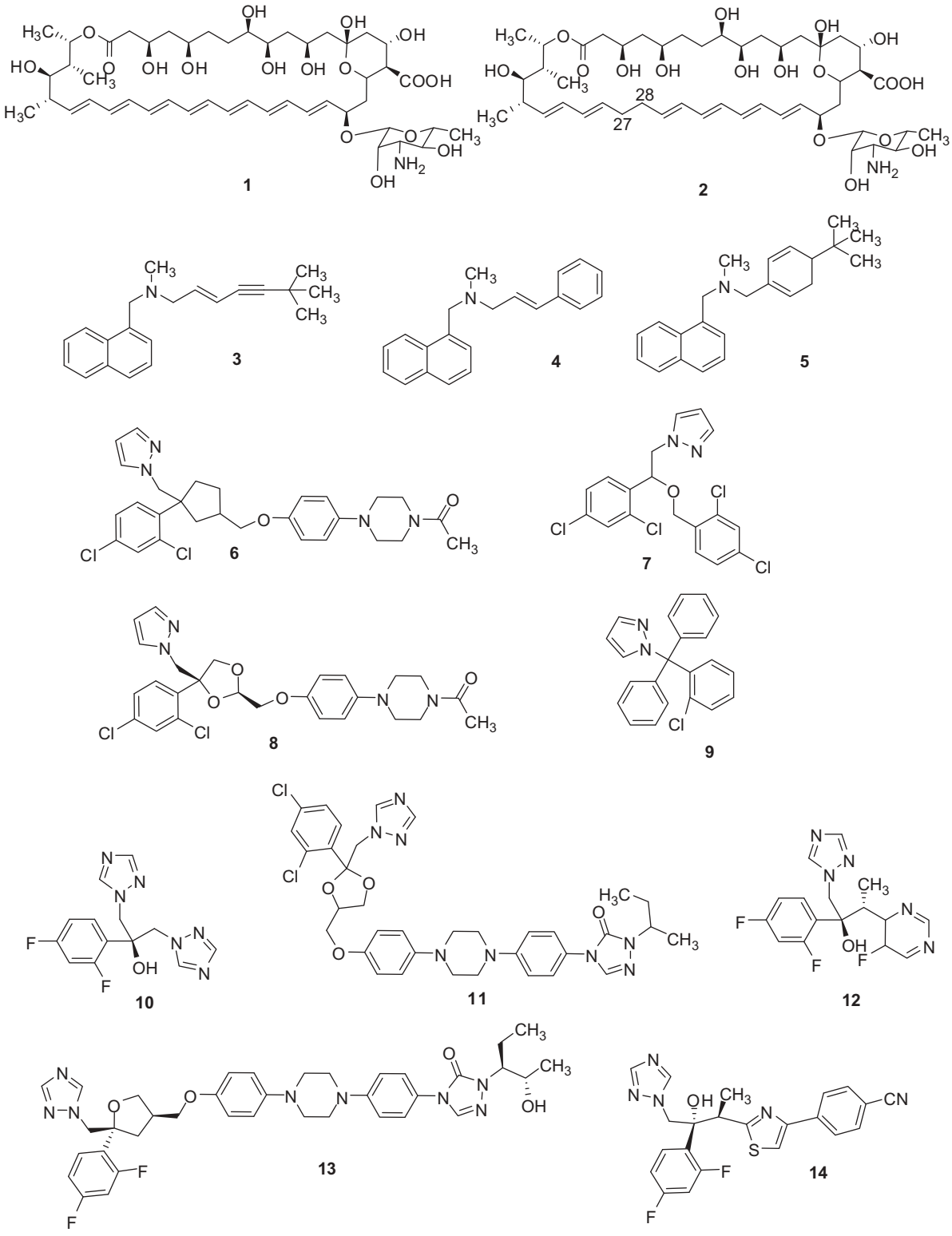


Figure 1. Structures of compounds 1 to 14.

1.3.1.2. Checkerboard design. This assay is performed in 96-well microplates (Figure 4) in which each row and each column contains twofold serial dilutions of substances X and Y, respectively, at concentrations ranging from 0 to slightly higher than their minimum inhibitory concentration (MIC), reaching a unique combination of the two substances in each well. Then, a quantified inoculum of the fungus is added to each well and the microplate is incubated at a proper temperature during a suitable time for each fungus. The concentrations of the first wells without visible growth along the stepwise boundary between inhibition and growth were used to calculate the Fractional Inhibitory Concentration Index (FICI).[36]

The FICI is calculated by the sum of the values of Fractional Inhibitory Concentration (FIC) (Equation (1)) that is defined for

