A Prenylated Flavanone from Dalea elegans Inhibits Rhodamine 6 G Efflux and Reverses Fluconazole-Resistance in Candida albicans

Key words  
- prenylated flavonoid  
- Dalea elegans  
- Fabaceae  
- rhodamine efflux inhibition  
- antifungal activity  
-azole-resistant Candida albicans  
-reversion of fluconazole resistance

Abstract

In previous studies, 2’,4’-dihydroxy-5’-(1″,1″’-dimethylallyl)-6-prenylpinocembrin, a prenylated flavonoid isolated from Dalea elegans roots, showed activity against multiresistant Staphylococcus aureus and Candida albicans, as well as an uncoupling effect on mitochondria and antioxidant activity. The aim of this study was to evaluate the inhibitory effects of 2’,4’-dihydroxy-5’-(1″,1″’-dimethylallyl)-6-prenylpinocembrin and fluconazole on the efflux of rhodamine 6 G in azole-resistant C. albicans 12–99 that expresses multidrug transporters Cdr1p, Cdr2p, and Mdr1p. The effect of fluconazole and 2’,4’-dihydroxy-5’-(1″,1″’-dimethylallyl)-6-prenylpinocembrin on rhodamine 6 G efflux was assessed in both azole-sensitive and azole-resistant C. albicans. Between 1 and 1000 µM, 2’,4’-dihydroxy-5’-(1″,1″’-dimethylallyl)-6-prenylpinocembrin inhibited rhodamine 6 G efflux only in azole-resistant C. albicans 12–99 in a concentration-dependent manner (IC₅₀ = 119 µM); a competitive effect was observed. It also showed selectivity of action in comparison with other flavonones (6-prenylpinocembrin, isolated from aerial parts of D. elegans, pinocembrin, naringenin, and hesperetin, all at 250 µM). To check the possible implications of the inhibition of azole efflux on cell growth, antifungal assays were conducted. Minimal inhibitory concentration values were 150 µM for 2’,4’-dihydroxy-5’-(1″,1″’-dimethylallyl)-6-prenylpinocembrin and higher than 400 µM for fluconazole. The combination of both compounds at either inhibitory or subinhibitory concentrations was significantly more effective than each compound separately. Minimal inhibitory concentration for fluconazole decreased by more than 400 times in the presence of 100 µM 2’,4’-dihydroxy-5’-(1″,1″’-dimethylallyl)-6-prenylpinocembrin, reversing azole resistance and giving values similar to those of azole-sensitive C. albicans. These data are consistent with a dual action of 2’,4’-dihydroxy-5’-(1″,1″’-dimethylallyl)-6-prenylpinocembrin: direct antifungal effect on azole-resistant C. albicans 12–99 and inhibition of azole transporters, which results in reversion of fluconazole resistance.

Abbreviations

- 6PP: 2’,4’-dihydroxy-5’-(1″,1″’-dimethylallyl)-6-prenylpinocembrin  
- Rh 6 G: rhodamine 6 G  
- RCA: azole-resistant Candida albicans strain  
- SCA: azole-sensitive Candida albicans strain  
- YPD: yeast peptone dextrose  
- ABC: ATP-binding cassette  
- Flz: fluconazole  
- MDR: multidrug resistance  
- MIC: minimal inhibitory concentration

Introduction

Dalea elegans Gillies ex Hook. et Arn., Fabaceae, belongs to an exclusively American genus and is the only species that grows in the province of Córdoba (Argentina). In previous articles we reported the isolation of two prenylated flavanones from D. elegans: 6PP 1 and its precursor 6-prenylpinocembrin 2 (Fig. 1) [1]. In bioactivity studies, the first compound showed antibacterial and antifungal properties, even against multiresistant strains [2,3]. In another biological characteriza-
tion, it demonstrated both antioxidant and antiradical activities; it also inhibited enzymatic lipid peroxidation and showed toxic effects against human tumor cells [4]. Some flavonoids have two synergistic functions: a direct action against microorganisms and the inhibition of pumps that extrude antimicrobials. In the latter, by increasing the concentration of antimicrobials within the microorganism, resistance can be reversed. Efflux pumps play a major role in azole susceptibility and may represent a new therapeutic target, particularly among immunocompromised patients, such as AIDS cases, in whom mycoses produced by *C. albicans* are very important causes of illness and complications [5].

