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Interactions of a prenylated flavonoid from *Dalea elegans* with fluconazole against azole- resistant *Candida albicans*

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ABSTRACT

Background: The prenylated flavonoid 2′, 4′-dihydroxy-5′-(1‴, 1‴-dimethylallyl)-8-prenylpinocembrin (8PP, formerly 6PP) shows antifungal activity, inhibits rhodamine 6G efflux and reverses fluconazole (FCZ) resistance in azole-resistant *Candida albicans* overexpressing cdr1, cdr2 and mdr1 transporters.
Purpose and design: In this paper, we tried to characterize 8PP *in vitro* interactions on the cell growth and lethality of *C. albicans*. We also initiated preliminary *in vivo* toxicological studies on mice.
Methods: The effects of 8PP and FCZ on cell growth and viability of *C. albicans* were evaluated by CLSI guidelines. The checkerboard assay was used to search for interactions on cell growth. The time-kill assay was used to study fungicidal effects. Acute toxicity was evaluated at a single dose schedules.
Results: From the checkerboard design, and using a starting inoculum of 10^3 CFU/ml, the fractional inhibitory concentration (FIC) of FCZ and 8PP could be determined as 0.11 and 0.50, respectively, with a FIC index value (FICI) of 0.61. This FICI and the isobologram showing a concave shape suggests an additive interaction between them. At a higher starting inoculum (10^5 CFU/ml), *C. albicans* growth and viability were decreased by FCZ, 8PP and their combination in a concentration-dependent way. For FCZ, minimum fungicidal concentration (MFC) and FC_{50} (the concentration that kills 50% of the fungal cells) were 4-fold reduced (280–70 μM) in combination with 125 μM 8PP. A decrease of 3 log units in viable counts with respect to control was reached (3.65 ± 1.05 ‰, $p < 0.0001$). Thus, both fungistatic compounds when combined achieved an almost complete fungicidal effect at lower concentrations respecting of each of them alone. In preliminary toxicological assessment, lethal dose 50% (LD_{50}) for 8PP by the i.p. route was 357 and 245 mg/kg, for female and male adult albino mice, respectively. FCZ LD_{50} was 785 and 650 mg/kg for female and male animals, respectively
Conclusions: In vitro results suggest additive interactions between 8PP and FCZ with respect to *C. albicans* cell growth. Besides killing *per se*, 8PP helps FCZ to achieve an almost complete fungicidal effect, which would be crucial to eradicate fungal infections.

Introduction

Opportunistic mycosis have increased in recent decades due to the increasing number of immunosupressed individuals such as those infected with HIV. *Candida albicans* is an opportunistic fungus that produces important mucosal and disseminated infections, particularly in patients with compromised immune system (Kim and Sudbery, 2011).

Treatment of candidiasis is generally carried out with azole antifungals and resistance to these drugs has been widely developed in the last times. Efflux pumps play a major role in resistance and may represent a new therapeutic target. In fact, by inhibiting such transporters, the concentration of antimicrobials within the microorganisms can be increased and therefore resistance can be reversed (Ghannoum and Rice, 1999).

Abbreviations: 8PP, 2′, 4′-dihydroxy-5′-(1‴, 1‴-dimethylallyl)-8-prenylpinocembrin; ABC, ATP-binding cassette; FCZ, fuconazole: FIC, fractional inhibitory concentration; FICI, fractional inhibitory concentration index; LD50, lethal dose 50 %; MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration; FC50, fungicidal concentration 50 %; YPD, yeast peptone dextrose; DMSO, dimethylsulphoxide

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The prenylated flavanone 2′,4′-dihydroxy-5′-(1‴,1‴-dimethylallyl)-8-prenylpinocembrin (8PP) inhibited ABC cdr transporters involved in azole extrusion and reversed FCZ resistance in *C. albicans* (Peralta et al., 2012). Formerly identified as flavanone 2′, 4′-dihydroxy-5′-(1‴, 1‴-dimethylallyl) – 6-prenylpinocembrin (6PP), this compound was isolated from the plant *Dalea elegans* Gillies ex Hook et Arn, (Fabaceae) (Cafaratti et al., 1994; Peralta et al., 2014). In bioactivity studies, it showed antibacterial and antifungal activities *per se* even against multiresistant strains (Pérez et al., 2003). In other studies, the flavonoid showed both antioxidant and antiradical activities; it also inhibited enzymatic lipid peroxidation, Krebs cycle, electron transport, proton potential, ATPsynthase and ATPase in rat hepatocytes and showed toxic effects against human tumor cells (Elingold et al., 2008).