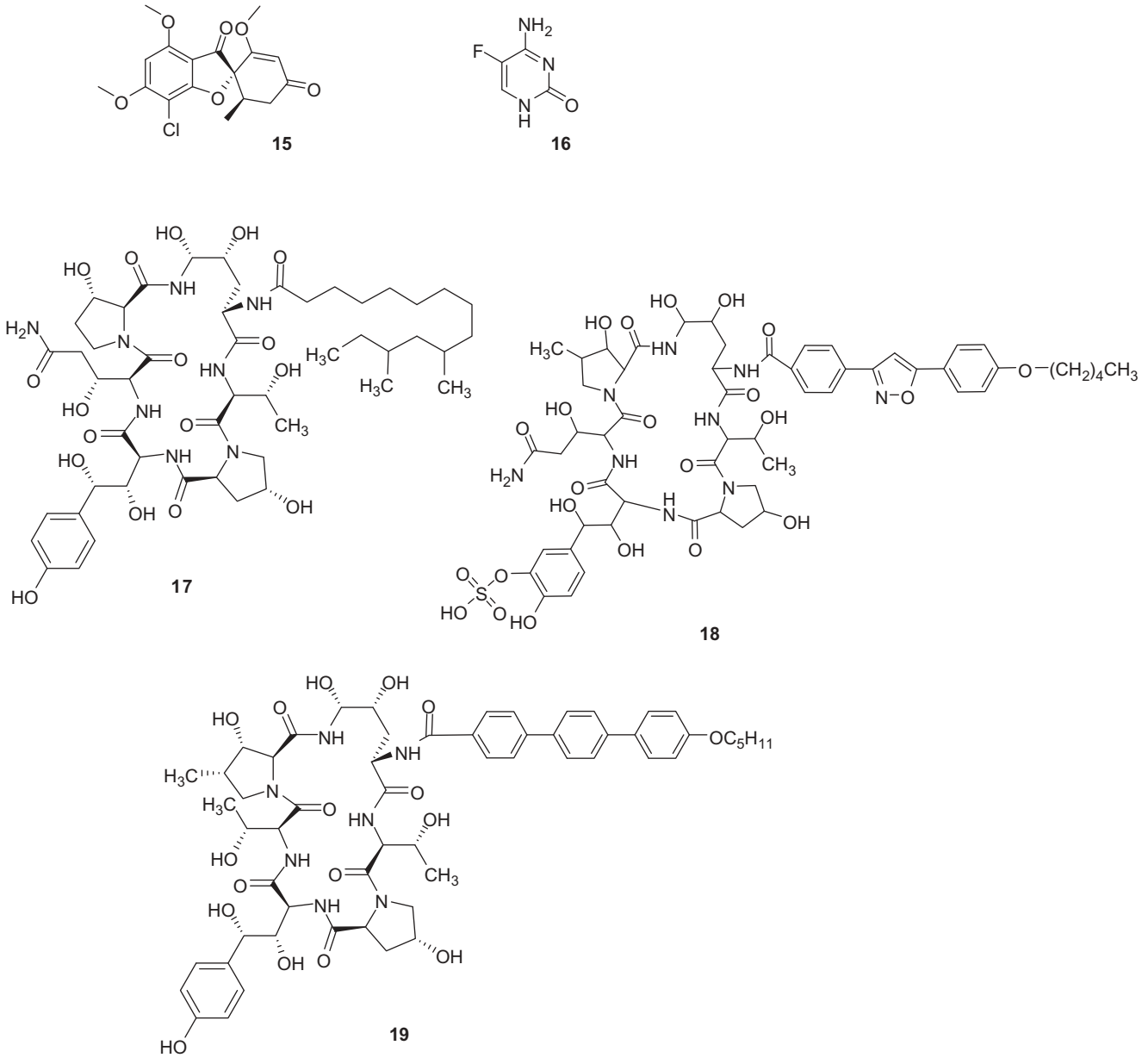


Figure 2. Structures of compounds **15** to **19**.

X as MIC of X in combination divided by MIC of X alone, and for Y as MIC of Y in combination divided by MIC of Y alone.

$$\mathrm{FICI} = \mathrm{FIC_X} + \mathrm{FIC_Y} = \frac{\text{MIC X in combination}}{\text{MIC X alone}} + \frac{\text{MIC Y in combination}}{\text{MIC Y alone}} \quad (1)$$

According to Odds, a FICI ≤ 0.5 is indicative of synergism; a FICI > 4.0 indicates antagonism and a FICI in the range of >0.5–4.0 is indicative of no interaction.[36] However, other authors consider other limits for FICI, and also describe the results that are neither synergistic nor antagonistic by using different terms such as 'additive', 'summation', 'no interaction', and 'indifference'.[29,37]

1.3.1.3. Isobolograms. An isobole is an 'iso-effect' curve (Figure 5),[38] obtained in a two-dimensional graphic [28] in which the *x* and *y* axes represent $\mathrm{FIC_X}$ and $\mathrm{FIC_Y}$ (obtained in the checkerboard assay), respectively. The line connecting the MICs of both compounds represents the line of no interaction. Synergistic mixtures fall below the line of indifference (FICI ≤ 0.5) and antagonistic ones fall above the line of no interaction (FICI ≥ 4).[39]

1.3.1.4. Time-kill studies. The time-kill method is used to get information about the time-dependent progression of the antimicrobial activity. In this method, synergy is defined as a 100-fold or 2-$\log_{10}$ decrease in colony count at 24 h produced by the combination, compared with the line produced by the most active single agent.[40] Figure 6 shows a time-kill graphic of a synergistic combination of A and B.

1.3.1.5. Other methods to assess synergism. Among the several sophisticated methods proposed for the analysis of

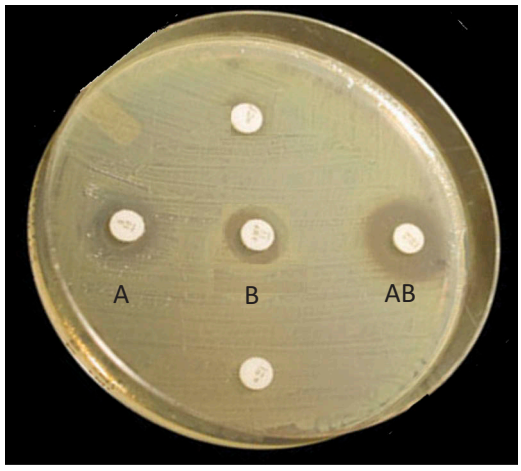


Figure 3. One of the methods using disk diffusion assay for the qualitative assessment of synergism. A: compound A alone; B: compound B alone; AB: both compounds in a sole disk.

interactions between agents [25,32,41–44] the method of Chou and Talalay [45] has become very popular in recent years. At first, a complete dose–response curve for each drug alone must be obtained, which should fit with the so-called 'median effect equation'

$$f_a/f_u = [(D)/(D_m)]^m (\text{where } f_a + f_u = 1) \quad (2)$$

in which the *ratio* of the fraction affected (*f*a) versus the fraction unaffected (*f*u) is equal to the dose (D) versus the median-effect dose (D_m) to the *m* power. D_m signifies potency and *m* signifies the sigmoidicity (shape) of the dose–effect curve. The combination index (CI) was introduced within the median-effect method.[32] Its characteristics are as follows: (i) it assesses the data from single ray fixed-*ratio* experiments, (ii) allows the identification of the optimal concentration (within the fixed-*ratio*) that will give the maximum synergy, and (iii) presents the results in a graphical form.

The CI mathematical expression for two drugs D_1 and D_2 that uses the median effect equation is detailed in Equation (3).

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} = \frac{(D)_1}{(D_m)_1[f_a/(1-f_a)]^{1/m_1}} + \frac{(D)_2}{(D_m)_2[f_a/(1-f_a)]^{1/m_2}} \quad (3)$$

$(D_x)_1$ is the concentration of D_1 alone that inhibits a system by *x*%, and $(D_x)_2$ is the concentration of D_2 alone that inhibits a system by *x*%, whereas in the numerator, $(D)_1$ and $(D)_2$ are the concentrations of D_1 or D_2 that, in combination, also inhibit *x* %. The CI value quantitatively defines the interaction as follows: CI < 1 synergism, CI = 1 additive effect, and CI > 1 antagonism.

A further improvement for this method is the MixLow design [44] which has the advantage over the median-effect design. It allows for the statistical comparison of the combinations' effects by providing accurate parameters of the dose–response curves and confidence intervals.

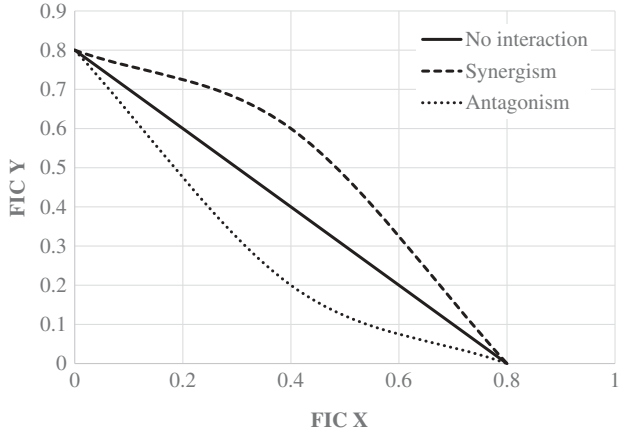


Figure 5. Isobologram.