In this context, we decided to explore 6PP’s effect on pumps involved in azole efflux in *Candida albicans*. Thus, we selected ABC transporters using Rh 6 G as a substrate and compared the activity of this compound with some related prenylated and non-prenylated flavanones. Since 6PP showed selectivity in inhibiting the efflux, in order to check the reversion of azole resistance, the effect of a combination of 6PP with fluconazole on RCa growth was studied. Verapamil was used as a transporter inhibitor standard.

### Materials and Methods

**Chemicals**

Rh 6 G (purity ≥ 99%), fluconazole (purity ≥ 98%), verapamil (purity ≥ 99%), and the flavanones pinocembrin (5,7-dihydroxyflavanone, 3; purity 95%), naringenin (5,7,4′-trihydroxyflavanone, 4; purity ≥ 95%), and hesperetin (5,7,3′-trihydroxy-4′-methoxyflavanone, 5; purity ≥ 95%) (Fig. 1) were from Sigma Aldrich.

**Microorganisms**

Two strains of *Candida albicans*, isolated from the oral cavity, were used. They were a kind gift from Dr. T. White (University of Washington, Seattle, WA, USA). The azole-resistant strain (12–99, hereafter RCa) overexpresses the transporter genes CDR1, CDR2, and MDR1, whereas the sensitive strain (2–76, SCa) lacks these [6]. For the experiments, both strains were cultured in YPD broth or Sabouraud agar medium and then resuspended according to the assay performed.

**Plant material**

*Dalea elegans* Gillies ex Hook. et Arn. (Fabaceae) was collected in February 2007, during the flowering period, in its natural habitat in hills near Cabalango (Córdoba, Argentina, GPS coordinates: latitude: 31°24′04.62″ south; longitude: 64°34′19.21″ west; height: 763 m). Plant material was identified by Dr. Gloria Barboza of the Botanical Museum, Universidad Nacional de Córdoba, Córdoba, Argentina (UNC). Roots and aerial parts were separated for further processing. A representative voucher specimen is on deposit as CORD Peralta 2 in the herbarium at the Botanical Museum (IMBIV, UNC).

**Extraction and isolation**

Roots and aerial parts from *D. elegans* were processed for extraction and isolation of 6PP (1; purity ≥ 98%) and 6-prenylpinocembrin (2, purity ≥ 98%), respectively (Fig. 1), and their structures were established by UV, NMR, and MS, according to Caffaratti et al. [1]. Additionally, for 6PP the optical rotation was determined as [α]D 25° − 67 (c 0.07, MeOH), which was coincident with the control sample.

**Rhodamine 6 G efflux**

The function of transporters was measured by flow cytometry using Rh 6 G as a fluorescent substrate, according to Vandeputte et al. [7]. This assay measures the efflux activity of individual cells, providing an analysis of the results in terms of distribution for cell size parameters, together with the intensity of fluorescence. A population of cells with similar size and complexity, recovered from the tube where they were incubated, was selected for evaluation in every assay. Fluorochrome efflux was measured in both RCa and SCa *Candida albicans*, in YPD medium containing 100 µM Rh 6 G at 525 nm in a FACSort (Becton Dickinson) flow...
cytometer. For Lineweaver-Burk analysis, different concentrations of Rh 6 G were used. In all experiments, a suspension of 10^7 yeasts/mL in YPD medium was analyzed in the cytometer. Fluconazole, verapamil, or flavanones were added from DMSO stock solutions to give different concentrations in the incubation medium. Uptake of fluorochrome was quantified after the first incubation of cells in YPD broth for 30 min. Then, after three washes to remove Rh 6 G, the cells were subjected to a second incubation for 15 min in YPD, and the residual fluorescence was quantified. Experiments were replicated twice. Efflux was calculated as the difference between the cell fluorescence content measured after the first and second incubations. Initial analysis was performed with CellQuest® software (Becton-Dickinson). Raw data were further analyzed and plotted with GraphPad Prism 5®.

