Abstract:

Doxycycline was found to act synergistically with the antifungal fluconazole against *Candida albicans*. Combination with doxycycline converts fluconazole from fungistatic to fungicidal, prevents the onset of drug resistance, and is also effective against a clinical isolate characterized by elevated resistance to fluconazole. Investigation on the interactions between the two drugs by way of checkerboard assays indicated that doxycycline had an influence on the MIC for fluconazole, as defined by CLSI standards, only at high concentrations (200 µg/ml). However, lower concentrations were effective in eliminating residual cell growth at supra-MIC concentrations of fluconazole. Using MIC-0, defined as a drug combination resulting in optically clear wells, as an endpoint, doxycycline was found to be synergistic with fluconazole at a concentration as low as 25 µg/ml, with a fractional inhibitory concentration index < 0.5.
Field of the invention

The present application relates to the field of antifungals, particularly antifungals against *Candida* species. It was found that antimicrobials from the tetracycline family potentiate the antifungal effect of fluconazole against *Candida*. Thus, these combinations are provided as medicament, particularly against Candida infections.

Introduction

Infections by *Candida albicans* have been on the rise in the past decades, mostly due to changes in the clinical practice, such as the increased use of immunosuppressants and broad-spectrum antibacterial agents (26, 29, 47). Fungal infections represent a challenge for clinicians, because of the scarcity and sometimes the limited efficacy of antifungal drugs (1, 44). Fluconazole and otherazole antifungals, targeting ergosterol biosynthesis (23), have been extensively used in the recent clinical practice thanks to their reduced toxicity and lower cost, compared to amphotericin B in its conventional and lipidic forms, respectively (44). The major disadvantage ofazole antifungals, however, is their fungistatic nature, an aspect that favors the onset of drug resistance. The development of isogenic strains of *C. albicans* characterized by stepwise increased tolerance to fluconazole in patients undergoing continued treatment with this drug has been documented in several cases (34, 56, 57). For these reasons, the conversion of fluconazole from fungistatic to fungicidal via combinations with other drugs is highly desirable.

In a different perspective, tools for the detailed study of the molecular genetics of *C. albicans* have been greatly improved in recent times (6, 7, 40). Regulatable promoters are available to the research community, to induce or repress gene expression in *C. albicans*. Most of them, such as the *PCK1* and the *MAL2* promoters, allow conditional expression/repression of the gene of interest upon incubation of cells in growth media containing the repressing or the activating carbon source (3, 28). The tetracycline direct and reverse systems, which allow
gene induction or repression by addition of doxycycline to the growth medium, have also been successfully used to regulate gene expression in *C. albicans* (38, 45). However, it was found that, compared to other organisms, significantly higher concentrations of doxycycline are necessary for full induction of tetracycline-inducible promoters in *C. albicans* (45).

**Summary**

Surprisingly we found that doxycycline, and to a lower extent tetracycline, two licensed antimicrobials that prevent bacterial protein synthesis, potentiate the antifungal activity of fluconazole against *Candida albicans* in a dosage-dependent manner. Doxycycline was found to act synergistically with the antifungal fluconazole against *Candida albicans*. Combination with doxycycline converts fluconazole from fungistatic to fungicidal, prevents the onset of drug resistance, and is also effective against a clinical isolate characterized by elevated resistance to fluconazole. Investigation on the interactions between the two drugs by way of checkerboard assays indicated that doxycycline had an influence on the MIC for fluconazole, as defined by CLSI standards, only at high concentrations (200 µg/ml). However, lower concentrations were effective in eliminating residual cell growth at supra-MIC concentrations of fluconazole. Using MIC-0, defined as a drug combination resulting in optically clear wells, as an endpoint, doxycycline was found to be synergistic with fluconazole at a concentration as low as 25 µg/ml, with a fractional inhibitory concentration index of < 0.5.

Doxycycline-mediated growth inhibition can be reversed by externally added iron, indicating that iron depletion may account for the synergism. Consistently, we confirmed old literature data about iron-chelating activity of doxycycline. Synergism of fluconazole with doxycycline does not appear to be mediated by calcineurin, since doxycycline further aggravates the susceptibility to fluconazole of mutants lacking the catalytic or the regulatory subunits of calcineurin. Growth in the presence of fluconazole and doxycycline is restored by elevated dosage of *ERG11* in *Saccharomyces cerevisiae* but not in *C. albicans*, despite full competence of the pathogen's protein to act as a suppressor in baker's yeast.
Thus, addition of doxycycline appears to have a major impact on prevention of fluconazole tolerance, defined as incomplete growth inhibition at supra-MIC concentrations of fluconazole. This finding is consistent with the role of doxycycline in converting fluconazole to a fungicidal drug, and may also have implications in the prevention of drug resistance and the combination of doxycycline and fluconazole can be used in treatment of candidiasis. The fact that we never recovered spontaneous mutants resistant to the combination of fluconazole and doxycycline indicates that multiple genetic alterations may be necessary for such resistance to occur, and makes this treatment especially attractive.

Accordingly, in a first aspect of the invention, a combination of a tetracycline antibiotic and fluconazole is provided. It is particularly envisaged that the tetracycline antibiotic is doxycycline. The combination can be provided as one composition, or as a kit of parts. According to a further aspect, the combination of the tetracycline and fluconazole is provided for use as a medicament. Most particularly, the combination is provided for use in treatment of Candida infections.

According to specific embodiments, this is equivalent to providing the use of a combination of a tetracycline and fluconazole for the manufacture of a medicament for treatment of Candida infections. Or according to further alternatives, it is equivalent as saying that methods of treatment of Candida infections are provided for subjects in need thereof, comprising:

administering a combination of a tetracycline and fluconazole to said subject.

According to particular embodiments, said Candida is Candida albicans. Even more particularly, the Candida albicans is a fluconazole resistant isolate of Candida albicans.

**BRIEF DESCRIPTION OF THE FIGURES**

**Fig. 1.** Doxycycline synergizes with fluconazole in a dosage-dependent and medium- and carbon source-independent manner.
Doxycycline synergizes with fluconazole in a dosage-dependent and medium- and carbon source-independent manner. Cells of the C. albicans reference strain SC5314 were spotted onto plates containing different drug combinations. Where indicated, fluconazole (Flu) is present at 10 \( \mu g/ml \), whereas the concentration of doxycycline (Dox) is expressed in \( \mu g/ml \). (A) SD plates with 3 days of incubation. (B) YPD plates with 1 day of incubation, RPMI-glucose plates and FBS plates with 2 days of incubation, and SEG plates with 5 days of incubation. (C) SC5314 cells were spotted onto SD plates containing fluconazole and either tetracycline (Tetr), gentamicin, or neomycin. The concentration of the antibiotics is indicated in \( \mu g/ml \). Plates were incubated for 2 days before being scanned.

**Fig. 2.** Doxycycline eliminates fluconazole tolerance.

(A) A 5-\( \mu l \) concentration of each well from one of the checkerboard assays was spotted onto a YPD plate after 48 h of incubation, to monitor the viability of drug-treated cells. The recovery plate was incubated for 2 days before being scanned. (B) Viable cell counts after 48 h of incubation in the presence of fluconazole alone or with 25, 50, or 100 \( \mu g/ml \) doxycycline added (corresponding to rows 7, 4, 3, and 2 in panel A, respectively). Circles, fluconazole alone; diamonds, fluconazole plus 25 \( \mu g/ml \) doxycycline; squares, fluconazole plus 50 \( \mu g/ml \) doxycycline; triangles, fluconazole plus 100 \( \mu g/ml \) doxycycline. Values falling below the detection limit of \( 10^1 \) CFU/ml were approximated to \( 0.5 \times 10^1 \) CFU/ml. Mean values from three independent experiments are shown. (C) E-test measurement of the fluconazole MIC of strain SC5314 on RPMI-glucose plates. The left panel shows medium without doxycycline, and the right panel shows medium with 50 \( \mu g/ml \) doxycycline.

**Fig. 3.** Doxycycline converts fluconazole from fungistatic to fungicidal and prevents the onset of drug resistance.

A: viable cell counts after 48 h or 96 h of incubation in the presence of fluconazole alone, or with 50 or 100 mg/ml doxycycline added. Open symbols: viable cell counts after 48 h of incubation. Filled symbols: viable cell counts after 96 h of incubation. Circles: fluconazole
alone; squares: fluconazole + 50 mg/ml doxycycline; triangles: fluconazole + 100 mg/ml doxycycline. Values falling below the detection limit of $10^1$ CFU/ml were approximated to 0.5 x $10^1$ CFU/ml. Mean values from three independent experiments are shown. B: emergence of fluconazole resistant strains on plates containing 128 mg/ml fluconazole, without (top) or with (bottom) 50 mg/ml doxycycline. Plates were incubated 4 days before being scanned.

**Fig.4.** The fluconazole+doxycycline combination is also effective on a clinical isolate of C. albicans characterized by elevated resistance to fluconazole.

A: Cells of the C. albicans reference strain SC5314, and of the clinical isolate FH5 were spotted on SD plates containing doxycycline, without (mid panel) or with fluconazole (right panel). Concentrations are expressed in mg/ml. Plates were incubated 2 days before being scanned. B: E-test measurement of fluconazole MIC of strain FH5 on RPMI-glucose plates. Left: medium without doxycycline; center: medium with 50 mg/ml doxycycline; right: medium with 200 mg/ml doxycycline.