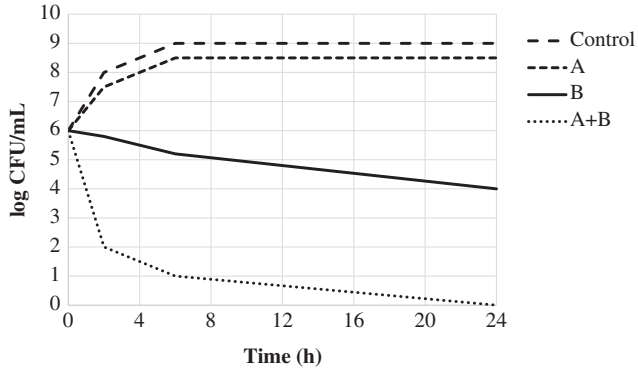


Figure 6. Time-to kill representation of A and B alone and its synergistic mixture.

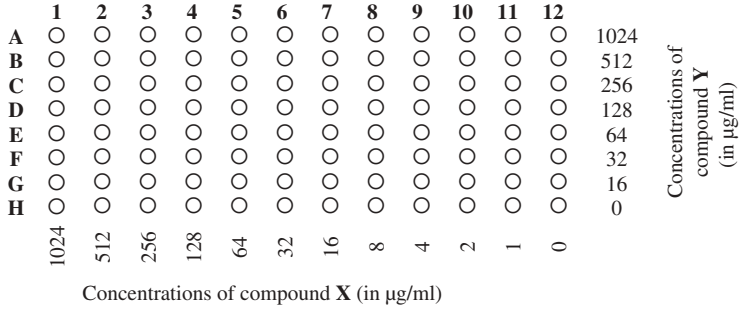


Figure 4. Scheme of a 96-well microplate used for the checkerboard assay.

2. Patents review

In view of the advantages of the combination therapy, we include in this review 24 patents on combinations for antifungal use that have been disclosed in the period 2000–2015. We organized the published patents according to the two mechanisms of action that were specified by the inventors in their claims: (1) pharmacodynamic potentiation and (2) pharmacokinetic enhancement of the antifungal effects.

2.1. Patents including pharmacodynamic potentiation of the antifungal effects

2.1.1. Combinations of two commercial antifungal drugs

Astellas Pharma Inc. published in 2008 [46] an invention comprising two known antifungal agents of either a polyene (**1** or **2**) and/or azole type (**6**–**14**), that, as stated above (Figure 1), target the fungal membrane. They were combined with another antifungal agent of lipopeptide structure that targets the fungal cell wall. The lipopeptide will have the general formula **20** wherein R_1 is an acyl group, R_2 and R_3 are H, OH, or a salt thereof, and can also include one or more optical or geometric isomers or mixtures of them (Figure 7). Both compounds can be administered simultaneously, separately, or sequentially.

The combination is claimed to be effective against fungi of the genera *Aspergillus, Candida, Fusarium*, dermatophytes, and many others, and to be synergistic, which was quantified with the FICI. As a positive example of the invention, the lipopeptide micafungin **18** (Figure 2) along with either AmpB **1**, itraconazole **11** (Figure 1), or nikkomycin **21** (Figure 7) showed synergism against *Aspergillus fumigatus* with FICIs of 0.50, 0.50, and 0.28, respectively.

2.1.2. Combination of azoles with the natural 3,5-dimethoxybenzoate derivative (+)-FKI-0076

Based on the previously demonstrated ability of the *Talaromyces flavus* soil fungus metabolite (+)-FKI-0076 **22** (International Union of Pure and Applied Chemistry [IUPAC] name: methyl 2-(6-(2-acetoxypropyl)-4-oxo-4H-pyran-3-

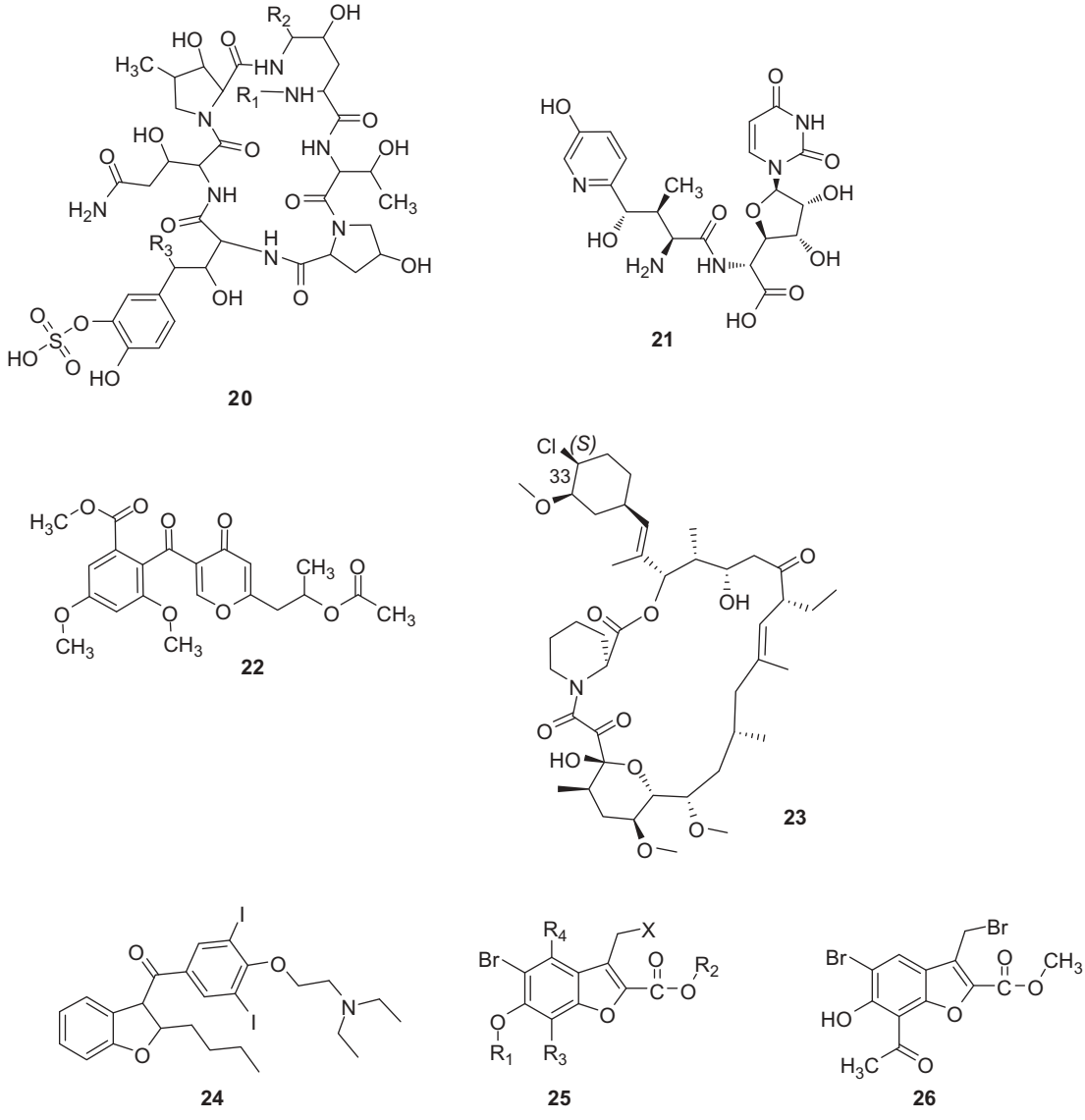


Figure 7. Structures of compounds **20** to **26**.

carbonyl)-3,5-dimethoxybenzoate) (Figure 7) for enhancing the antifungal activity of azoles,[47] the Kitasato Institute (Japan) patented in 2004 a process for its production. The method comprises the culture of *T. flavus* in a special liquid medium (described therein) that allows a particularly high concentration of the fungal metabolite **22**, and thus the isolation of a good amount of the compound from the mycelia and from the filtrate afterward.[48] The enhanced activity of azoles was demonstrated with the disk diffusion assay in the presence of **22** (Figure 7).