Since cooperative interactions between 8PP and FCZ were suggested by some results (Peralta et al., 2012; 2015; Elingold et al., 2008; Barceló et al., 2015), in this work we decided to check this hypothesis on cell growth. Since no relevant fungicidal effect has been reported by FCZ *per se* (Klepser et al., 1997), we wanted to dilucidate whether the combination with 8PP could improve it. This fact would be crucial to eradicate fungal infections, overcoming the major disadvantage of azole antifungals (Pfaller et al., 2004; Meletiadis et al., 2007; Fiori and van Dijck, 2012). To continue the search for effective and less toxic antifungals, we also conducted preliminary *in vivo* studies on acute toxicity on experimental animals.

Materials and methods

Compounds

The compound 8PP was obtained from roots and aerial parts of *D. elegans* according to Cafaratti et al. (1994). Its structure was characterized by UV, NMR1H and 13C and HRMS, according to Peralta et al. (2014). The purity of 8PP was determined as 95% by using a Varian (Palo Alto, CA) Pro Star HPLC equipment which was coupled with a UV detector (Varian). The elution was carried out in a Varian C18 column (Ø250 mm × 4.6 mm), in order to analyze the purity of 8PP, under two different conditions. Method 1: mobile phase: (A) H_2O, (B) MeOH; elution program: linear gradient from 50% B to 85% Bin 60 min, followed by 100% B maintained for 10 min and finally linear gradient from 100% B to 50% B in 10 min. Method 2: mobile phase was composed of acetonitrile with 1% acetic acid (A) and H_2O with 1% acetic acid (B) with gradient elution system: 0–10 min, 10–40% A; 10–15 min, 40–50% A, maintained for 5 min; 20–25 min, 50–60% A; 25–30 min, 60–30% A; 30–35 min, 30–10% A, maintained for 5 min. Both methods were performed at a flow rate of 1.0 ml/min; detection wavelength: 290 nm; injection volume: 20 µl; temperature: 30 °C.

FCZ (purity higher than 98%) was obtained from Sigma Aldrich (Saint-Louis, MO, USA). Amphotericin B (activity equivalent to 5 mg per ml, 98% purity) was a gift from Laboratorios Richet S.A. (Buenos Aires, Argentina).

Microorganisms

An azole-resistant *C. albicans,* isolated from the oral cavity, was used. It was a gift from Dr. T. White (University of Washington, Seattle, USA). It has been numbered as 12–99 and overexpresses the transporter genes CDR1, CDR2 and MDR1 (White et al., 2002). For the experiments, it was cultured in yeast peptone dextrose (YPD) broth or Sabouraud agar medium and then resuspended according to the assay performed.

C. albicans growth assay

It was evaluated according to the CLSI-approved standard M27-A3 (CLSI, 2008) as described by Peralta et al. (2012) with some modifications in starting inoculum or incubation time. As consigned for different experiments, a starting inoculum of 10^3 or 10^5 colony forming units per ml (CFU/ml) was cultured in liquid medium using a microtiter 96-well plate. In some experiments cellular growth was followed through different incubation times using a starting inoculum of 10^3 CFU/ml.

The compounds were added from dimethylsulphoxide (DMSO) stock 20 mM preparations and diluted in saline solution to give different concentrations in the incubation medium. Samples were compared to the respective control containing the same solvent. Absorbance was measured at 540 nm with a *MicroQuant* microplate spectrophotometer, (Winooski, VT, USA).

Checkerboard microtitter plate testing

The checkerboard method was chosen to determine antimicrobial interactions such as synergism and antagonism between 8PP and FCZ on *C. albicans* cell growth. It was carried out according to the CLSI-approved standard M27-A3 (CLSI, 2008) as described by Iten et al. (2009).