**Fig.5.** Overexpression of ERG11 suppresses fluconazole+doxycycline susceptibility in S. cerevisiae but not in C. albicans.

A: Transformants of the S. cerevisiae reference strain BY4742 with plasmid pAFC88 (ERG11) or with an empty plasmid (control) were spotted on SD plates containing combinations of fluconazole and doxycycline, at the indicated concentrations (mg/ml). Plates were incubated 4 days before being scanned. B: Top panel: cells of the C. albicans strains AFA60 (control) and AFA59b (ACT1p-CaERG11) were spotted on SD plates containing the indicated concentrations of fluconazole+doxycycline. Bottom panel: cells of strains AFA21 (control) and AFA63a (PCK1p-CaERG11) were spotted on SCAA plates with or without fluconazole+doxycycline. Plates were incubated 4 days before being scanned. C: quantitative real-time PCR assessment of expression of CaERG11 in strains carrying an extra copy of the gene under the control of the constitutive ACT1, or of the inducible PCK1 promoter. The graph shows mean values with standard deviations from two independent experiments. D:
Transformants of the *S. cerevisiae* reference strain BY4742 with plasmid pAFC99a (GAL1,10p-CaERG11) or with an empty plasmid (control) were spotted on SGal plates containing combinations of fluconazole and doxycycline, at the indicated concentrations (mg/ml). Plates were incubated 5 days before being scanned.

**Fig. 6.** Doxycycline synergizes with fluconazole via iron sequestration.

**A:** Cells of *C. albicans* SC5314 were spotted on SD plates containing combinations of fluconazole and doxycycline at standard concentrations, with and without ferric citrate or ferric chloride added at the indicated concentrations. Plates were incubated 2 days before being scanned. **B:** Cells of *S. cerevisiae* BY4742 were similarly spotted on SD plates. Plate scans were taken after 2 days. **C:** Colorimetric assessment of iron chelating activity of doxycycline, tetracycline and fluconazole. Iron chelation is expressed as a reduction of absorbance at 630 nm. Filled circles: doxycycline, open circles: tetracycline, filled squares: fluconazole, open squares: water. Mean values with standard deviations from three independent experiments are shown. **D:** Cells of *C. albicans* SC5314 were spotted on SD plates containing combinations of fluconazole (10 mg/ml) and BPS (20 mg/ml). Plates were incubated 4 days before being scanned.

**Fig. 7.** Synergism between doxycycline and fluconazole is not mediated by calcineurin.

Cells of the indicated *C. albicans* strains were spotted on SD plates containing combinations of fluconazole and doxycycline. Fluconazole was maintained at the standard 10 µg/ml concentration, whereas the concentration of doxycycline was decreased as indicated (Mg/ml) to provide a sensitized background for the assay. Plates were incubated 3 days before being scanned.

**Detailed description**

In a first aspect of the invention, a combination of a tetracycline antibiotic and fluconazole is provided. A "tetracycline antibiotic" or "a tetracycline" as used herein refers to the broad-
spectrum polyketide antibiotic produced by the Streptomyces genus of Actinobacteria, indicated for use against many bacterial infections, or semisynthetic derivatives or related substances with antimicrobial activity, characterized by containing the same four-ring system. They can also be indicated as 'a subclass of polyketides having an octahydrotetracene-2-carboxamide skeleton' or as 'derivatives of polycyclic naphthacene carboxamide'. Examples of tetracyclines include, but are not limited to: tetracycline, chlortetracycline, oxytetracycline, demeclocycline, doxycycline, lymecycline, meclocycline, methacycline, minocycline, rolitetracycline, tigecycline, penimepicycline and pipacycline.

It is particularly envisaged that the tetracycline antibiotic is doxycycline.

According to a further aspect, the combination of the tetracycline and fluconazole is provided for use as a medicament. Particularly, the combination is provided for use in treatment of Candida infections. Candida is a genus of yeasts, and common Candida species that may cause an infection include, but are not limited to Candida albicans, Candida glabrata, Candida tropicalis, Candida krusei and Candida parapsilosis.

According to particular embodiments, said Candida is Candida albicans. Even more particularly, the Candida albicans is a fluconazole resistant isolate of Candida albicans.

The combination can be provided as one composition, or as a kit of parts. The pharmaceutical compositions containing a combination of a tetracycline antibiotic and fluconazole can be utilized to achieve the desired pharmacological effect by administration to a patient (or subject) in need thereof. A patient, for the purpose of this invention, is a mammal, including a human, in need of treatment for the particular condition or disease. According to particular embodiments, the patient (or subject) is immunocompromised.

Therefore, the present invention includes pharmaceutical compositions that are comprised of a pharmaceutically acceptable carrier and a pharmaceutically effective amount of a tetracycline antibiotic (e.g. doxycycline) and fluconazole, or salt thereof. A pharmaceutically acceptable carrier is preferably a carrier that is relatively non-toxic and innocuous to a patient at concentrations consistent with effective activity of the active ingredient so that any side effects...
ascribable to the carrier do not vitiate the beneficial effects of the active ingredient. A pharmaceutically effective amount of the combination is preferably that amount which produces a result or exerts an influence on the particular condition being treated, e.g. one which reduces *Candida* growth or reproduction. The compositions of the present invention can be administered with pharmaceutically-acceptable carriers well known in the art using any effective conventional dosage unit forms, including immediate, slow and timed release preparations, orally, parenterally, topically, nasally, ophthalmically, optically, sublingually, rectally, vaginally, and the like. According to specific embodiments, the compositions are foreseen for topical administration (as opposed to systemic administration). Nevertheless, according to alternative embodiments, the compositions are provided for systemic administration.

For oral administration, the compositions can be formulated into solid or liquid preparations such as capsules, pills, tablets, troches, lozenges, melts, powders, solutions, suspensions, or emulsions, and may be prepared according to methods known to the art for the manufacture of pharmaceutical compositions. The solid unit dosage forms can be a capsule that can be of the ordinary hard- or soft-shelled gelatin type containing, for example, surfactants, lubricants, and inert fillers such as lactose, sucrose, calcium phosphate, and corn starch.

In another embodiment, the compositions of this invention may be tableted with conventional tablet bases such as lactose, sucrose and cornstarch in combination with binders such as acacia, corn starch or gelatin, disintegrating agents intended to assist the break-up and dissolution of the tablet following administration such as potato starch, alginic acid, corn starch, and guar gum, gum tragacanth, acacia, lubricants intended to improve the flow of tablet granulation and to prevent the adhesion of tablet material to the surfaces of the tablet dies and punches, for example talc, stearic acid, or magnesium, calcium or zinc stearate, dyes, coloring agents, and flavoring agents such as peppermint, oil of wintergreen, or cherry flavoring, intended to enhance the aesthetic qualities of the tablets and make them more acceptable to the patient. Suitable excipients for use in oral liquid dosage forms include dicalcium phosphate and diluents such as water and alcohols, for example, ethanol, benzyl alcohol, and
polyethylene alcohols, either with or without the addition of a pharmaceutically acceptable surfactant, suspending agent or emulsifying agent. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance tablets, pills or capsules may be coated with shellac, sugar or both.

Dispersible powders and granules are suitable for the preparation of an aqueous suspension. They provide the active ingredient (i.e. the tetracycline, the fluconazole or both) in admixture with a dispersing or wetting agent, a suspending agent and one or more preservatives. Suitable dispersing or wetting agents and suspending agents are exemplified by those already mentioned above. Additional excipients, for example those sweetening, flavoring and coloring agents described above, may also be present.

The pharmaceutical compositions of this invention may also be in the form of oil-in-water emulsions. The oily phase may be a vegetable oil such as liquid paraffin or a mixture of vegetable oils. Suitable emulsifying agents may be (1) naturally occurring gums such as gum acacia and gum tragacanth, (2) naturally occurring phosphatides such as soy bean and lecithin, (3) esters or partial esters derived from fatty acids and hexitol anhydrides, for example, sorbitan monooleate, (4) condensation products of said partial esters with ethylene oxide, for example, polyoxyethylene sorbitan monooleate. The emulsions may also contain sweetening and flavoring agents.

Oily suspensions may be formulated by suspending the active ingredient in a vegetable oil such as, for example, arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oily suspensions may contain a thickening agent such as, for example, beeswax, hard paraffin, or cetyl alcohol. The suspensions may also contain one or more preservatives, for example, ethyl or n-propyl p-hydroxybenzoate; one or more coloring agents; one or more flavoring agents; and one or more sweetening agents such as sucrose or saccharin. Syrups and elixirs may be formulated with sweetening agents such as, for example, glycerol, propylene glycol, sorbitol or sucrose. Such formulations may also contain a demulcident, and preservative, such as methyl and propyl parabens and flavoring and coloring agents. The compositions of this invention may also be administered parenterally, that is,
subcutaneously, intravenously, intraocularly, intrasynovially, intramuscularly, or intraperitoneally, as injectable dosages of the composition or combination in preferably a physiologically acceptable diluent with a pharmaceutical carrier which can be a sterile liquid or mixture of liquids such as water, saline, aqueous dextrose and related sugar solutions, an alcohol such as ethanol, isopropanol, or hexadecyl alcohol, glycols such as propylene glycol or polyethylene glycol, glycerol ketalts such as 2,2-dimethyl-1, 1-dioxolane-4-methanol, ethers such as poly(ethylene glycol) 400, an oil, a fatty acid, a fatty acid ester or, a fatty acid glyceride, or an acetylated fatty acid glyceride, with or without the addition of a pharmaceutically acceptable surfactant such as a soap or a detergent, suspending agent such as pectin, carbomers, methycellulose, hydroxypropylmethylcellulose, or carboxymethylcellulose, or emulsifying agent and other pharmaceutical adjuvants.