**Antifungal activity**

To characterize and quantify the antifungal activity, the growth of R. _candida_ and S. _cerevisiae_ were measured in the absence and presence of different concentrations of fluconazole, verapamil, 6PP, or their combinations. Absorbance was measured at 540 nm with a MicroQuant microplate spectrophotometer (BioTek Instruments). A checkerboard microtiter 96-well plate format was used. Experiments were performed according to the NCCLS-approved standard M27-A [8] as described by Marchetti et al. [9]. 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% or less with respect to that of the growth control measured at 540 nm in a microplate reader.

**Statistical analysis**

GraphPad Prism version 5.00 software (GraphPad Software) 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) with 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 ± SEM.

**Supporting information**

Data regarding rhodamine 6 G efflux in _Candida albicans_ are shown as Supporting Information.

**Results**

In Rh 6 G efflux assays, both strains of _C. albicans_ (SCa and RCa) acted as homogeneous populations, showing unimodal curves. Autofluorescence in RCa was only 3% of the first incubation control and 11% compared to the second one, reflecting the high sensitivity of the method used. The rhodamine 6 G fluorescence retained after the second incubation was significantly lower in RCa: (37.0 ± 10.5%) than in SCa (81.0 ± 14.6%), reflecting the operation of efflux transporters in RCa. DMSO 5% v/v was chosen as the solvent for 6PP and other drugs because it did not modify the intracellular rhodamine content, either in the first or second incubation controls (Fig. 15, Supporting Information). Consistent with the involvement of ABC transporters, verapamil (250 µM) reversed Rca rhodamine efflux (left plot in Fig. 2A). As expected, no effect was observed in azole-sensitive _C. albicans_ (right plot in Fig. 2A).

As all these data indicated a high selectivity in the method used, it was decided to evaluate the effect of 6PP in RCa transporters. Incubation for 15 min with 250 µM 6PP or verapamil reversed Rh 6 G efflux (Fig. 2B). In another set of experiments, a short incubation with 6PP did not affect Rca viability, as assessed by growth curves (data not shown).

To further characterize the effect of 6PP on Rh 6 G efflux, the concentration-response relationship was studied. Between 1 and 1000 µM, 6PP inhibited Rh 6 G efflux in a concentration-dependent manner in azole-resistant _Candida albicans_. As expected, fluconazole (standard substrate of azole transporters) was also able to decrease the efflux. The results are compatible with an inhibition of Rh 6 G efflux by both 6PP and fluconazole, with IC_{50} values of 121 ± 1 and 756 ± 1 µM, respectively (p < 0.0001), as calculated from inhibitor concentration vs. normalized response analysis (Fig. 3). The Lineweaver-Burk plot revealed that 6PP competitively inhibits Rh 6 G efflux and produces a fourfold increase in the apparent K_m (11.75 ± 2.45 towards 49.29 ± 10.76 µM) but presents no effect on the V_{max} (Fig. 4). It is noteworthy that IC_{50} values for 6PP were 100 µM to inhibit ATP synthetase in rat liver mitochondrial systems and between 20 and 400 µM to produce a toxic effect on HEp 2 cells, depending on the presence of albumin [4].

With the aim of screening selectivity in the efflux inhibitory activity of 6PP and highlighting some structural implications of this active compound from _D. elegans_, the efflux of Rh 6 G C in resistant _C. albicans_ was evaluated for comparative purposes with other flavanones: pinocembrin, 6-prenylpinocembrin, naringenin, and hesperetin, at 250 µM (Fig. 1). The higher activity of 6PP compared with the other flavanones is shown in Fig. 5. The low activity observed for 6-prenylpinocembrin enables the implications of a B-ring substitution pattern to be estimated in relation to efflux inhibition of 6PP. Also, the lack of a significant difference between pinocembrin and 6-prenylpinocembrin demonstrated that the prenyl group at A-ring is not very important in the activity. In this context, naringenin and hesperetin, having a different substitution pattern at the B-ring, did not inhibit the efflux; so the dimethylallyl group attached at the B-ring might be involved in this activity.