The upper most row (A) of a 96-well microtiter plate contained 8PP in a concentration of about four times the MIC of azole-resistant *Candida albicans* (500 µM). Each following row (B-H) contained half the concentrations of the previous one. The same procedure was carried out along the columns (1–12) with FCZ. So, each well contained a unique combination of 8PP and FCZ. The compounds were added from stock solutions as above described in the *C. albicans* growth section.

At least 100 µl of a starting inoculum of 10^3 CFU/ml were added to the wells and the plate was incubated at 37 °C for 24 h. Absorbance was measured at 540 nm. The concentration of the first wells without visible growth along the stepwise boundary between inhibition and growth were used to calculate the FICI values.

MIC, FIC and FICI were calculated. MIC for a compound was defined as the lowest concentration able to produce a growth inhibition higher than 90% when the viable counts were compared with those of the control conducted in its absence. In a spectrophotometer, it corresponds to the concentration producing an optical density of 50% (IC_{50}) or less with respect to that of the growth control when a 10^3 CFU/ml starting inoculum is used (CLSI, 2008; Marchetti et al. 2000; Peralta et al. 2012). FIC was calculated as MIC of FCZ or 8PP when are combined divided by the MIC of the compound alone. FICI was the sum of the FIC of each substance (CLSI, 2008).

Isobologram

As described by Iten et al. (2009), this plot despicts the results of the checkerboard assay and the FICI values. The FIC values of 8PP were graphed as a function of FIC values of FCZ. The line connecting the maximum value located on each axis represents no interaction (line of indifference). Below this line we find the area of additive and synergistic effects. Above the line of indifference are the combination with subtractive and antagonistic effects.

Fungicidal activity. Concentration-response curves

The activity was evaluated by counting the CFU/ml as a measure of cell viability of azole-resistant *C. albicans* The assay was performed in a microtiter 96-well using the standard CLSI M27-A3 for MIC determination (CLSI, 2008), with the exception of that a larger inoculum of 10^5 CFU/ml was used (Marchetti et al., 2000). Plates were incubated at 37 °C during 24 h. Variable concentrations of 8PP, FCZ, or their combinations were used. The content of each well was further submitted to serial dilutions and 100 µl of each resulting sample were cultured on Sabouraud agar plates. The CFU/ml were counted after 24 h. MFC and FC_{50}were calculated.

Time-kill assay

This method was carried out to get information about the time-dependent progression of the fungicidal activity, according to the CLSI-approved standard M27-A3 (CLSI, 2008) and basically as described in the previous section. A starting inoculum of 10^5 CFU/ml was cultured at different times in liquid medium using a microtiter 96-well plate at 37 °C. FCZ, 8PP or their combination were used, at a concentration of 125 μM of each compound. Viable count checks were carried out by plating and incubating as described in the previous section.

In vivo toxicological assessment

Acute toxicity was evaluated at a single dose schedules. Six-month old CF1 male and female adult albino mice, weighing 30–50 g, were used to determine the LD_{50} by Dixon's method as previously described (Nuñez Montoya et al., 2003; Dixon, 1965). The flavonoid was dissolved in 100% dimethylsulphoxide to give a 8PP concentration of 80 mg/ml to inject by the peritoneal way. The initial level was 100 mg/kg of body weight and animals were controlled for 7 days; control mice were injected with DMSO alone (app. 130 μl *per* mouse). Since DMSO is a toxic vehicle, we took the precaution of injecting doses smaller than its LD_{50} (Nuñez Montoya et al., 2003).

At the doses tested, the symptoms observed were categorized as either safety or toxicity, death, decreased spontaneous activity, trembling, loss of posture, rigidity, violet coloring of the abdomen, etc. The LD_{50} for low number of samples was calculated by Dixon´s method modified as previously reported (Nuñez Montoya et al., 2003). The principles of laboratory animal care were followed according to regulations of the University of Buenos Aires (approval number and date 003/15, 04/09/15, respectively).

Statistical analysis

GraphPad Prism version 5.00 software (GraphPad Software San Diego, CA, USA) was used to calculate the standard errors of independent experiments involving duplicate or triplicate analyses for each sample conditions. Statistical analysis was made with either one-way analysis of variance (ANOVA), Newman-Keuls multiple comparison test or the unpaired t test for two groups, as appropriate. Significance was accepted at $p < 0.05$. Data are presented as means ± S.E.M.