Illustrative of oils which can be used in the parenteral formulations of this invention are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, sesame oil, cottonseed oil, corn oil, olive oil, petrolatum and mineral oil. Suitable fatty acids include oleic acid, stearic acid, isostearic acid and myristic acid. Suitable fatty acid esters are, for example, ethyl oleate and isopropyl myristate. Suitable soaps include fatty acid alkali metal, ammonium, and triethanolamine salts and suitable detergents include cationic detergents, for example dimethyl dialkyl ammonium halides, alkyl pyridinium halides, and alkylamine acetates; anionic detergents, for example, alkyl, aryl, and olefin sulfonates, alkyl, olefin, ether, and monoglyceride sulfates, and sulfosuccinates; non-ionic detergents, for example, fatty amine oxides, fatty acid alkanolamides, and poly(oxyethylene-oxypropylene)s or ethylene oxide or propylene oxide copolymers; and amphoteric detergents, for example, alkyl-beta-aminopropionates, and 2-alkylimidazoline quaternary ammonium salts, as well as mixtures.

The parenteral compositions of this invention will typically contain from about 0.5% to about 25% by weight of the active ingredient in solution. Preservatives and buffers may also be used advantageously. In order to minimize or eliminate irritation at the site of injection, such compositions may contain a non-ionic surfactant having a hydrophile-lipophile balance (HLB) preferably of from about 12 to about 17. The quantity of surfactant in such formulation
preferably ranges from about 5% to about 15% by weight. The surfactant can be a single component having the above HLB or can be a mixture of two or more components having the desired HLB. Illustrative of surfactants used in parenteral formulations are the class of polyethylene sorbitan fatty acid esters, for example, sorbitan monooleate and the high molecular weight adducts of ethylene oxide with a hydrophobic base, formed by the condensation of propylene oxide with propylene glycol.

The pharmaceutical compositions may be in the form of sterile injectable aqueous suspensions. Such suspensions may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents such as, for example, sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethyl-cellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents which may be a naturally occurring phosphatide such as lecithin, a condensation product of an alkylene oxide with a fatty acid, for example, polyoxyethylene stearate, a condensation product of ethylene oxide with a long chain aliphatic alcohol, for example, heptadeca-ethyleneoxycetanol, a condensation product of ethylene oxide with a partial ester derived from a fatty acid and a hexitol such as polyoxyethylene sorbitol monooleate, or a condensation product of an ethylene oxide with a partial ester derived from a fatty acid and a hexitol anhydride, for example polyoxyethylene sorbitan monooleate.

The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent. Diluents and solvents that may be employed are, for example, water, Ringer's solution, isotonic sodium chloride solutions and isotonic glucose solutions. In addition, sterile fixed oils are conventionally employed as solvents or suspending media. For this purpose, any bland, fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid can be used in the preparation of injectables.

A composition of the invention may also be administered in the form of suppositories for rectal administration of the drug. These compositions can be prepared by mixing the drug with a suitable non-irritation excipient which is solid at ordinary temperatures but liquid at the rectal
temperature and will therefore melt in the rectum to release the drug. Such materials are, for example, cocoa butter and polyethylene glycol.

Another formulation employed in the methods of the present invention employs transdermal delivery devices ("patches"). Such transdermal patches may be used to provide continuous or discontinuous infusion of the compositions of the present invention in controlled amounts. The construction and use of transdermal patches for the delivery of pharmaceutical agents is well known in the art (see for example US 5,023,252). Such patches may be constructed for continuous, pulsatile, or on demand delivery of pharmaceutical agents. Controlled release formulations for parenteral administration include liposomal, polymeric microsphere and polymeric gel formulations that are known in the art. It may be desirable or necessary to introduce the pharmaceutical composition to the patient via a mechanical delivery device. The construction and use of mechanical delivery devices for the delivery of pharmaceutical agents is well known in the art. One such implantable delivery system, used for the transport of agents to specific anatomical regions of the body, is described in US 5,01 1,472.


_Dose and administration:

Based upon standard laboratory techniques known to evaluate compositions useful for the
treatment of infectious disease, in particular fungal infections, in particular *Candida albicans* infections, by standard toxicity tests and by standard pharmacological assays for the determination of treatment of the conditions identified above in mammals, and by comparison of these results with the results of known medicaments that are used to treat these conditions, the effective dosage of the compositions of this invention can readily be determined for treatment of each desired indication. The amount of the active ingredient to be administered in the treatment of one of these conditions can vary widely according to such considerations as the particular respective amounts of the combination, the tetracycline used, the dosage unit employed, the mode of administration, the period of treatment, the age and sex of the patient treated, and the nature and extent of the condition treated.

The total amount of the active ingredient to be administered will generally range from about 0.001 mg/kg to about 200 mg/kg body weight per day, and preferably from about 0.01 mg/kg to about 50 mg/kg body weight per day. Clinically useful dosing schedules will range from one to three times a day dosing to once every four weeks dosing. In addition, "drug holidays" in which a patient is not dosed with a drug for a certain period of time, may be beneficial to the overall balance between pharmacological effect and tolerability. A unit dosage may contain from about 0.5 mg to about 1500 mg of active ingredient, and can be administered one or more times per day or less than once a day. The average daily dosage for administration by injection, including intravenous, intramuscular, subcutaneous and parenteral injections, and use of infusion techniques will preferably be from 0.01 to 200 mg/kg of total body weight. The average daily rectal dosage regimen will preferably be from 0.01 to 200 mg/kg of total body weight. The average daily vaginal dosage regimen will preferably be from 0.01 to 200 mg/kg of total body weight. The average daily topical dosage regimen will preferably be from 0.1 to 200 mg administered between one to four times daily. The transdermal concentration will preferably be that required to maintain a daily dose of from 0.01 to 200 mg/kg. The average daily inhalation dosage regimen will preferably be from 0.01 to 100 mg/kg of total body weight. Preferably, compositions for inhalation are presented for administration to the respiratory tract as a snuff or an aerosol or solution for a nebulizer, or as a microfine powder for insufflation, alone or in
combination with an inert carrier such as lactose. In such a case the particles of active combination suitably have diameters of less than 50 microns, preferably less than 10 microns, for example between 1 and 5 microns, such as between 2 and 5 microns. Alternatively, coated nanoparticles can be used, with a particle size between 30 and 500 nm. A favored inhaled dose will be in the range of 0.05 to 2 mg, for example 0.05 to 0.5 mg, 0.1 to 1 mg or 0.5 to 2 mg.

It is evident for the skilled artisan that the specific initial and continuing dosage regimen for each patient will vary according to the nature and severity of the condition as determined by the attending diagnostician, the activity of the specific combination employed, the age and general condition of the patient, time of administration, route of administration, rate of excretion of the drug, drug combinations, and the like. The desired mode of treatment and number of doses of the combination of the present invention or a pharmaceutically acceptable salt or ester or composition thereof can be ascertained by those skilled in the art using conventional treatment tests.

As is common practice, the compositions will usually be accompanied by written or printed directions for use in the medical treatment concerned.

The tetracycline and fluconazole can be provided as one composition, or as different compositions which are intended for combination therapy (i.e. for simultaneous, concomitant or immediate subsequent administration). In the latter case, they will typically (although not exclusively be offered as kits.

Kits typically will comprise at least a combination of a tetracycline antibiotic and fluconazole as described herein and at least a suitable buffer. The kits may contain any of the pharmaceutical compositions or agrochemical compositions described herein. The kit may additionally contain instructions for use. Also, the kits will optionally contain devices for administration purposes (e.g. a syringe, a container with spraying nozzle).
According to specific embodiments, the use is provided of a combination of a tetracycline and fluconazole for the manufacture of a medicament for treatment of Candida infections. According to further alternatives, it is equivalent as saying that methods of treatment of Candida infections are provided for subjects in need thereof, comprising:

5 administering a combination of a tetracycline and fluconazole to said subject.

Typically, the administration of the tetracycline (e.g. doxycycline) and fluconazole will result in treatment of the Candida infection. This can be by stopping or reducing growth of the Candida, by stopping or reducing reproduction of the Candida, by stopping or reducing biofilm formation by the Candida, and/or by killing the Candida.

Administrating the combination of a tetracycline and fluconazole may be simultaneous administration, concomitant administration, subsequent administration, but it is envisaged that both substances will be simultaneously present and active in the subject for at least an amount of time necessary to result in an effect on the Candida species.

15 It is to be understood that although particular embodiments, specific configurations as well as materials and/or molecules, have been discussed herein for cells and methods according to the present invention, various changes or modifications in form and detail may be made without departing from the scope and spirit of this invention. The following examples are provided to better illustrate particular embodiments, and they should not be considered limiting the application. The application is limited only by the claims.