2.1.3. Combination of an antifungal agent targeting squalene epoxidase with an immunomodulatory antifungal agent

Novartis AG patented in 2005 a combination of an antifungal drug that is an inhibitor of squalene epoxidase (i.e. allylamines) and a macrolide, such as 33-epichloro-33-desoxyascomycin **23** (Figure 7) that has both antifungal and T-cell immunomodulating properties.[49] The macrolides are natural compounds characterized by a large macrocyclic ring including a lactone or lactame unit that are currently used in clinical practice and/or have been tested in clinical trials.[50–53] The patent claims that when a compound with this activity is coadministered with squalene epoxidase inhibitors, the mixture acts synergistically. Combinations with azoles targeting 14-α-demethylase, such as fluconazole **10** (Figure 1), has no significant positive interaction with such macrolides or instead, interaction results in antagonism. This composition is indicated in mycoses caused by *Candida, Malassezia*, and dermatophytes and intends to be useful for the treatment of mycoses in which fungal colonization plays a role in the fungal resistance. The nature of the interactions between **23** and **10** was defined with the FICI that was determined with the checkerboard design.

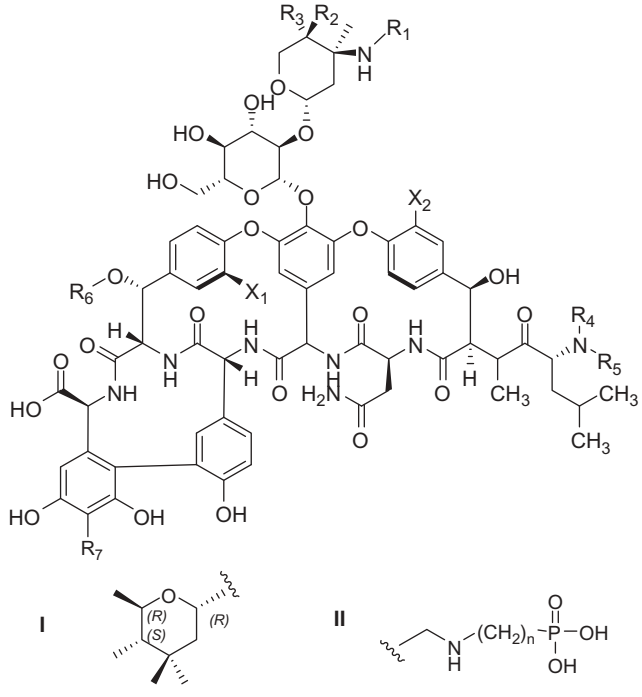
2.1.4. Combination of amiodarone and a benzofurane

Courchesne and others published in 2012 a patent application [54] on combinations of amiodarone **24** with an antifungal benzofurane of formula **25** (Figure 7).

Compound **24** was developed over 30 years ago as an antianginal and antiarrhythmic agent, but it was recently identified as an antifungal agent.[55] Regarding the structure of **25** (Figure 7), R_1, R_2, and R_4 are either H or an alkyl group having one to six optionally substituted carbon atoms, or both R_1 and R_2 represent negative charges on the oxygen, which result in mono- and di-salts; R_3 can be H, alkyl, COR, OCOR, NRCOR, $CON(R)_2$, or CO_2R, where each R and R′ are independently selected from H or an alkyl group having one to six optionally substituted carbon atoms. The invention is useful for treating fungal infections produced by *Cryptococcus, Aspergillus*, or *Candida* spp. In one embodiment, the combination of the benzofuran **26** with **24** showed a 20-fold higher growth inhibition for *Aspergillus* than either drug on its own.[56]

2.1.5. Combination of an antibacterial glycopeptide and either an echinocandin-type or polyene-type antifungal agent

Theravance Inc. patented two combinations between an antifungal agent of the echinocandin [57] or the polyene types [58] and an antibacterial glycopeptide of the general formula **27** in which X_1 and X_2 are independently H or Cl; R_1 must have at least eight nonsubstituted or substituted carbons; R_2 and R_3 are OH and H, respectively; R_4 and R_5 are independently H or CH_3; R_6 is H or a group '**I**' and R_7 is H or a group '**II**' (Figure 8).



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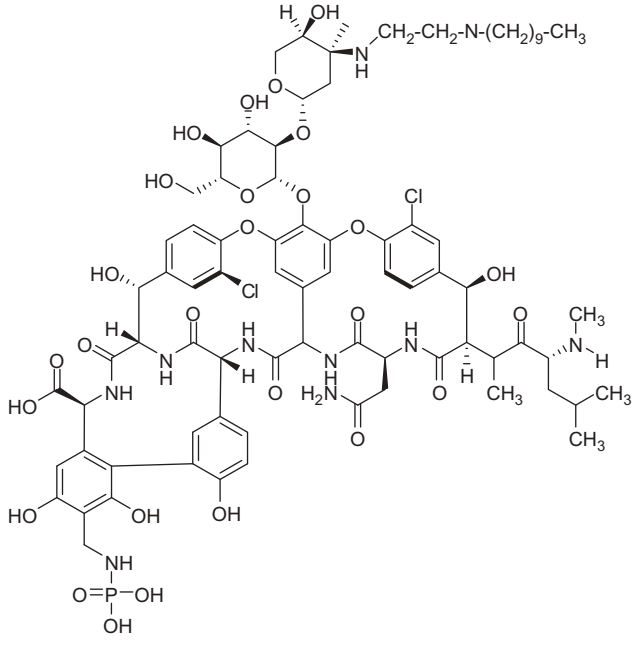

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Figure 8. Structures of compounds **27** and **28**.

The interactions were evaluated with the checkerboard method. In one embodiment, the inventors obtained synergistic effects with the mixture of **16, 1**, or **2** with the glycopeptide telavancin **28** (Figure 8) against different fungi.[57,58]

2.1.6. Combination of an antifungal drug with a tetracycline compound

In 2006, Paratek Pharmaceuticals, Inc. (Boston, MA) patented a combination of an antifungal agent with a tetracycline compound with the formula shown in **29** [59] in which X can be a substituted Carbon, N, S or O; R_2, $R_{2'}$, R_5, R_7, and R_9 are each independently a hydrogen, an alkyl, or an alkenyl among others, or a prodrug moiety; R_4 is NR, alkyl, alkenyl, or others; R_3, R_{10}, R_{11}, and R_{12} are each hydrogen or a prodrug moiety (Figure 9).

The antifungal agent can be, but is not limited to, azoles, polyenes, echinocandins, or analogues. The combination is synergistic against yeasts and filamentous fungi such as *A. fumigatus, A. flavus, A. nidulans, Candida albicans, C. glabrata*, and many others. The patent does not inform on the method used for assessing synergism.