Results

The chemical structure of compound 8PP, used in this study, is shown in Fig. 1. In preliminary kinetic studies on *C. albicans* growth, an incubation period of 24 h was selected for further assays, since it

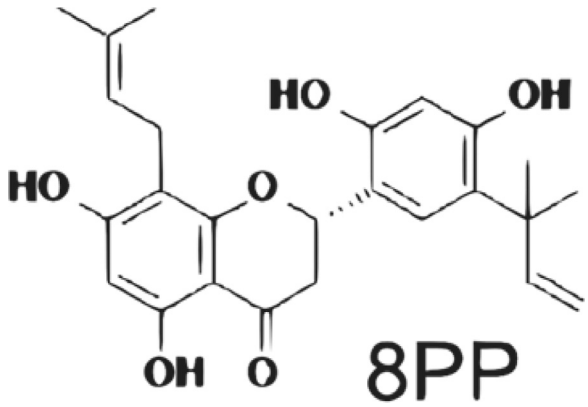

Fig. 1. Chemical structure of the compound 2′,4′-dihydroxy-5′-(1‴,1‴-dimethylallyl) – 8-prenyl-pinocembrin (8PP).

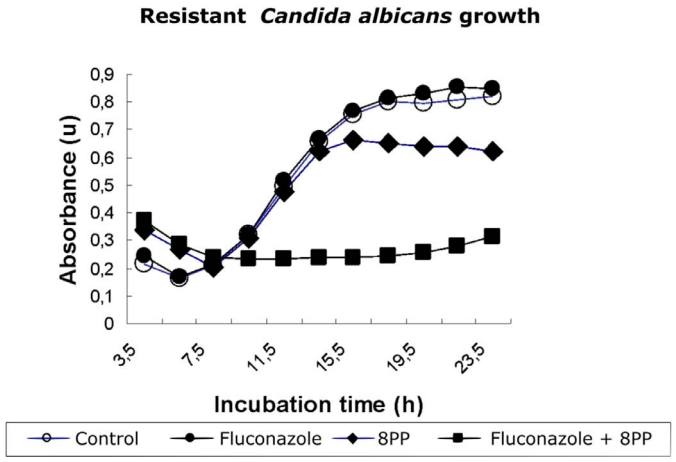

Fig. 2. Effect of 8PP, FCZ and their combination on the time course of azole-resistant *Candida albicans* growth. Cellular growth was followed through different incubation times. Absorbance was measured at 540 nm at different concentrations of 8PP, FCZ or their combination. A typical experiment is shown. It was conducted according to the CLSI-approved standard M27-A3 (CLSI, 2008). A starting inoculum of 10^3 CFU/ml was used.

allowed to reach the maximum activity of compounds (Fig. 2; other data, not shown). It should be noted that similar kinetic results were reported by Neelofar et al. (2011) for the spice curcumin against *Candida glabrata.*

Then, studies on azole-resistant *C. albicans* growth at different concentrations of FCZ, 8PP or their combinations were performed using the checkerboard model. A 10^3 CFU/ml starting inoculum was used, according to CLSI standard M 27-A3 (CLSI, 2008). Isoboles plot (Fig. 3) and Table 1 show FIC for FCZ and 8PP which could be determined as 0.11 and 0.50, respectively, with a FICI of 0.61. This value of FICI (higher than 0.5 but lower than 1) together with the isobologram showing a concave shape suggests an additive interaction (Iten et al., 2009).

In previous reports we had reported that 8PP inhibits azole ABC cdr efflux pumps and thereby reverses fluconazole resistance concerning *C. albicans* growth (Peralta et al., 2012). In this work we wanted to know if the inhibition of those transporters could affect the potential fungicidal effect of FCZ. So, in order to ensure the inhibition of FCZ efflux in the culture medium, we selected a concentration of 8PP of 125 μM, which is close to its IC_{50} (121 μM) on cdr transporters, as measured in the rhodamine 6 G efflux assay and to its MIC (150 μM), detected previously