**EXAMPLES**

**Materials and methods to the examples**

25 **Strains and media.** Strains used in this study are listed in Table 1. Most experiments were conducted with the Candida albicans reference strain SC5314 (18). Yeast and C. albicans strains were routinely refreshed from frozen stocks at -80°C and maintained on YPD plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar). Experiments were carried out using
minimal medium (1.7 g/l Difco Yeast Nitrogen Base w/o ammonium sulphate, 5 g/l ammonium sulphate) supplemented with 2% glucose (SD), 3% glycerol and 3% ethanol (SEG), 2% galactose (SGal), or 2% Casamino acids (SCAA). Minimal medium was supplemented with uridine when \textit{ura3D/ura3D} strains of \textit{C. albicans} were used. RPMI 1640 medium with L-glutamine without sodium bicarbonate (Sigma) was buffered with 0.165 M morpholinepropanesulfonic acid (MOPS). Glucose at the final concentration of 2% was added only to RPMI-agar medium. FBS medium consisted of 10% fetal bovine serum in sterile milliQ water (Millipore). Media were solidified with 2% agar. Sabouraud dextrose agar medium was bought from Fluka. Minimal medium for yeast experiments was supplemented with histidine, lysine, leucine and uracil at appropriate concentrations (59). Leucine and uracyl were omitted from media for experiments shown in Fig. 5A and 5D, respectively. All plate tests for \textit{C. albicans} were carried out at 37°C; plate tests for \textit{S. cerevisiae} were carried out at 30°C. The standard concentration of fluconazole on plates was 10 µg/ml, whereas that of doxycycline was 50 µg/ml. Indications are given in the text for experiments conducted with different drug concentrations.

Fluconazole, doxycycline, tetracycline, gentamycin, neomycin, potassium cyanide, ferric chloride hexahydrate, ferric citrate, zinc sulphate, bathophenanthroline disulfonic acid, were added, starting from concentrated solutions, to autoclave-sterilized, pre-cooled agar media. With the exception of doxycycline, which was dissolved in 50% ethanol, all above-mentioned chemicals were dissolved in milliQ water.

\textbf{Table 1.} Fungal strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Comment</th>
<th>Source or Reference</th>
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</thead>
<tbody>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>SC5314</td>
<td>Wild type</td>
<td></td>
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<tr>
<td>FH5</td>
<td>Clinical isolate, highly resistant to fluconazole</td>
<td>Marr et al., 1997; Selmecki et al., 2008</td>
<td></td>
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**Saccharomyces cerevisiae**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Description</th>
<th>Notes</th>
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</table>
| BY4742    | MATa; his3A1; leu2A0; lys2A0; ura3A0 | Reference strain Eurosearfl
**MIC testing in broth microdilution assays.** Fluconazole MIC was determined according to the approved CLSI standard reference method for broth dilution antifungal susceptibility testing of yeasts M27-A3 (10). Inocula of *C. albicans* SC5314 and FH5 were prepared from cultures grown overnight on Sabouraud medium, adjusted to obtain final cell suspensions of $0.5 \times 10^3$ to $2.5 \times 10^3$ CFU/ml. Viable counts of the inocula were verified by plating serial dilutions on YPD plates. MIC plates were incubated at 35°C for 48 h for the reference strain SC5314, and for 24 h for strain FH5. MIC endpoints were defined as the lowest concentration of fluconazole causing a $> 50\%$ decrease in optical density (prominent decrease in turbidity, or score 2, according to CLSI guidelines), and a $> 90\%$ decrease in viability as compared to the drug-free control. Optical densities were recorded at 540 nm using a Molecular Devices SpectraMax Plus384 Absorbance Microplate Reader. Viable counts were measured plating 100 ml of $10^{-1}$ to $10^{-5}$ serial dilutions on YPD plates. For viable count checks of optically clear wells, 100 ml of undiluted wells content were also plated. Where appropriate, comparison of results obtained from plating $10^{-1}/10^{-2}$ dilutions and from 100 ml of undiluted wells content did not evidence a significant influence of drugs carry over in our experimental conditions. Experiments were repeated for a minimum of three times.

**Checkerboard assays.** Interaction of doxycycline with fluconazole was investigated by way of checkerboard tests on 96-well plates, performed according to the CLSI approved standard M27-A3 (10), with 48 h incubations for strain SC5314 and 24 h incubations for strain FH5. For SC5314, fluconazole and doxycycline concentrations tested ranged from 0.03 to 32 µg/ml and from 3.125 to 200 µg/ml, respectively. For FH5, fluconazole concentrations ranged from 0.125 to 128 µg/ml and doxycycline concentrations ranged from 25 to 800 µg/ml. Concentrations of doxycycline higher than 200 µg/ml were repeatedly found to cause cloudiness of the RPMI medium, altering spectrophotometric readings and the general outcome of experiments (our unpublished observations). Results obtained with such concentrations of doxycycline were therefore not considered further. Viable counts were performed as described above. Elimination of fluconazole tolerance, defined as incomplete growth inhibition at supra-MIC concentrations of fluconazole, by doxycycline, allowed the use of MIC-0 as an endpoint. MIC-0
was defined as the lowest drugs combination resulting in optically clear wells. The Fractional Inhibitory Concentration (FIC) index was calculated as the sum of the FICs of either drugs (MICfluconazole with doxycycline/MICfluconazole + MICdoxycycline with fluconazole/MICdoxycycline). Drug interactions were classified as synergistic when the FIC value was \( \leq 0.5 \) and antagonistic when the FIC value was \( > 4 \) (22, 41). FIC values \( > 0.5 \) but \( \leq 4 \) were not considered informative for the establishment of interactions (22, 41). The MIC for doxycycline, which was \( > 200 \ \mu g/ml \) experimentally, was considered to be 400 \( \mu g/ml \) for calculation purposes. Similarly, the MIC-0 for fluconazole and doxycycline, which resulted \( > 32 \ \mu g/ml \) and \( > 200 \ \mu g/ml \), respectively, were considered to be 64 and 400 \( \mu g/ml \). Experiments were repeated for a minimum of three times.

**MIC testing using E-test strips.** Determination of fluconazole MICs using E-test strips (AB Biodisk) was carried out on RPMI-glucose plates. Overnight cultures in YPD were diluted in sterile saline to \( OD_{600}=0.1 \) and plated using sterile swabs, following directions from the manufacturer. Plates were incubated at 37°C for 48 h after the overlay of strips. Results of E-test measurement are normally considered in agreement with those of broth microdilution tests when discrepancies between MIC values are not higher than two dilutions (48).

**Spot tests and resistance to fluconazole assays.** Cultures were grown overnight in minimal glucose medium, diluted to \( OD_{600}=1 \) and serially diluted in pre-sterilized, flat bottom 96-well plates. Tenfold (C. albicans) or fivefold (S. cerevisiae) dilutions were spotted onto indicated media using a spotter (Sigma). The spot test presented in Fig. 2A was obtained spotting 5 \( \mu l \) of each microtiter well using a multichannel pipette. Plates were incubated for the time indicated on figure legends at 37°C (C. albicans) or 30°C (S. cerevisiae). Plates containing fluconazole and/or doxycycline were used within 24 hours from preparation. All spot tests presented in this work were repeated for a minimum of three times. For experiments on resistance to fluconazole, \( 10^4 \) cells of C. albicans SC5314 were spread on SD medium containing 128 \( \mu g/ml \) fluconazole, with or without 50 \( \mu g/ml \) doxycycline. Plates were incubated at 37°C for 4 days.

**Determination of fungicidal activity.** Determination of fungicidal activity was performed in microtiter plates using the standard CLSI M27-A3 procedure for MIC determination (10), with the exception that larger inocula of \( 10^4 \) CFU/ml were used (8, 49). Plates were incubated for
either 48 or 96 h. Viable count checks were carried out plating 100 µl of appropriate dilutions or 100 ml of undiluted wells content (for optically clear wells) on YPD plates. Both kind of experiments were repeated three times.

Iron chelation assay. Iron chelation was assayed using the colorimetric SideroTec Assay kit from Emergen Bio (Maynooth, Ireland), following instructions from the manufacturer. Briefly, fluconazole, tetracycline and doxycycline at the concentrations of 25, 50, 100, 200 and 400 µg/ml were mixed with the provided reagents in flat-bottom 96 well plates, and incubated at room temperature for 15 minutes. Plates were read at 630 nm on the above-mentioned plate reader. Decreases in absorbance are inversely proportional to iron chelation activity. milliQ water was substituted for the chemicals in the negative control.

Genomic screen for multicopy suppressors of susceptibility to fluconazole + doxycycline. We transformed the S. cerevisiae genomic library in vector pFL44 constructed in the lab of F. Lacroute (60) in the yeast strain BY4742 by the lithium acetate procedure. One aliquot of each transformation was plated on SD medium containing histidine, leucine and lysine for estimation of the transformation efficiency. The remaining transformation mixture was outgrown 4 hours in liquid YPD before being spread on plates same as above containing 10 µg/ml fluconazole and 50 µg/ml doxycycline. True resistant transformants were separated from false positives by further re-streaking them in selective conditions, and by co-segregation analysis of resistance to fluconazole+doxycycline with the presence of a transforming plasmid. Interesting plasmids were extracted from yeast, transformed in Escherichia coli and retransformed into BY4742 for confirmation of their suppressor activity. Genomic inserts of suppressor plasmids were sequenced using oligonucleotides M13F (5'-GTAAAACGACGTCGAC-3') (SEQ ID NO:1) and M13R (5'-CAGGAAACGTTCGAC-3') (SEQ ID NO:2), identifying a -5.4 kb fragment containing incomplete SOD2, YHR007, ERG11 and incomplete STP2. Reintroduction of pAFC88 (see further) into BY4742 and subsequent suppression of the susceptibility to fluconazole+doxycycline of the resulting transformants confirmed ERG11 as the suppressor gene.