2.1.7. Combination of any commercial antifungal agent with indolizine

Birch et al. published a patent application in 2011 for a combination of any antifungal agent and an indolizine compound of the general structure **30** [60] wherein X is either a bond or NR_8, O, S; and R_1–R_6 independently represent an H or unsubstituted or substituted group selected from C_6–C_{10} aryl, a 5- to 12-membered heterocyclic group, among other substituents (Figure 9). The tested fungi were *Absidia corymbifera, A. flavus, A. fumigatus, A. niger, A. terreus, C. albicans, C. glabrata*, and many others. The combined effects were assessed with the FICI.

2.1.8. Combination of an antifungal drug and an amphoteric phospholipid for body and hair cleansing products

Janssen Pharmaceutica published two inventions comprising body and hair cleansing products, in particular shampoos, useful for dandruff, seborrheic dermatitis, psoriasis, oil, or sebum production of the scalp. In the first patent,[61] published in 2001, the formulation comprises one or more

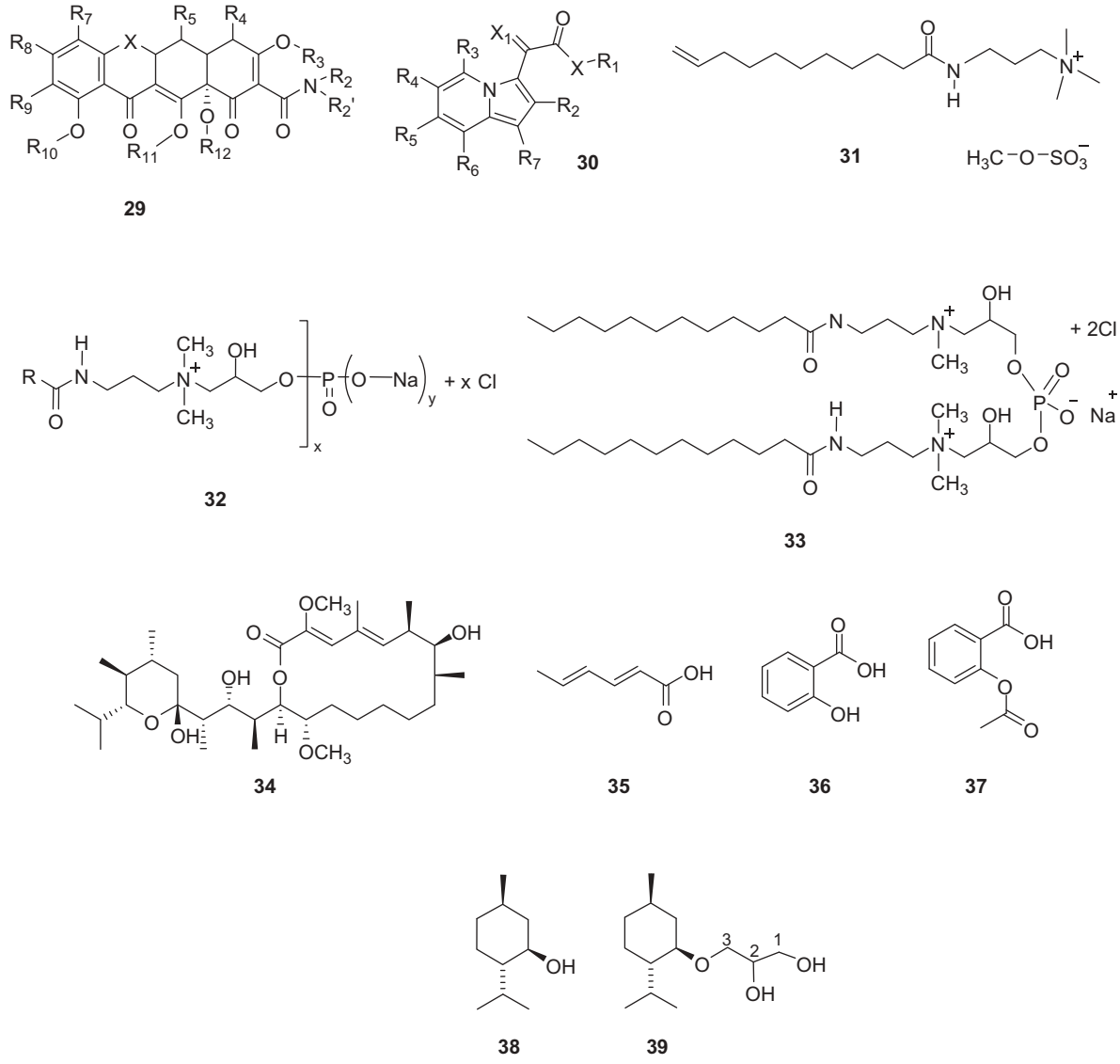

Figure 9. Structures of compounds **29** to **39**.

antifungal inhibitors of the ergosterol biosynthesis and the cationic surface active ingredient 10′-undecen-3-oyl-aminopropyl trimethylammonium methylsulfate **31** (Figure 9) that produces mutual synergistic effect in particular on several isolates of *Malassezia furfur* evaluated with the FICI.

The second patent was published in 2005 [62] and consists essentially of one or more antifungals inhibiting fungal ergosterol biosynthesis and a synthetic amphoteric phospholipid having the general formula **32** (Figure 9) wherein R represents a straight, saturated, monounsaturated, or poly-unsaturated C_7–C_{19} alkyl group. Cocamidopropyl phosphatidyl PG-dimonium chloride **33** (Figure 9) is the most preferred partner for the mixture with antifungal drugs which showed a synergistic effect on the inhibition of the growth of either *M. furfur* or *Epidermophyton, Microsporum*, and *Trichophyton* spp.

The shampoos of the present invention proved to respond well, in contrast to those containing only one active ingredient.[63]

2.1.9. Combinations of a commercial antifungal drug and a proton homeostasis modifier

Kemijski Inštitut published a patent's application [64] that relates to a pharmaceutical combination of any antifungal drug and a proton homeostasis modifier that improves the performance of an antifungal substance through the lowering of intracellular pH.[65,66] Modifiers of proton homeostasis are compounds that either inhibit enzymes that maintain the intracellular pH within the physiological range, such as ATPases, ion channels, ion exchangers, or oxidative phosphorylation (e.g. bafilomycine **34**, Figure 9) or compounds that directly affect intracellular pH such as weak carboxylic and hydroxycarboxylic acids (e.g. sorbic acid **35**, Figure 9). The combination is suitable for fungal infections produced by pathogenic or opportunistic yeasts or filamentous fungi.

The effects were tested by measuring the metabolic activity of fungi with XTT (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) and confocal microscopy employing the dyes SynaptoRed, that stained the cell membrane, and SYTOX, that emits fluorescence only after binding to deoxyribonucleic acid (DNA), indicating damage to cell membranes.

A similar patent [67] relates to the combination between salicylic **36** or acetylsalicylic **37** acids (Figure 9) and an antifungal drug in difficult-to-treat infections.