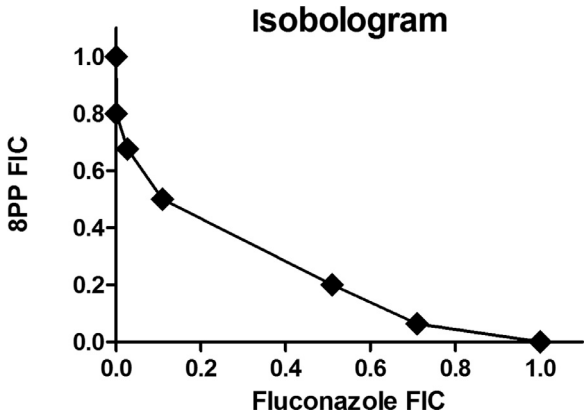

Fig. 3. Isoboles plot of azole-resistant *Candida albicans* growth. The fractional inhibitory concentration of each compound was calculated as the MIC of it in combination divided by the MIC of the compound alone. The MIC of 8PP, FCZ or their combinations on *C. albicans* growth was evaluated by the checkerboard microtiter plate test, as described in Materials and methods. A starting inoculum of 10^3 CFU/ml was used. Growth was measured as absorbance at 540 nm. Data come from 7 independent experiments made by triplicate.

Table 1
MIC, FIC and FICI of 8PP, FCZ and their combinations on *Candida albicans* growth.

MIC of Flz (μM)		MIC of 8PP (μM)				
alone	combined with 8PP	alone	combined with Flz	FIC of Flz	FIC of 8PP	FICI
1126	125	150	75	0.11	0.5	0.61

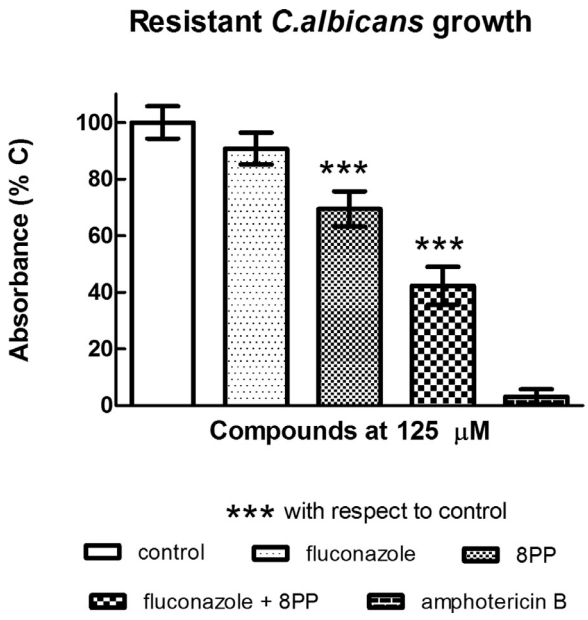

Fig. 4. Effect of 8PP, FCZ and their combination on azole-resistant *Candida albicans* growth Cellular growth was measured as absorbance at 540 nm. FCZ, 8PP or their combination were used, at a concentration of 125 μM of each compound. Data are means ± SEM from 3 independent experiments made by triplicate as described in Materials and Methods. Statistical analysis was conducted with one way analysis of variance Newman–Keuls comparison test for 3 or more groups. The combination of FCZ and 8PP was different from both control and 8PP (***) regarding their effects on absorbance.

with a starting inoculum of 10^3 CFU/ml (Peralta et al., 2012).

Since viability would be measured using starting inocula higher than those of the checkerboard assay (Marchetti et al., 2000), and considering that some compounds are inoculum-dependent, we wanted to compare the effects of the drugs on cell growth in both experimental conditions.

The combination of 8PP with FCZ at 125 μM was active, even with less efficacy than that obtained using 10^3 CFU/ml as a starting inoculum. The decrease in cell growth was 48% with respect to control, whereas 8PP inhibited 31% and FCZ only 9% (Fig. 4). The low activity of FCZ was consistent with *C. albicans* resistance. Its concentration was chosen as a reference starting point lower than its MIC (1126 μM; Peralta et al., 2012), and following criteria of Klepser et al. (1997).

In time-kill studies, after 24 h or 48 h incubation in liquid medium with the antifungal compounds tested followed by incubation in Sabouraud agar, the presence of either FCZ, 8PP or their combination increased the CFU/ml with respect to the control analyzed before incubation in liquid medium (time: 0 h). In contrast, no survival was detected with the fungicidal standard amphotericin B (Fig. 5), which would act within 3 h (Marchetti et al., 2000).