Plasmids used in this study. A HindIII-Smal fragment, containing ERG11 surrounded by
-730 nucleotides at its 5' and -670 nucleotides at its 3', was excised from the Lacroute library suppressor plasmid, and cloned into Hindlll-Smal YEplac181 (17), creating plasmid pAFC88. For overexpression studies in C. albicans, CaERG11 was amplified from genomic DNA of strain SC5314 using primers CaERGHs (5'-CCCAAGCTTATGGCTATTGAAACTGTCA-3') (SEQ ID NO:3) and CaERG11as (5'-GCGGCTAGCTGAATCGAAAGAAAGTTGCCG-3') (SEQ ID NO:4). The amplified fragment was subcloned Hindlll-Nhel, replacing the Luciferase gene, in plasmid Cip10::ACT1p-gLUC59 (15), and in plasmid pPCK1-GFP (5), replacing GFP. The resulting plasmids are named pAFC89b (ACT1p-CaERG11) and pAFC92a (PCK1p-CaERG11). Cloned CaERG11 was sequenced to verify the absence of mutations.

Quantitative real-time PCR. Cells of the AFA59b, AFA60a and AFA63a strains were grown to mid-log phase in minimal glucose medium before their RNA was extracted. For AFA63a, cells were collected, split, and each half was resuspended in either SD or SCAA media, and outgrown for 4 hours before RNA extraction. Complementary DNA was prepared from DNase-treated RNA samples with the Reverse Transcription kit A3500 (Promega). Quantitative PCR was performed on a StepOnePlus real-time PCR system (Applied Biosystems) using the Kapa SYBR Fast kit (Kapabiosystems). The fold regulation of each target gene was calculated using the DDCi method, using expression of the TEF1 to normalize data. The Ct value of TEF1 was subtracted from that of CaERG11 to calculate the DCf. The DCf value of AFA60a was used as a reference, and was therefore subtracted from the DCf value of the other samples, to obtain DDCi values. Gene expression levels relative to the reference were expressed as 2-^DDCi^. Primers used are: CaERG1 1up (5'-TTACCTCATTATTGGAGACGTGATG-3') (SEQ ID NO:5), CaERG1 1down (5'-CACGTTCTCTTCTCAGTTTAATTTCTTTC-3') (SEQ ID NO:6), TEF1a-fw (5'-CCACTGAAGTCAAGTCCGTTGA-3') (SEQ ID NO:7), and TEF1a-rv (5'-CACCTTCAGCCAATTGTTCTG-3') (SEQ ID NO:8).
Example 1: Doxycycline acts synergistically with fluconazole in a dosage-dependent manner.

Growth of wild-type Candida albicans SC53 14 cells is prevented in minimal medium containing a combination of fluconazole (10 μg/ml) and doxycycline (50 μg/ml) at concentrations at which these chemicals cause either no growth defects (doxycycline) or only have a mild one (fluconazole) when used separately (Fig. 1). Growth inhibition exerted by doxycycline in the presence of fluconazole is dosage dependent (Fig. 1A), suggesting that the action of doxycycline is specific. No growth of any kind was observed on fluconazole-doxycycline plates containing 50 μg/ml doxycycline, even when these were incubated for up to 10 days (our unpublished observations). Tests on different media, such as RPMI-glucose, YPD, and fetal bovine serum-containing medium, produced identical results (Fig. 1B), demonstrating that growth inhibition is not dependent on the composition of the medium. Similar results were also obtained when glucose was replaced with glycerol and ethanol in the medium (Fig. 1B), indicating that growth inhibition is not dependent on the carbon source. Finally, tests in liquid media produced the same results as those on solid media (our unpublished observations).

Example 2: Tetracycline acts synergistically with fluconazole with minor efficiency.

Tetracyclines are antibacterial drugs that prevent the association of acylated tRNAs with the ribosome, therefore inhibiting translation (9). Since doxycycline is a derivative of tetracycline with improved pharmacokinetic properties, we decided to test whether amelioration of the antifungal properties of fluconazole was an exclusive feature of doxycycline or whether it could be extended to tetracycline.

Tetracycline proved to be also synergistic with fluconazole, albeit with reduced potency (Fig. 1C). In fact, even though an inhibitory effect of fluconazole plus tetracycline could be observed against C. albicans, this was less robust than that observed with doxycycline, given that a higher concentration of tetracycline was required to prevent fungal growth. Notably, the 200-μg/ml tetracycline concentration that appeared to completely prevent growth of C. albicans on solid medium was considerably less efficient in liquid medium (our unpublished observations).
Gentamicin and neomycin, two unrelated antibiotics that also prevent bacterial translation, showed no effect in combination with fluconazole against *C. albicans* (Fig. 1C).

**Example 3: Doxycycline eliminates fluconazole tolerance.**

Using the CLSI standard reference method (M27-A3) for broth dilution antifungal susceptibility testing of yeasts (10), the MIC for fluconazole of standard wild-type strain SC5314 was 0.5 µg/ml, whereas the MIC of doxycycline alone was >200 µg/ml. In order to verify the nature of interactions between the two drugs, fluconazole and doxycycline at concentrations ranging from 0.03 to 32 µg/ml and from 6.25 to 200 µg/ml, respectively, were tested in all possible concentration permutations by using checkerboard microtiter assays.

Doxycycline reduced the MIC for fluconazole 1-fold (0.25 µg/ml) when added at 200 µg/ml (not shown) and led to no detectable changes at lower concentrations (described further below). Hence, calculation of the fractional inhibitory concentration index (see Materials and Methods) produced values of >0.5 for concentrations of doxycycline that were <200 µg/ml, failing to indicate synergism with fluconazole (22, 41).

Growth of strain SC5314 at supra-MICs of fluconazole, a phenomenon referred to as "tolerance" (55), is commonly observed, fluconazole being only mildly fungistatic against this strain (33). These observations were confirmed in our experiments: Figure 2A shows robust recovery of cells incubated for 48 h with fluconazole alone at all concentrations tested (bottom row of cells) and decreases in viability in the presence of doxycycline. Starting from inocula of 10³ CFU/ml, viable counts indicated increases of 3 and 2 logs, respectively, for cells incubated in the absence or presence of 0.5 µg/ml fluconazole (Fig. 2B). In contrast, doxycycline further reduced growth in the presence of fluconazole by 1 log, and resulted in optically clear wells (Fig. 2B). Addition of 25 µg/ml doxycycline stably reduced the viable counts by 1 log and was associated with a strong potentiation of the fungistatic power of fluconazole (Fig. 2B). Higher concentrations of doxycycline caused further reductions in viable counts at supra-MICs of
fluconazole, to levels just above the detection limit of $10^1$ CFU/ml (50 µg/ml doxycycline) (Fig. 2B) or below that (100 µg/ml doxycycline) (Fig. 2B).

These observations indicate that tolerance to fluconazole is lost in the presence of doxycycline and that killing of *C. albicans* at supra-MICs of fluconazole occurs in the presence of >25 µg/ml doxycycline. Since in these experiments, determination of the 99.9% killing activity that classically defines the minimal fungicidal concentration (MFC) (16) was not possible, the MFC could not be used as an endpoint. Nevertheless, the ~3-log decrease in cell viability, compared to that of the drug-free control, observed at supra-MICs of fluconazole in the presence of 25 µg/ml doxycycline (Fig. 2B) met the requirements that define optically clear wells according to the CLSI guidelines (10), making possible the use of MIC-0 as an endpoint.

Given the MIC-0 value of fluconazole alone as >32 µg/ml and the MIC-0 value when tested in combination with doxycycline as 2 µg/ml, and with the MIC-0 values of doxycycline as >200 µg/ml alone and as 25 µg/ml in combination with fluconazole, recalculation of the FICs resulted in values of 0.031 for fluconazole and 0.063 for doxycycline. The recalculated FIC index of 0.094 indicates synergism between fluconazole and doxycycline.

Fluconazole MIC testing using Etest strips has been reported to deliver results comparable to those obtained using the broth microdilution method, within a 2-dilution margin of discrepancy (48). Consistent with the results reported above for broth microdilution MIC determinations, 50 µg/ml doxycycline did not affect the fluconazole MIC of strain SC5314 in Etest measurements (Fig. 2C). However, the robust growth of smaller colonies within the fluconazole inhibition ellipse that is normally observed in these assays (48) was completely eliminated in the presence of the antibacterial (Fig. 2C). Clearing of the subpopulation of cells growing inside the fluconazole inhibition ellipse by doxycycline represents another piece of evidence of the interference of doxycycline with cell growth at supra-MIC fluconazole concentrations.

Taken together, results obtained by broth microdilution tests and Etests indicate that doxycycline potentiates the activity of fluconazole abrogating tolerance to the antifungal.
Example 4: Doxycycline converts the action of fluconazole from fungistatic to fungicidal.