2.1.10. Combinations of an antifungal drug of the polyene or echinocandin type with an antibody or an antigen-binding fragment

NeuTec Pharma Plc published two patent applications. The first one, in 2008,[68] relates a composition comprising an antibody or an antigen-binding fragment and an antifungal agent of the polyene or echinocandin types. These antibodies may be specific for one or more epitopes of a fungal stress protein, comprising the sequence SEQ ID NO:1 generated by standard methods known in the art. Examples of antibodies include (but are not limited to) polyclonal, monoclonal, chimeric, single-chain, fragment antigen-binding (Fab) fragments, fragments produced by a Fab expression library, and antigen-binding fragments of antibodies. They were produced in a range of hosts (rabbits, rats, human, or others) which were immunized with heat shock protein from the *Candida* genus, for example, hsp90 or any fragment or oligopeptide. In a previous study, Matthews et al. demonstrated [69] in an animal model that the sera containing these antibodies can protect against systemic candidiasis due to their humoral immunity which is important in preventing dissemination of candidiasis beyond the mucosa.[70] According to the claims of the invention, the combined preparations are useful for infections by species not only of *Candida* but also of *Cryptococcus, Histoplasma, Aspergillus, Mucor, Blastomyces, Coccidioides*, or *Paracoccidioides* genera.

In one of the embodiments, hsp90 and **1** (Figure 1) showed synergism in checkerboard assays against fluconazole-sensitive but also fluconazole-resistant strains of *C. albicans* and also against other yeasts such as *C. glabrata* and *C. krusei* which is intrinsically resistant to fluconazole.[68] This synergy was corroborated in *in vivo* experiments.[68] In the second invention, in 2008, NeuTec Pharma Plc patented, a combination [71] comprising an azole and an antibody or antigen-binding fragment, which is active against *Aspergillus* spp. that showed synergism against a wide variety of pathologically important *Aspergillus* strains.

2.1.11. Combination of an antifungal drug targeting the fungal membrane and a cell-wall degrading enzyme

In 2003,the Cornell Research Foundation Inc. patented [72] combinations for topical and internal application in medicine (as well as in agriculture), acting by inhibiting either the fungal germination or the fungal growth. The combinations comprise the following: (a) a cell-wall degrading chitinolytic or glucanolytic enzyme and (b) a cell-membrane-affecting antifungal compound that is not expressed by the same organism as the fungal cell-wall degrading enzyme in nature. The chitinolytic enzymes include endochitinases, chitin 1,4-β-chitobiosidases, β-*N*-acetylglucosaminidases, and nagase that can be obtained from fungi (e.g. *Trichoderma, Gliocladium, Lycoperdon*, and *Calvatia* genera), bacteria (e.g. *Streptomyces, Vibrio, Serratia*, and *Bacillus*), and higher plants (e.g. *Nicotiana, Cucumis*, and *Phaseolus*). The glucanolytic enzymes comprise 1,3-β-glucosidases.

The cell-membrane-affecting antifungals comprise azoles or polyenes. The synergistic interaction was assessed with the following equation [73]: $Ee = X + Y - XY/100$, where *Ee* is the expected effect from additive responses of the chemicals, and *X* and *Y* are the percentage inhibition of the chemicals, each one on its own. So, if *X* provides 20% of inhibition and *Y* provides 30%, the expected additive effect is $20 + 30 - (20 \times 30)/100 = 44\%$. According to this equation, any value greater than 44% is evidence of synergy. Although all examples are given for phytopathogenic fungi, the invention applies to antifungals for human beings as stated above.

2.1.12. Combination of an allylamine-type antifungal drug and menthol or its derivatives

In 2010, Hisamitsu Pharmaceutical Co. published an external preparation for athlete's foot, also known as *tinea pedis* commonly caused by *Trichophyton rubrum, T. mentagrophytes*, and

Epidermophyton floccosum. The treatment comprises a mixture of **5** (Figure 1) with either L-menthol **38** or 3-L-menthoxypropane-1,2-diol **39** (Figure 9). This mixture should be accompanied by at least one local anesthetic (dibucaine hydrochloride, lidocaine, or its salt), an antihistamine (chlorpheniramine maleate, diphenhydramine, or its salt), and an anti-inflammatory drug (allantoin, glycyrrhetinic acid, or its salt).[74] Although **5** has an excellent activity on its own against fungi of the *Trichophyton* genus and *C. albicans*, the combination could be highly useful having effects on both the enhancement of patients' compliance and the reduction of rubefaction's symptoms produced by the mycoses.

2.1.13. Combination of essential oils and herbal extracts, both displaying antifungal activities when acting alone

In 2000, Farmo-Nat Ltd. [75] published an invention that describes a combination consisting of an essential oil (EO) that has antimicrobial activity (antibacterial and/or antifungal) on its own and a herbal extract (HE) that shows lower or no antimicrobial activity when acting alone. The EO can be any one of a list given by the inventors (e.g. cinnamon, eucalyptus, lavender, lemon oils) but not limited to them. HEs include, but are not limited to, species of the genera *Plantago, Echinacea, Hypericum, Commiphora, Phytolacca, Salvia*, and many others. They are preferably prepared in hydroalcoholic solvents according to the British Herbal Pharmacopeia [76] or the British Herbal Compendium.[77] The additive, synergistic, or antagonistic activities of the combination were determined by calculating the FICI which was interpreted as synergistic, additive, or antagonist when it takes the values <1, =1, or >1, respectively.

2.1.14. Combination of an antifungal drug with a phenolic compound with activity against biofilms

In 2012, the Board of Trustees of the University of Arkansas published a patent application on antibiofilm synergistic compositions [78] comprising at least one phenolic compound of natural origin (ellagic acid **40**) as well as its glycosylated derivatives such as **41–43** (Figure 10) and one antibiofilm agent.

The antibiofilm agent can be any antifungal drug such as polyenes **1, 2**, allylamines **3**–**5**, azoles **7**–**14** (Figure 1), griseofulvin **15**, 5-FC **16**, and echinocandins **17**–**19** (Figure 2). The biofilm's location can be either on a surface of an implantable chemical device (catheter) or within a subject (an animal, a person) or a fresh or processed food product.

2.1.15. Combination of an antifungal drug with an inhibitor of the adhesion of epithelial or endothelial cells

In 2014, Noe and Noe-Letschnig [79] published a patent that relates to the combination of an antifungal agent with an inhibitor of the adhesion of epithelial or endothelial cells for topical use. Any antifungal drug can be used in combination but regarding the adhesion inhibitors, they are preferably selected from (a) a nonsteroidal anti-inflammatory drugs (NSAID) (i.e. diclofenac **44**, ibuprofen **45**, lornoxicam **46**, mefenamic acid **47**); (b) a prostacyclin **48** or its analogues; or (c) an inhibitor of the expression of adhesion molecules (AM) such as ticlopidin **49** and clopidogrel **50** (Figure 10).

This invention is based on the assumption that the first step in a fungal infection process consists in the fungal attachment to the plasma membrane (epithelium and endothelium) of a cell, *via* the interaction with AM, thus becoming pathogenic. These AM are the same that play a decisive role in the course of blood coagulation during the adhesion of thrombocytes and whose expression is induced by derivatives of the arachidonic acid metabolism (*inter alia* von Willebrand factor, vitronectin, fibronectin, integrins).