As in *C. albicans* growth, the combination of FCZ with 8PP reduced considerably cell viability. In fact, after 24 h incubation and using 125 μM of each compound, the ‰ reduction of survival values with respect to control was 21; 371; 715 for FCZ, 8PP and their combination, respectively (Fig. 6). As similar results were obtained after 48 h incubation, in the following assays a shorter incubation time of 24 h was

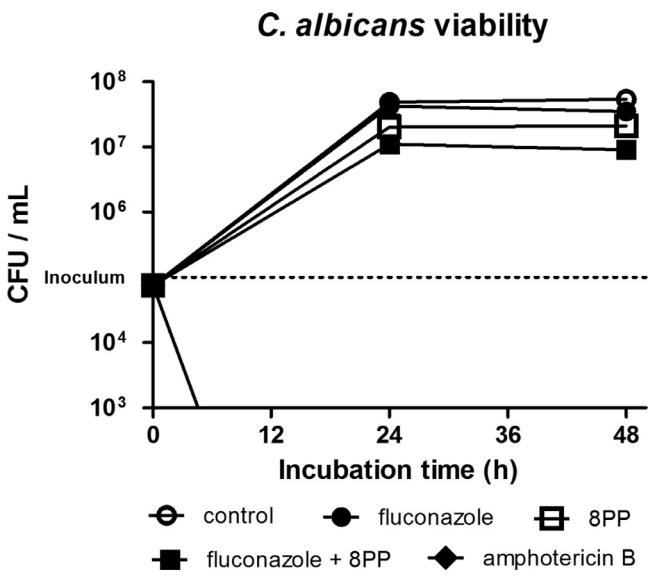

Fig. 5. Effect of 8PP, FCZ and their combination on the time-kill curves of azole-resistant *Candida albicans*. Data correspond to means and SEM from 2 to 4 statistical data corresponding to a typical experiment. Viability was evaluated as CFU/ml according to the CLSI-approved standard M27-A3 (CLSI, 2008). A starting inoculum of 10^5 CFU/ml was cultured in liquid during 24 h using a microtiter 96-well plate at 37 °C. The content of each well was further submitted to serial dilutions, which were cultured on Sabouraud agar plates. The resulting CFU/ml were counted after 24 h. FCZ, 8PP or their combination were used, at a concentration of 125 μM of each compound. Amphotericin B at 0.5 μg/ml was used as a fungicidal standard.

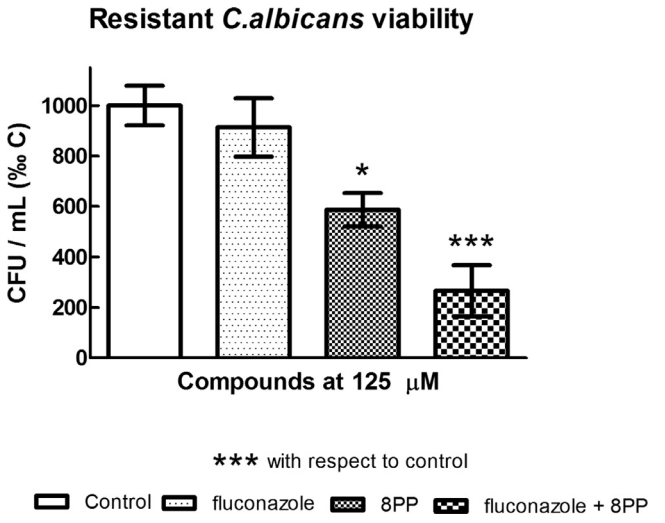

Fig. 6. Fungicidal activity of 8PP, FCZ and their combination on azole-resistant *Candida albicans*. Viability was measured after 24 h incubation, as described in Fig. 5. A starting inoculum of 10^5 CFU/ml was used. The combination of FCZ and 8PP is different from control (***) and 8PP (*) concerning their effects on CFU/ml). No survival was observed with 0.5 μg/ml amphotericin B, which was used as a fungicidal standard.

chosen.