Taking 6PP IC_{50} values into account, the growth of RCa was evaluated in the presence of 100 µM of this prenylated flavanone, of fluconazole, or a combination of both. Fig. 6a shows that the combination is significantly more effective than each compound separately. As expected, fluconazole (100 µM) alone did not affect RCa growth, whereas 6PP at the same concentration decreased it significantly by 50%. The association of fluconazole and 6PP resulted in a higher inhibition of RCa growth (78% with respect to control culture), suggesting some kind of synergistic interaction [10]. In order to better understand the importance of transporter inhibition for the reversal of fluconazole resistance in the growth inhibition of _C. albicans_, a control experiment with verapamil and fluconazole was performed. As expected, verapamil, at a concentration that would produce significant inhibition of transporters, inhibited _C. albicans_ growth in presence of fluconazole by 37%, but did not produce any effect per se (Fig. 6b).

These results led us to evaluate the effect of 100 µM 6PP or 300 µM verapamil combined with different concentrations of flu-
400-fold with respect to fluconazole and reached a value similar to that of the azole-sensitive C. albicans strain (data not shown). Some data fitted inhibitor concentration-response curves yielded IC₅₀ values of 1126 ± 3 µM (flz) and 209 ± 1 µM (flz combined with 300 µM verapamil). Thus, the IC₅₀ of flz was decreased 5-fold by verapamil (p < 0.0001).

The higher effect of 6PP in comparison with verapamil on concentration-response curves for fluconazole might be explained by the fact that 6PP, in addition to blocking fluconazole efflux pumps (thus increasing its antifungal action), inhibits C. albicans growth per se. Notably, when 6PP is added at concentrations below 10 µM, which are practically subinhibitory with respect to flz efflux (Fig. 3) and yeast growth, a qualitatively similar result is obtained (Fig. 6c). These results suggest that other additional effects of 6PP could be involved.

Discussion

Our previous results showed that, among several compounds, 6PP is an interesting prenylated flavonoid isolated from the South American plant Dalea elegans. It showed in vitro antibacterial and antifungal activity against nosocomial multiresistant strains [2, 3]. On the other hand, other flavonoids and extracts from different species of the Dalea genus, such as D. versicolor and D. scandens var. paucifolia (growing in North America), have been reported as antimicrobial agents [11, 12].

conazole. Fig. 6D and E demonstrate that the interaction observed is also found with other fluconazole concentrations, resulting in the reversion of azole resistance. MIC values were 150 µM and higher than 400 µM for 6PP and fluconazole, respectively. Thus, MIC for the combination was decreased more than...
Since different flavonoids from the Dalea genus are inhibitors of pumps that export antimicrobials [11], it was decided to study 6PP’s effect on azole transport by using rhodamine 6 G as a fluorescent substrate [13]. Like fluconazole (a standard substrate of azole transporters), 6PP inhibited rhodamine 6 G efflux in azole-resistant Candida albicans. Results are consistent with a competitive inhibitory action of 6PP on transporters that extrude azole antifungals. 6PP could thus be a putative substrate of CDR pumps which extrude rhodamine 6 G [13] and are called ATP-binding cassettes (ABC) because they depend on ATP energy. CDR1 and CDR2 are ABC transporter genes, overexpressed in the resistant Candida strain. The proteins Cdr1p and Cdr2p, encoded by these genes, confer resistance to azole and other antifungal agents, playing an important role in Candida MDR [14]. In the last ten years, a number of studies have exhaustively investigated natural and hemisynthetic flavonoids as modulators of ABC transporters, such as P-glycoprotein [15], MRP1, and BCRP [16], which are responsible for the failure of anticaner chemotherapy and the related multidrug transporter in yeast, pdr5p [17]. Studies of the structure-activity relationship have demonstrated that the presence of prenyl groups at the A-ring of the flavonoid structure increased their ability to inhibit these transporters [16]. As far as we know, no correlation between flavonoid structure and CDR inhibitory activity has been demonstrated and this is the first report of a prenyl flavonoid as an inhibitor of Candida albicans MDR. Moreover, in order to estimate structural specificity of the efflux inhibitory activity of 6PP, other structurally related prenylated and non-prenylated flavanones were evaluated. Our results provide evidence that the increased activity of 6PP, compared with the above-mentioned derivatives, could be related to the substitution pattern at the B-ring.