Given its fungistatic nature against C. albicans, fluconazole is unable to eradicate fungal infections, and the presence of elevated levels of the drug tends to favor the onset of drug resistance. Experiments shown in Fig. 2 indicated that killing of C. albicans cells occurred upon treatment with supra-MICs of fluconazole in the presence of 50 and 100 µg/ml doxycycline (96.5% and >99% killing, respectively, with an initial inoculum of 103 CFU/ml). In order to test whether killing of C. albicans by the fluconazole-doxycycline combination could reach the minimal fungicidal concentration (MFC) defined by 99.9% killing (16), cell survival was tested using the CLSI procedure for MIC determinations, with the modification proposed by Canton et al. (8). This consisted of using initial inocula of 104 CFU/ml. Sampling of 100 µl of the well’s volume under these conditions allows the detection of survival rates up to 101 CFU/ml, corresponding to a decrease in viability of 3 logs (99.9% killing) (49). Using this procedure, no CFU could be recovered from wells containing fluconazole at ≥1 µg/ml in the presence of 100 µg/ml doxycycline (Fig. 3A), indicating a killing activity of >99.9% of initial cells. Under similar fluconazole conditions, 50 µg/ml doxycycline led to an average detection of 2 x 101 to 4 x 101 CFU/ml (Fig. 3A), indicating 99.6 to 99.8% killing activity. However, it has to be noted that a long incubation period (96 h) was necessary to obtain such a high percentage of killing. At standard 48-h incubations, a strong fungistatic effect was observed for 50 µg/ml doxycycline (Fig. 3A), whereas for 100 µg/ml doxycycline, cell viability was already below the initial inoculum (Fig. 3A). These results corroborate those presented in Fig. 2 and demonstrate that addition of doxycycline renders fluconazole fungicidal.

Example 5: Doxycycline prevents the onset of resistance to fluconazole.

In Candida albicans, resistance to fluconazole has been documented to arise via different mechanisms, including mutations in ERG11 (encoding the drug target protein lanosterol 14a-demethylase) or increased expression of multidrug transporters and efflux pump-encoding genes such as CDR1, CDR2, and MDR1 (reviewed in references 1, 36, and 58). In recent
years, it has become clear that in patients, resistance of \textit{C. albicans} to fluconazole develops as the result of multiple genetic alterations (30, 57).

To test whether the presence of doxycycline could also influence the onset of resistance to fluconazole, we plated large numbers of \textit{C. albicans} cells on media containing a high concentration of fluconazole (128 µg/ml), with and without doxycycline. As observed in Fig. 3B, a large number of colonies became visible on plates containing fluconazole only. However, no resistant colonies were present on plates that also contained doxycycline after 4 days (Fig. 3B) nor after 10 days of incubation (our unpublished observations).

Thus, the fluconazole-doxycycline combination is instrumental in the prevention of cellular resistance to fluconazole, a result that is likely to arise from the conversion of fluconazole to a fungicidal drug. In other words, cells of \textit{C. albicans} may be killed before resistance-conferring mutations can be fixed by cell proliferation.

\textbf{Example 6: The fluconazole + doxycycline combination is also effective on a clinical isolate of \textit{Candida albicans} highly resistant to fluconazole.}

Clinical isolate FH5, isolated from a patient who had undergone marrow transplantation (34), was described as extremely resistant to fluconazole (57). Resistance in this strain is considered to be the result of isochromosome 5L duplication and loss of heterozygosity at the \textit{TAC1} gene locus, events that generated four copies of a nonhyperactive \textit{ERG11} allele and four copies of the hyperactive \textit{TAC1}-7 allele (57). When FH5 was tested for growth in fluconazole-doxycycline-containing medium, it proved able to grow in the presence of the standard concentrations of 10 µg/ml fluconazole and 50 µg/ml doxycycline (Fig. 4A). However, growth inhibition was observed when the concentration of both chemicals was raised 4-fold (Fig. 4A), although it has to be noted that, under these conditions, growth was severely retarded but not completely abolished (data not shown). Interestingly, while stepwise increases in the concentration of doxycycline alone did not affect the fitness of strain FH5, they imparted detectable growth retardation on the reference strain SC5314 (Fig. 4A) (see Discussion).
Interactions between fluconazole and doxycycline were tested by way of checkerboard tests also with strain FH5. In broth microdilution assays, 50 to 100 μg/ml doxycycline had little effect on the MIC for fluconazole of this strain, whereas a reduction from 64 to 16 μg/ml was observed in the presence of 200 μg/ml doxycycline (data not shown). Similar results were obtained by measuring the MIC for fluconazole by way of Etest strips on RPMI-glucose (Fig. 4B). The effect of doxycycline on growth at supra-MICs of fluconazole could not be established, since this phenomenon is only minimally present in FH5 (our unpublished observations).

These observations indicate that the fluconazole-doxycycline combination can also be effective against strains with increased resistance to fluconazole.

Example 7: Genome-wide screen for suppressors of fluconazole-doxycycline susceptibility in *Saccharomyces cerevisiae* reveals suppression by increased dosage of *ERG11*.

*Saccharomyces cerevisiae* and *Candida albicans* are estimated to have diverged ~800 million years ago (21). Baker's yeast offers a number of tools for genetic studies that cannot be performed with *C. albicans*, because of the diploid nature of the latter and the absence of a known sexual cycle. For this reason, a number of studies of pathogenic fungi, including susceptibility/resistance to azole antifungals, have been conducted using *S. cerevisiae* as a tool, with the aim of translating the obtained results to pathogenic species (36, 58). In order to verify whether we could use baker's yeast for genetic studies on the susceptibility to fluconazole-doxycycline, we checked whether yeast was also susceptible to this combination. Addition of doxycycline to fluconazole plates inhibited growth of *S. cerevisiae* wild-type strain BY4742, with some differences with respect to *C. albicans*. First, a dosage of chemicals higher than the standard 10 μg/ml fluconazole and 50 μg/ml doxycycline was necessary to obtain more sensitized experimental conditions (i.e., to eliminate background growth) (Fig. 5A); second, growth in fluconazole-doxycycline was severely retarded but not completely abolished,
as in the case of the pathogen (our unpublished observations). Furthermore, while we never observed the onset of stable clones of *C. albicans* resistant to fluconazole plus doxycycline, colonies of *S. cerevisiae* appeared at high frequency on plates containing this drug combination. These colonies proved to be true resistant mutants at a further analysis, since their fluconazole-doxycycline-resistant phenotype was maintained after repeated passages under nonselective conditions (absence of fluconazole plus doxycycline) (our unpublished observations).

In order to identify multicopy suppressors of fluconazole-doxycycline susceptibility, BY4742 was transformed with a yeast genomic library in a 2µ plasmid (60), and transformations were plated on minimal medium containing fluconazole plus doxycycline. Putative suppressors were separated from false positives by restreaking them on the same medium and by allowing them to lose the transforming plasmids upon growth under nonselective conditions. Strains for which uracil prototrophy cosegregated with resistance to fluconazole plus doxycycline were analyzed further: their plasmids were extracted, amplified in *E. coli*, and retransformed into yeast to confirm plasmid-linked suppression. Two plasmids that reproducibly conferred resistance to fluconazole plus doxycycline were isolated, sequenced, and found to contain the same insert (see Materials and Methods). The insert DNA was subcloned so that the second-generation plasmid contained a yeast genomic fragment carrying a single gene. By use of this procedure, the suppressor gene was identified as *ERG11* (Fig. 5A), encoding the target of fluconazole.

Overexpressed *ERG11* is already known to confer resistance to fluconazole and itraconazole in *S. cerevisiae* (24, 32), a result that was confirmed here (Fig. 5A). Hence, these results suggest that, compatibly with the synergistic hypothesis, doxycycline enhances the toxicity of fluconazole rather than adding toxicity via a different mechanism.

**Example 8: Overexpression of CaERG11 is not sufficient for resistance to fluconazole and doxycycline in *C. albicans*.**

Isolation of *ERG11* as a multicopy suppressor in *S. cerevisiae* prompted us to test whether the corresponding gene of *C. albicans* was also capable of suppression. The *Candida* Genome
database (http://www.candidagenome.org) indicated ORF19.922 and ORF19.8538 as the C. albicans ERG11 alleles (hereafter referred to as CaERG11), with the corresponding predicted proteins differing at two amino acids only. We amplified ORF1 9.922 and cloned it in two Clp10-derived plasmids (37), so as to obtain expression of CaERG11 under the control of the strong constitutive ACT1 promoter (15) or the strong, gluconeogenic carbon source-inducible PCK1 promoter PCK1p (5). Both plasmids were integrated at the RPS10 locus of the wild-type CAI4 strain of C. albicans and tested for suppression. The corresponding strains failed to grow in the presence of the fluconazole-doxycycline combination (Fig. 5B). Furthermore, no difference between growth of these strains and their parental ones was evidenced, even when smaller amounts of doxycycline were used (Fig. 5B) (our unpublished observations), nor was any difference in the MIC for fluconazole observed (our unpublished data). Overexpression of ERG11 in these transformants was verified via quantitative real-time PCR and shown to be ~3-fold higher in overexpression strains than that in the controls (Fig. 5C).