Fig. 7 shows that FCZ in combination with 125 μM 8PP reduces *C. albicans* survival in a concentration-dependent way with higher potency than each compound alone. The presence of 8PP produces a shift to left on the FCZ curve. Estimated values of FC50 were 280, 150 and 70 μM for FCZ, 8PP and its combination, respectively. Thus, FCZ potency was increased by 4 times in combination with 8PP, reaching values lower than one hundred. At higher concentrations, like 500 μM FCZ, a decrease of 3 log units of viability with respect to control was reached (3.65 ± 1.05, $p < 0.0001$). In this way, both fungistatic compounds, when combined, achieve an almost complete fungicidal effect that only would reach by separate at much higher concentrations. The fungistatic

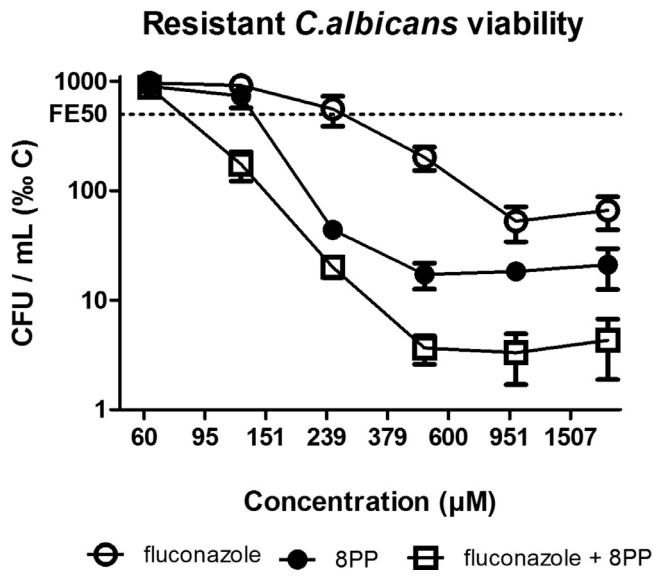

Fig. 7. Fungicidal activity of 8PP, FCZ and their combination on azole-resistant *Candida albicans*. Concentration- response curves. Viability was measured as CFU/ml, as described in Figs. 5 and 6, as described in the Materials and methods section. A starting inoculum of 10^5 CFU/ml was used. Different concentrations of 8PP, FCZ alone or combined with 125 μM 8PP were tested. Data are means ± SEM from 7 independent experiments made by triplicate as described in Materials and methods. Statistical analysis was conducted with one way analysis of variance Newman-Keuls comparison test for 3 or more groups. All the response values are different from control ($p < 0.0001$). Fungicidal effect 50% (FE_{50}) is marked.

nature of FCZ and 8PP is indicated by the fact that their MFC/MIC ratio are higher than 4 (Klepser et al., 1997; Pfaller et al., 2004; Meletiadis et al., 2007).

Since this *in vitro* fungicidal efficacy is crucial to eradicate infections, we decide to check *in vivo* effects of 8PP, and therefore initiated studies on toxicity. The intraperitoneal (i.p.) route was chosen because it offers both a big irrigation and a wide surface for absorption of substances. It is similar to the intravenous (i.v.) way for rapid entry of medicaments into blood circulation in experimental animals (Jin et al., 2015).

Preliminary results on acute toxicity at a single dose schedule revealed a relatively low toxicity in albino mice, as suggested by LD_{50} values. In fact, the LD_{50} was 357 and 245 mg/kg, for female and male animals, respectively. Depending on the dose tried, several symptoms were observed. A decreased spontaneous activity was observed only in 75% female mice. LD_{50} of FCZ was 785 and 650 mg/kg for female and male animals, respectively. At a doses equivalent to half of LD_{50}, 8PP did not show toxicity symptoms on different organs or cavities, whereas FCZ appears to produce hepatic damage (data not shown).

Discussion

Our previous results showed that 8PP is an interesting prenylated flavonoid with *in vitro* antibacterial and antifungal activity against nosocomial multi-resistant strains (Ortega et al., 1996; Pérez et al., 2003). They include resistant *C. albicans,* which plays an important role in oral infections in immune suppressed individuals, as above mentioned (Kim and Sudbery, 2011).