In turn, it is known that under the influence of prostaglandins (that are derivatives of arachidonic acid), *Candida* strains undergo a transformation from budding to hyphae shape that clearly intensifies the fungal pathogenicity by forming a closer layer that prevents the attack of the usual locally applied antimycotic agents.[80]

The effect of this combination is based on (i) suppression of the pathogen growth, the adhesion of the pathogen to the host cell, the acute inflammation, and the pain symptoms; and (ii) prevention of the adhesion of the pathogen to the host cell upon transformation, thus preventing the aggravation of the clinical picture and avoiding the chronification of the infection.

2.1.16. Combination of an antifungal agent targeting the (1,3)β-D-glucan synthesis of the fungal wall and an hemopoietic growth factor

Oblon, Spivak, Mcclelland, Maier, and Neustadt, RC published in 2004 a patent application [81] describing the antifungal combination of a hemopoietic growth factor, such as granulocyte-colony stimulating factor (G-CSF) with a cyclic antifungal hexapeptide of the formula **20** (Figure 7) (including all the optical and geometrical isomers), that targets the cell-wall (1,3)β-D-glucan synthesis. In X, R_1 is an acyl group and R_2 and R_3 are hydrogen or hydroxyl. G-CSFs (molecular weight = 1.8–2.2 million) are naturally occurring cytokines that have key roles in granulocyte production/maturation and neutrophil functions, including phagocytosis, chemotaxis, and the production of reactive oxygen intermediates, thus playing antimicrobial functions.[82] They are also used to treat cancer patients whose white blood cell levels are adversely affected by chemotherapy or radiation. This invention is intended for infections caused by dermatophytes or other fungi of the genera *Cryptococcus, Candida, Aspergillus, Histoplasma*, and others.

2.2. Patents including pharmacokinetic potentiation of the antifungal effects

2.2.1. Combination of an antifungal agent containing a benzylamine or an allylamine moiety and a steroidal anti-inflammatory agent

Penederm, Inc. patented in 2000 an invention [83] comprising a stable topical formulation useful for treating fungal diseases and their related inflammation. It contains an antifungal agent lacking an imidazole group, particularly those including a benzylamine or allylamine moiety, and a steroidal type anti-inflammatory compound. The selection of a non-imidazole antifungal compound was due to the disadvantages reported by a combination of an imidazole-antifungal agent with a corticosteroid,[84] such as skin atrophy and suppression of the hypothalamic–pituitary–adrenal axis, among others, since

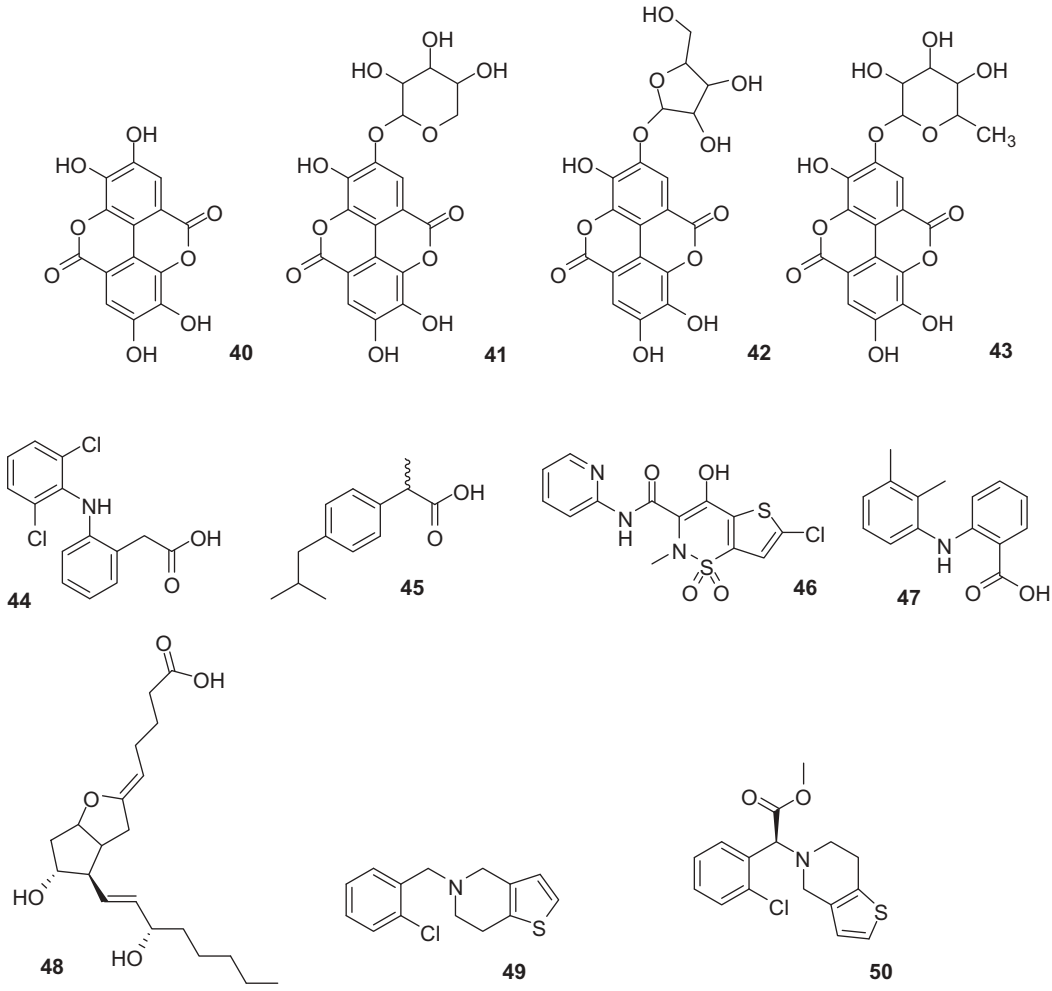

Figure 10. Structures of compounds **40** to **50**.

steroidal compounds sometimes function as deactivating agents.[85] So, an improved formulation should ideally deliver the antifungal agent and the steroid to the skin and maintain the combination on the skin for the minimum period necessary for an effective treatment. The anti-inflammatory steroid, whose purpose is to alleviate the symptoms of erythema and the related itching that are normally associated with fungal infections,[86] could be one of a long list, with preference of betamethasone or its propionate, fluocinonide, hydrocortisone, methylprednisolone, clobetasol, and beclomethasone. The steroids proposed here do not contain an ester-bearing steroid group, which is hydrolyzed at a much lower rate than expected by the components of the topical formulation, thus providing increased shelf life for the formulation.[87,88]

The combination proposed in this invention demonstrated a synergistic antifungal effect that greatly reduces the doses necessary for complete eradication of the disease, and minimizes the penetration avoiding the potential side effects of prolonged steroid use.