There has been some speculation about possible structure-activity relationships regarding the substituents and the substitution patterns for various classes of flavonoids in different ABC transporters. Considering that prenyl substituents at the B-ring have not been assessed regarding their positive or negative effect on the inhibition of ABC transporters, a more comprehensive evaluation, on a much larger set of related compounds, will be required in order to define which structural parameters are related to the potency, efficacy, and specificity of 6PP with respect to CDR transporters. However, in view of the good relative activity of 6PP, its lipophilic characteristics, and the derived potential pharmacokinetic features, it was decided to continue studying this compound. It seems to mimic verapamil, which was used as a positive control because it inhibits P-glycoprotein (P-gp, ABC transport in mammalian cells) [18] and azole efflux pumps in C. albicans [19]. Bearing in mind that ATP energy is necessary for the operation of ABC transporters, in addition to directly inhibiting transporters in yeasts, 6PP might act at similar concentrations as a blocker of mitochondrial ATP synthesis in C. albicans, as some of us reported previously for liver and tumor cells [4].

The probability that the modulation of ABC transporter function would result in increases in the intracellular concentration of

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Fig. 4  Lineweaver-Burk plot of Rh 6 G efflux in the absence and presence of 6PP. White and filled circles represent 0 and 200 µM 6PP, respectively. Efflux was calculated as the difference of fluorescence retention between the first and the second incubation. Data are means and SEM from two independent experiments.

Fig. 5  Comparison of the activity of different flavanones (their chemical structures are shown in Fig. 1) at a 250 µM concentration on rhodamine 6 G efflux in azole-resistant C. albicans. Data are means and SEM from four independent experiments. Statistical analysis was conducted by one-way analysis of variance with Newman-Keuls multiple comparison test for 3 or more groups. Significance was accepted at p < 0.05 (*). 6PP activity was considered as unity, and the other compounds were referenced to this. The figure shows significance in relation to 6PP. Significance with respect to control was found only in 6PP *** p < 0.0001 and pinocembrin ** p < 0.01.
drugs to toxic levels, thus reversing fungus resistance, is the most attractive application of ABC transporter inhibitors. However, it was reported that another prenylated flavanone, 8-prenylnarigenin, was able to increase rhodamine accumulation in doxorubicin-resistant human adenocarcinoma cells by inhibiting P-glycoprotein, but it was not able to increase doxorubicin toxicity to resistant cells [20]. Curcumin, another plant compound, inhibits rhodamine 6 G efflux and shows synergism with antifungals in S. cerevisiae overexpressing C. albicans ABC transporters, but it has no antimicrobial activity per se [10]. In our case, the presence of 6PP was able to reverse cell resistance to fluconazole, as became apparent from growth studies. When 6PP was used in combination, it shifted fluconazole MIC values towards that of azole-sensitive C. albicans. Moreover, it inhibited cell growth per se, adding an extra interaction that could improve the treatment. In conclusion, 6PP inhibits Rh 6 G efflux and reverses fluconazole resistance in the RCa. Both effects seem to be mutually related at high concentrations, as supported by control experiments with verapamil. However, the synergistic effects of low concentrations of 6PP and fluconazole cannot be explained by the inhibition of efflux transporters or by the antifungal effect of 6PP. Thus, another effect might be involved; further studies should be required to elucidate it. Nevertheless, if similar results were obtained in humans in vivo, a combined therapy of 6PP with azoles could be clinically relevant.

In this context, this potential synergistic interaction between 6PP and azole antifungics deserves further research, particularly considering 6PP’s lipophilicity, derived from its molecular characteristics, which would facilitate the passage through biological membranes.

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Conflict of Interest

No conflicts of interest exist for any of the authors.
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