In addition, 8PP inhibits ABC efflux pumps and produces mitochondrial toxicity on azole-resistant *C. albicans* (Peralta et al., 2012, 2015; Barceló et al., 2015). *In silico* studies seem to indicate that 8PP may also act on two sites of the ABC cdr1 and cdr2 transporters. Part of this presumption is based on the similarities in chemical structure with 6PP (Barceló et al., 2014; 2016).

As positive cooperative interactions between 8PP and FCZ are suggested by our previous studies, we conducted checkerboard studies to check this hypothesis. According to criteria of Odds (2003), a FICI of 0.61 (Table 1) would indicate absence of interaction between 8PP and FCZ. However, as shown in Fig. 3, the concave curve with that FICI would indicate additive effects, according to criteria of Iten et al. (2009) and Joray et al. (2011), who reported this type of interactions. It should be noted that the FICI value is close to 0.5, which separates the synergistic and additive effects (Iten et al., 2009). Moreover, the notion of a synergistic cooperative interaction would be supported by the fact that 8PP might produce effects on additional targets as the above mentioned (Peralta et al., 2012; 2015; Barceló et al., 2015; 2016), according to criteria from Berthroud (2013).

In this context, we decided to find out if 8PP interactions were extensive to other pharmacological effects such as the fungicidal one. Our results show that 8PP cooperates with FCZ to kill azole-resistant *C. albicans,* besides doing it by itself (Fig. 7). As observed, 8PP can increase by 4 times FCZ potency and efficacy to reach the maximum fungicidal effect. This fact would be crucial to eradicate fungal infections, since the major disadvantage of azole antifungals is their fungistatic nature, that favours the onset of drug resistance. For these reasons, the achievement of high fungicidal efficacy *via* combination with other compounds is highly desirable (Fiori and van Dijck, 2012).

As mentioned in the Results section, 8PP would be defined as a fungistatic agent. Similar cases in which fungistatic agents cooperate with FCZ to kill *C. albicans in vitro* were reported for cyclosporine and berberine (Marchetti et al., 2000; Lidd, 2013). Flavonoids are other examples of plant compounds that interact cooperatively with antimicrobials (Nanayakkara et al., 2002).

Summarizing, 8PP shows additive interactions with FCZ regarding cell growth. These interactions could be reflected in the fungicidal effect. Respecting of it, 8PP shows a dual effect: killing *per se* and by increasing FCZ potency and efficacy. This last effect would involve the inhibition of cdr transporters, with the subsequent increase in intracellular azole concentrations and responses, in a similar way as that reported previously for growth cell (Peralta et al., 2012).

These data, taken together with other previously reported on 8PP toxicity in rat liver mitochondria and human HEp-2 cancer cells (Elingold et al., 2008), led us to check *in vivo* effects, in spite they require big quantities of 8PP and its availability at present is scarce.

In this context, 8PP lipophilicity would help it to freely diffuse through membranes and enter cells of different tissues and organs. As expected, *in vivo* effects were observed in preliminary acute toxicity studies on mice. These studies are usually conducted in the search for new effective and innocuous medicaments (Nuñez Montoya et al., 2003; Pérez et al., 1988). At a single dose schedule, LD_{50} values for the i.p. route suggest an acceptable level of safety for the prenylated flavonoid, in comparison with other compounds. Even when LD_{50} values indicate that 8PP is more potent than FCZ to kill mice, it would not induce hepatotoxicity, which is one of the most frequent azole side effects (Tachibana et al., 1987). These data are consistent with other results obtained by some of us (Carral et al., manuscript in preparation). In this context, no evidence of 8PP toxicity was found in a preliminary testing of toxoplasmosis in the NMR1 strain of mice with gavage administration. Even when 8PP, up to 37 mg/kg.day. p.o. during 10 days did not decrease any of the parameters associated to infection burden or mortality, it did not produce any toxicity on non infected controls. These results were obtained in the Kahn's model of infection (Kahn et al., 2001), using mice infected with *Toxoplasma gondii* and pyrimethamine as a positive standard.

Further studies will be necessary to determine whether the toxicity detected is also found against normal human cells. Taken all together, the results suggest that the continuation of pharmacological evaluation of 8PP would be worthy of attention.

Conflicts of interest

No conflict of interest exist for any of the authors.

Acknowledgments

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