2.2.2. Combination of an antifungal agent with a keratinolytic agent

Browdy and Neimark P.L.L.C. patented in 2010 [89] a composition useful for the full clearance of a superficial fungal infection, containing an antifungal agent (that can be, but it is not limited to, an azole) in combination with at least two keratinolytic agents and a polar solvent (such as a short-chain mono-alcohol and/or a diol of not more than 5 C).[90] Keratinolytic agents are compounds that loosen and remove the stratum corneum of the skin or alter the structure of the keratin layer where the fungus resides, initiating the formation of collagen, having anti-inflammatory properties, and possessing skin-hydration properties. Suitable keratinolytic agents include α-hydroxy acids (lactic, glycolic, malic, citric, and tartaric acids; dihydroxybenzene; and urea and derivatives), β-hydroxy acids (salicylic acid **30**, Figure 9), short-chain carboxylic acids (formic, acetic, propionic, and butyric acids), phenolic compounds (resorcinol, hydroquinone), and urea and derivatives.

The invention is useful for the treatment of dermal infections (produced by dermatophytes of the *Trichophyton, Microsporum*, and *Epidermophyton* genera) which concurrently involves hyperkeratosis. The invention provides a kit, consisting of the keratinolytic agents in conjunction with an occlusive device that is placed in contact with the skin from 10 min to 2 h. After its removal, the effect of the keratinolytic agents is noticed by desquamation or peeling of the outer layers of the skin, allowing the antifungal effect to be synergistically enhanced.

2.2.3. Combination of an antifungal or anti-inflammatory agent with a cleanser

Pennie & Edmons LLP patented in 2003 [91] a composition that contains hydrogen peroxide as a cleanser, a moisturizing agent, and one or more dermatological agents selected from either an antifungal or an anti-inflammatory agent (or a combination thereof). The invention is claimed to be used for treating, preventing, or managing scalp, hair, and nail conditions, thus cleansing the dermatological surfaces and facilitating the penetration of antifungal agents. The antifungal drug can be an azole, a polyene, an allylamine, or a combination of them. The anti-inflammatory drug can be a nonsteroidal or a steroidal compound or a combination thereof. The composition further comprises an amphoteric surfactant or citric acid in sufficient amounts to inhibit decomposition of hydrogen peroxide for at least 3 months. The effectiveness of this invention was evaluated by employing the pour plate technique consisting of the enumeration of the target organisms immediately after inoculation.

2.2.4. Combination of an essential oil containing tri-unsaturated monoterpenes with a solid bioadhesive carrier inserted into a sticker for local release

Axiomedic Ltd. patented in 2011 an absorbable solid composition, in the form of a topical self-adhesive sticker that adheres to the oral tissue surface, for treating oral mucosal disorders such as aphthous stomatitis, inflammation, mucosal microbial infection, and toothache.[92] The sticker may contain a minimally effective amount of a bioactive plant extract or EO, incorporated as a powder onto a pharmaceutical inert bio-adhesive carrier, which is then the mixture compressed into tablet stickers. The bioactive extract can be an active EO (citronella, lemon, citron, rosmarinus oils, or others) which should contain at least one monoterpene of three unsaturations such as limonene, myrcene, sabinene, or others. The sticker is placed over the oral mucosal lesions and it is expected to release a safe and effective amount of tri-unsaturated monoterpenes to the oral activity, stopping the progression of the lesion in any phase of its development.

3. Expert opinion

The present antifungal armamentarium for the treatment of fungal infections is limited, and has important problems such as toxicity, low potency, narrow spectrum of activity, or inappropriate bioavailability and pharmacokinetics.

Antifungal combination therapy has emerged as one of the available strategies to provide clinicians with effective tools. Achievement of synergy is one of the major theoretical justifications for combination therapy, since the desired therapeutic effect would be gained with lower quantities of each substance than when acting alone, thus reducing their toxic side effects, improving safety and tolerability, increasing the spectrum of antimicrobial activity, and preventing treatment failure when antimicrobial resistance is suspected.

As new antifungal alternatives, several patents on antifungal combinations have been disclosed between 2000 and 2015, which constitute a fortress for the development of new antifungal agents that intend to surpass the drawbacks of the antifungal drugs in clinical use. The common characteristics of the published patents (2000–2015) are that in almost all of them at least one of the partners is a commercial antifungal drug and the evaluation of synergism is always made with simple methods (mainly disk diffusion and checkerboard) or there is no mention on the design of the method or the analysis of the results. This constitutes a weakness of the disclosed patents since it is well known that a reliable assessment of synergy must be done with a sophisticated method such as that of Chou and Talalay [45] or with a statistically supported method such as the Mixlow.[44]

The soil fungus metabolite **22** (Figure 7), patented by the Kitasato Institute [48] as a partner of azoles, appears to be a promising compound since several highly important antifungals in clinical use have been isolated from fungi. AmpB **1** and nystatin **2** (Figure 1) were isolated from *Streptomyces* spp. and griseofulvin **15** (Figure 2) was isolated from *Penicillium griseofulvum*. Caspofungin **17** (Merck & Co., Inc., Figure 2) is a semisynthetic compound derived from pneumocandin B, which was isolated from *Glarea lozoyensis*, a fungus found in water.[93] In turn, micafungin sodium **18** (Fujisawa, Astellas Pharmaceuticals, 2002, Figure 2) is derived from the lipopeptide FR901379 which was isolated from the fungus *Coleophoma empetri*, a plant pathogen associated with postharvest fruit rot in cranberries.[94] Anidulafungin **19** (Vicuron and Pfizer, 2006, Figure 2) is a synthetic derivative of echinocandin B, a naturally occurring lipopeptide obtained by fermentation from *A. nidulans*.[95]

Regarding innovative combinations, Kemijski Inštitut [64] prepares a combination containing an antifungal drug and a modifier of proton homeostasis such as the macrolactone bafilomicine **34** (Figure 9), thus improving the performance of an antifungal drug through the lowering of the intracellular pH; NeuTec Pharma Plc. [68] proposes the use of an antibody (monoclonal, chimeric, single-chain Fab fragments, or others), specific for one or more epitopes of fungal stress, as the partner of an antifungal drug; Noe and Noe-Letschnig [79] combines an antifungal agent with an inhibitor of the adhesion of epithelial or endothelial cells that can be an NSAID or a piperidin-thiophene like ticlopidin **49** or clopidogrel **50** (Figure 10) and Browdy and Neimark P.L.L.C. [89] proposes the mixing of an antifungal agent with a keratinolytic agent, such as α-hydroxy acids, in order to improve the penetration of the antifungal drug into the skin.

The selected innovative patents as well as the less innovative ones open big avenues for the development of new combinations which, with lower doses than when used alone, showed enhanced antifungal activity and thus they can be new alternatives for the difficult-to-treat fungal infections. However, it is surprising that none of them analyzes the toxicity of the new inventions, taking into account that one of the main objectives of the combinations is the lowering of the toxicity of the existing antifungal drugs.

Another concern of the disclosed patents is the lack of *in vivo* studies, since it is well known that the *in vitro* tests do not predict the *in vivo* behavior. The lack of details of the methods to assess synergism is another drawback of the disclosed patents.

Due to the above considerations, the combination antifungal therapy, which has been demonstrated to work well in other diseases such as human immunodeficiency virus (HIV),[96] cancer,[97] and microbial infections,[98–100] among others, will surely be the focus of intensive studies over the coming years. This trend is due to the existing antifungal agents not meeting the expectations of eradicating the mycoses mainly in immunocompromised hosts.

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