Finally, since overexpression of CaERG11 failed to suppress susceptibility to the fluconazole-doxycycline combination in C. albicans, we decided to investigate whether this failure was to be attributed to a functional divergence of the CaErg11 versus Erg11 protein or to a wider difference in the physiology of C. albicans versus S. cerevisiae. For this purpose, the CaERG11 gene was cloned in a yeast vector under the control of a galactose-inducible promoter, and the corresponding transformants were tested for suppression of fluconazole and fluconazole-doxycycline susceptibility. As shown in Fig. 5D, yeast cells overexpressing CaERG11 were less susceptible to fluconazole and fluconazole plus doxycycline than control cells, demonstrating that increased dosage of CaErg11 can suppress fluconazole susceptibility in yeast but not in C. albicans, and, as a consequence, that the suppression activity is retained by the CaERGH gene. Taken together, the results on overexpression of ERG11 in the two yeast species underline the difference existing between them in terms of their responses to the antifungal fluconazole.
Example 9: Doxycycline potentiates the action of fluconazole via iron chelation.

Metal chelation by tetracycline antibiotics in vitro, with a high affinity for Fe$^{3+}$ ions, has been described in the past (reviewed in reference 39). Grenier and collaborators (19) proposed that the activity of tetracyclines against *Actinobacillus actinomycetemcomitans* may be due not only to their antibacterial properties, but also to their iron-chelating activity. In addition, doxycycline is also known to be an inhibitor of mammalian matrix metalloproteases, an action mediated by chelation of the catalytic Zn$^{2+}$ ions of these enzymes (20, 53).

Recovery of Erg11, a hemoprotein, as a multicopy suppressor of hypersusceptibility of baker's yeast to fluconazole plus doxycycline led us to hypothesize that perturbation of iron homeostasis in the presence of doxycycline may play a role in establishing the hypersusceptibility of *C. albicans* to fluconazole. To this aim, we tested whether addition of ferric iron could reverse the doxycycline effect. As can be observed in Fig. 6A and B, addition of ferric chloride or ferric citrate restored growth in the presence of fluconazole plus doxycycline, rescuing *C. albicans* as well as baker's yeast from hypersusceptibility.

Importantly, growth in the presence of fluconazole plus doxycycline was not restored by the addition of zinc, nor did addition of Fe$^{3+}$ alter growth of *C. albicans* in the presence of fluconazole alone (our unpublished observations).

Iron-chelating activity of tetracyclines was confirmed using a colorimetric assay. We could demonstrate that both tetracycline and doxycycline chelate iron, with doxycycline showing higher affinity than tetracycline (Fig. 6C), whereas fluconazole showed no intrinsic iron-binding activity. Notably, the stronger iron-chelating activity of doxycycline correlated with its stronger synergism with fluconazole.

Potentiation of the action of fluconazole against *C. albicans* by iron depletion has been demonstrated by Prasad et al. (50), via the addition of the iron chelator bathophenanthroline disulfonic acid (BPS) to the growth medium. While we confirmed this observation (Fig. 6D), we also noticed that a concentration of BPS of as little as 20 µg/ml had a negative effect on cell growth even when used alone (Fig. 6D), as also observed in *S. cerevisiae* (12).
Taken together, the observations described here indicate that iron depletion by doxycycline may underlie its synergism with fluconazole.

**Example 10: Synergism between fluconazole and doxycycline is not mediated by Calcineurin.**

Despite showing no intrinsic antifungal activity of their own, the immunosuppressants cyclosporine (CsA) and FK506 were reported to be synergistic with fluconazole against *Candida albicans* (31, 33), converting fluconazole to a fungicidal drug and eliminating trailing growth (33). The synergistic action of fluconazole with CsA and FK506 is mediated by inhibition of the phosphatase activity of calcineurin by the immunosuppressants (13, 55).

Given the similarities between the combined action of CsA/FK506 and doxycycline with fluconazole, we decided to investigate on the possibility that a decrease in or abrogation of calcineurin activity may also result from the presence of doxycycline. For this purpose, we made use of *cna* Δ/*cna* Δ mutants lacking subunit A of calcineurin (55). If doxycycline exerts its action via inhibition of calcineurin, one would expect the presence of doxycycline to have no effect on the hypersusceptibility of these mutants to fluconazole. Hypersusceptibility of *cna* Δ/*cna* Δ mutants to fluconazole (55) was confirmed in our tests (Fig. 7). However, growth of these mutants in the presence of the antifungal was completely inhibited by concentrations of doxycycline that only mildly affected wild-type cells (Fig. 7), indicating that doxycycline further compounds the hypersusceptibility of *cna* Δ/*cna* Δ mutants to fluconazole.

Identical results were obtained using *cnb* Δ/*cnb* Δ mutants (13) lacking subunit B of calcineurin (our unpublished data).

Calcineurin is inactive under normal conditions, being activated only in the presence of certain external cues (52). A strain of *C. albicans* in which calcineurin was permanently activated by removal of the self-inhibitory C-terminal domain of Cna1 was less susceptible to fluconazole (55). While we confirmed a moderately increased tolerance to fluconazole in this strain (our unpublished observations), we also found this strain to be as susceptible to the fluconazole-doxycycline combination as an isogenic control strain carrying a reintegrated copy of wild-
type CNA1 (Fig. 7), demonstrating that constitutively active calcineurin is incapable of rescuing the cells' susceptibility to fluconazole plus doxycycline. Taken together, these observations suggest that the synergism of doxycycline with fluconazole is not mediated by inhibition of calcineurin activity.

Discussion

We found that doxycycline, and to a lesser extent tetracycline, two licensed antimicrobials that prevent bacterial protein synthesis, potentiate the antifungal activity of fluconazole against Candida albicans in a dosage-dependent manner. Doxycycline converts the action of fluconazole from fungistatic to fungicidal and prevents the onset of drug resistance. Addition of doxycycline appears to have a major impact on prevention of fluconazole tolerance, defined as incomplete growth inhibition at supra-MICs of fluconazole. This finding is consistent with the role of doxycycline in converting fluconazole to a fungicidal drug and may also have implications in the prevention of drug resistance.

Other authors have reported on alteration of C. albicans susceptibility to fluconazole in the presence of tetracyclines (42, 43). Odds and collaborators (42) observed a strong reduction of the MIC for fluconazole and elimination of trailing growth by doxycycline when they used diagnostic susceptibility test agar medium, but not when tissue culture-based medium was used. On the other hand, Oliver and collaborators (43) reported on a positive effect of tetracycline on a fluconazole MIC of strain SC5314 in broth microdilution tests and a negative one when MIC was measured using Etest strips. Given the similarity between the bacterial and mitochondrial ribosomes, these authors proposed that increased susceptibility to fluconazole might result from the action of tetracycline against mitochondrial translation. Consistently, they reported inhibition of respiratory growth of C. albicans in the presence of 200 \( \mu g/ml \) tetracycline (43). We report here that the doxycycline MIC is higher than 200 \( \mu g/ml \). Furthermore, the 50-\( \mu g/ml \) concentration of doxycycline used throughout this study does not affect growth of C. albicans on any medium tested, including medium containing ethanol and glycerol, carbon sources that can only be metabolized via mitochondrial respiration. It therefore appears
unlikely that the synergistic effect of fluconazole plus doxycycline can be attributed to a perturbation of respiration by doxycycline. Furthermore, we tested whether inhibition of mitochondrial respiration could also be synergistic with fluconazole. Although a substantial growth defect could be observed in the presence of fluconazole when respiration was blocked by cyanide, this growth impairment appeared to be the sum of individual contributions of either chemical to the cells’ fitness, rather than a synergistic effect, and did not resemble the strong effect of fluconazole plus doxycycline (our unpublished data).

Addition of iron to the growth medium restored growth of C. albicans, as well as that of S. cerevisiae, in the presence of fluconazole plus doxycycline, suggesting that hypersusceptibility to the drugs in combination could be due to antibiotic-mediated titration of iron. Consistent with this hypothesis, we confirmed that doxycycline and, to a minor extent, tetracycline act as iron chelators. Interestingly, while iron was found to be a chemical suppressor of fluconazole-doxycycline hypersusceptibility in baker’s yeast as well as in C. albicans, an increased dosage of ERG1 1 rescued only the growth defect of S. cerevisiae. Iron depletion by doxycycline may result in lower incorporation of heme in the Erg1 1 protein in yeast, and an increase in the copy number of Erg1 1 may help the protein compete for heme. Alternatively, since iron deprivation has been demonstrated to result in downregulation of ERG1 1 in C. albicans (25, 50), it is possible that a similar situation occurs in S. cerevisiae and that ERG1 1 overexpression may simply restore gene expression to wild-type levels. Whatever the mechanism in baker’s yeast, the situation appears to be more complex in C. albicans. In this organism, iron depletion as the major cause of growth inhibition has been demonstrated by reversal upon addition of ferric iron to the medium. However, the failure of an increased dosage of CaERG1 1 to suppress growth inhibition in C. albicans, despite full competence of the corresponding protein to act as a suppressor in yeast, suggests that doxycycline-mediated iron depletion may have more severe effects in this fungus. This interpretation is also corroborated by the higher susceptibility to fluconazole plus doxycycline observed in C. albicans with respect to S. cerevisiae (see the text above and the panels with the fluconazole-doxycycline combination in Fig. 5A and andB).B). Alternatively, lower levels of gene overexpression obtained in C. albicans compared to those
achieved in the yeast system could provide a simpler explanation for our observations. In fact, we found that overexpression of CaERG11 in our C. albicans strains was 3-fold higher than that under normal conditions (Fig. 5C), whereas overexpression in yeast transformants carrying ERG11 on a multicopy plasmid was ~10- to 11-fold higher than that in the untransformed control (our unpublished data). However, several observations argue against this simple threshold hypothesis for failed suppression by CaERG11 in C. albicans. First, the C. albicans overexpressers show no signs of suppression even when mild, low-stringency conditions of fluconazole plus doxycycline are applied (Fig. 5B) (our unpublished observations). Second, if high levels of CaERG11 were necessary and sufficient per se to confer a resistant phenotype, one would expect the onset of stable, spontaneous mutants of C. albicans resistant to fluconazole plus doxycycline at high frequency, as predicted from the frequency of mutations in single genetic loci. Mutations in CaFKSI conferring resistance to the antifungal caspofungin, for instance, have been reported to occur at a frequency of \(<10^{-8}\) mutations per cell per generation (4). The fact that we never recovered spontaneous mutants resistant to fluconazole plus doxycycline (our unpublished observations) favors the hypothesis that multiple genetic alterations may be necessary for such resistance to occur. On the other hand, while strong suppression of susceptibility to fluconazole upon overexpression of ERG11 in baker's yeast is reported in this article and has also been described by others (24, 32), a similar clear-cut correlation has not been established in C. albicans. The only published paper, to our knowledge, describing resistance to fluconazole upon overexpression of CaERG11 in C. albicans reports a moderate resistance in cells with ~11-fold increased levels of CaERG11 (14). Although certain mutations in CaERG11 (54), as well as increased CaERG11 mRNA levels (46, 61), have been associated with resistance to fluconazole in C. albicans, it has become increasingly clear in recent years that decreased susceptibility to fluconazole in this fungus derives from complex genetic alterations that comprise and are possibly not limited to genomic rearrangements and deregulated expression of several genes (11, 30, 57).

Iron depletion has been proposed to decrease ergosterol content in C. albicans, leading to higher fluidity in cell membranes, with consequent increased passive diffusion of fluconazole.
Some of the results reported in this paper appear similar to those obtained using BPS as the iron chelator: addition of 200 µM BPS lowered the MIC of C. albicans for fluconazole (50), and so did that for 200 µg/ml doxycycline. Importantly, the iron chelation properties of the two chemicals at the indicated concentrations are similar (our unpublished observations). Minor differences could be due to different experimental conditions.

We report here that fluconazole and doxycycline are also effective against a clinical strain of C. albicans with previously acquired resistance to fluconazole, although a higher dosage of the chemicals is required in this case. While elevated dosage of doxycycline alone has a negative impact on growth of the reference strain SC5314 (without reaching the MIC), presumably by iron depletion, clinical strain FH5 is insensitive to these conditions, suggesting that the ability of this strain to thrive in a low-iron environment and resistance to fluconazole may be linked. Interestingly, transcription of the iron transporters FRE9 and FTR1—the latter required for growth under low-iron conditions (51)—is upregulated in response to exposure to fluconazole (27).

Recently, a role for C. albicans Als3 in iron acquisition from host ferritin has been proposed (2). We therefore tested whether als3Δ/als3Δ mutants were differentially susceptible to fluconazole plus doxycycline and found them to be as susceptible as their wild-type counterpart (our unpublished observations), a result that argues against a role for Als3 in the establishment of doxycycline-mediated potentiation of fluconazole activity. Similarly, our experiments argue against a role for calcineurin in this process.

From a practical point of view, since experiments with doxycycline-regulated promoters are an established reality in the field of C. albicans molecular genetics, we propose that the interesting results obtained using this system should be further confirmed by experiments performed using media with additional iron, in order to discriminate between phenotypes that can be attributed to altered dosage of the gene of interest and phenotypes that may have arisen from doxycycline-mediated perturbation of iron homeostasis.
REFERENCES


CLAIMS

1. A combination of a tetracycline antibiotic and fluconazole.
2. The combination according to claim 1, wherein the tetracycline antibiotic is doxycycline.
3. The combination according to claim 1 or 2 for use as a medicament.
4. The combination according to claim 1 or 2 for use in treatment of Candida infections.
5. Use of a combination of a tetracycline and fluconazole, particularly of doxycycline and fluconazole, for the manufacture of a medicament for treatment of Candida infections.
6. A method of treating a Candida infection in a subject in need thereof, comprising:
   - administering to the subject a combination of a tetracycline and fluconazole, thereby treating the Candida infection.
7. The method according to claim 6, wherein the tetracycline is doxycycline.
8. The combination for use according to claim 4, the use according to claim 5, or the method according to claim 6 or 7, wherein said Candida is Candida albicans.
9. The combination for use, use or method according to claim 8, wherein said Candida albicans is a fluconazole resistant Candida albicans.
10. A composition comprising a combination of a tetracycline and fluconazole, particularly a combination of doxycycline and fluconazole.
11. A kit comprising:
   - a tetracycline and a pharmaceutically acceptable excipient;
   - fluconazole and a pharmaceutically acceptable excipient.
Figure 1

A

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Figure 2

A

![Doxycycline and Fluconazole Interaction](image1)

B

![Fluconazole Concentration vs. CFU/mL](image2)

C

![PET Plate Comparison](image3)
Figure 3

A

[Graph showing the effect of Fluconazole on log_{10} CFU/ml over different concentrations. The graph includes lines for different concentrations and markers for inoculum and limit of detection.]

B

without Dox

[Image of a petri dish with no visible growth.]

with Dox

[Image of a petri dish with visible growth.]
Figure 5

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C

![Graph showing relative fold variation](image)

D

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Figure 7

SD | Dox 50
---|---
SC5314  
cna1Δ/cna1Δ (DSY2091)  
cna1Δ/cna1Δ; CNA1 (DSY2146)  
cna1Δ/cna1Δ; CNA (DSY2115)

Flu | Flu Dox 10
---|---

Flu Dox 25
### A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K31/4196 A61K31/65 A61P31/10

### ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

**A61K**

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

**EPO-Internal**, **BIOSIS**

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>CN 102 626 415 A (QIANFOSHAN HOSPITAL SHANDON) 8 August 2012 (2012-08-08) Synergistic combination of doxycycline and fluconazole for the treatment of fungal infections, in particular induced by Candida. (See claims 1-3, 7, figure 4, Section on &quot;Summary of the invention&quot;, page 5)</td>
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Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:

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**P** document member of the same patent family

Date of the actual completion of the international search: 8 May 2013

Date of mailing of the international search report: 05/06/2013

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Fax: (+31-70) 340-3016

Authorized officer

Veronese, Andrea
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| X,P      | FIORI ALESSANDRO ET AL: "Potent synergic effect of doxycycline with fluconazole against Candida albicans is mediated by interference with iron hostasis."
          | ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 56, no. 7, July 2012 (2012-07), pages 3785-3796, XP002696554, ISSN: 1098-6596
          | Doxycycline acts synergically with the anti fungal fluconazole against Candida Albicans, transforms fluconazole from fungi static to fungicidal, and prevents the onset of resistances. |
| X        | MICELI MARISA H ET AL: "In vitro analyses of the combination of high-dose doxycycline and anti fungal agents against Candida albicans biofilms.", INTERNATIONAL JOURNAL OF ANTIMICROBIAL AGENTS, vol. 34, no. 4, October 2009 (2009-10), pages 326-332, XP002696555, ISSN: 1872-7913
          | Combinations of doxycycline and fluconazole show synergistic activity on Candida Albicans biofilms: see abstract, figure 2, results, and in particular page 327, right hand column, and page 331 |
| Y        | YOUNGSAYE WILLMEN ET AL: "Overcoming fungal resistance in Candida albicans clinical isolates with tetracycline indoles.", BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, vol. 22, no. 9, 22 February 2012 (2012-02-22), pages 3362-3365, XP002696556, ISSN: 1464-3405
<pre><code>      | Tetracycline indoles synergize stably potentiate the activity of fluconazole, converting it from a fungicidal agent, and overcoming fungal resistances: see abstract, figures, tables and page 3364, right hand column. |
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<td>YOUNGSAYE WILLMEN ET AL: &quot;Pi perazi nyl quinolines as chemosensitizers to increase fluconazole susceptibility of Candida albicans clinical isolates .&quot;, BIOORGANIC &amp; MEDICINAL CHEMISTRY LETTERS, vol. 21, no. 18, 15 September 2011 (2011-09-15), pages 5502-5505, XP002696557, ISSN: 1464-3405 Pi perazi nyl quinolines synergistically potentiate the activity of fluconazole, converting it from a fungi static to a fungicidal agent, and overcoming resistance: see abstract, figures, tables and page 5504, right hand column</td>
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<td>UPPULURI PRIYA ET AL: &quot;Synergistic effect of calci neurin inhibitors and fluconazole against Candida albicans biofilms .&quot;, ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 52, no. 3, March 2008 (2008-03), pages 1127-1132, XP002696558, ISSN: 0066-4804 Calci neurin inhibitors synergistically potentiate the activity of fluconazole, converting it from a fungi static to a fungicidal agent, and prevent biofilms: see abstract, figures, tables, results and discussion right hand column</td>
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