

Inhibition of *Saccharomyces cerevisiae* Pdr5p by a natural compound extracted from Brazilian Red Propolis

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Abstract: Multidrug resistance of cancer cells and pathogenic microorganisms leading to the treatment failure of some forms of cancer or life-threatening bacterial or fungal infections is often caused by the overexpression of multidrug efflux pumps belonging to the ATP-binding cassette transporters superfamily. The multidrug resistance of fungal cells often involves the overexpression of efflux pumps belonging to the pleiotropic drug resistance (PDR) family of ABC transporters. Possibly the best-studied fungal PDR transporter is the multidrug resistance transporter Pdr5p of *Saccharomyces cerevisiae*. Some research groups have been searching for new inhibitors of these efflux pumps in order to alleviate resistance. Natural products are a great source for the discovery of new compounds with biological activity. Propolis is a complex resinous material collected by honeybees from exudates and buds of certain plant sources and this material is thought to serve as a defense substance for bee hives. Propolis is widely used in traditional medicine and is reported to have a broad spectrum of pharmacological properties. Literature reported some biological functionalities of propolis, such as antibacterial, antiviral, fungicidal, anti-inflammatory and anti-carcinogenic activities. The chemical composition of propolis is qualitatively and quantitatively variable. Components isolated from methanolic extract of red Brazilian propolis (Alagoas, Northeast of Brazil) are isoflavonoids (including pterocarpan, isoflavans, isoflavones), flavanones and polyprenylated benzophenones. In this work we demonstrated the effects of five different isolated compounds on the ATPase activity of Pdr5p. Out of all five substances tested, only BRP-1 was able to completely abolish the enzymatic activity while others worked as positive modulators of the enzyme activity. BRP-1 also inhibited the efflux of Rhodamine 6G from yeast cells overexpressing Pdr5p. Taken together, these results demonstrate that Brazilian propolis could be a source of promising compounds that can alleviate the MDR phenomenon, particularly in some fungi, where it could be used as an adjuvant for the treatment with azoles.

Introduction

The overexpression of pleiotropic drug resistance (PDR) efflux pumps of the ATP-binding cassette (ABC) transporter superfamily, similar to the human multidrug resistance protein P-glycoprotein (ABCB1), frequently correlates with multidrug resistance of fungal cells. Multidrug resistance leads to the resistance to several structurally and functionally unrelated drugs and can be the cause of treatment failure of anticancer, antimicrobial and antifungal

chemotherapy (Lamping et al., 2010). The pleiotropic drug resistant protein Pdr5p is a plasma membrane associated ATP-binding cassette (ABC) transporter and the major multidrug efflux pump of *Saccharomyces cerevisiae*. The overexpression of multidrug efflux pumps has been reported to cause multidrug resistance in several pathogenic microorganisms such as fungi (Morschhäuser, 2010; Ernst et al., 2005), parasites (Pradines et al., 2005), as well many pathogenic bacteria (Hawkey & Jones, 2009).

The emergence and spread of multidrug

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resistance (MDR) poses a severe and increasing public health threat, since new pharmaceutical drugs, especially drugs, show moderate to severe toxicity and/or side effects (Shukla et al., 2008). Efforts to identify natural products as inhibitors of MDR exporters have the potential to provide a large number of novel drug leads since many of these products have been used for centuries without harmful side effects (Wang et al., 2010).

Propolis is a complex resinous material collected by honeybees (*Apis mellifera*) from exudates and buds of certain plant sources and this material is thought to serve as defense substance for bee hives (Pietta et al., 2002). It is used to block holes and cracks, to repair combs, to strengthen the thin borders of the comb and for making the entrance of the hive weathertight or easier to defend. Propolis is also used as an "embalming" substance to cover hive invaders which bees have killed but cannot transport out of the hive (Ghisalberti, 1978). The action against microorganisms is an essential characteristic of propolis and it has been used by human beings since ancient times for its pharmaceutical properties. For all types of propolis a broad spectrum of pharmacological properties is reported. Literature reported some biological functionalities of propolis, such as antibacterial and antiviral properties and many other beneficial biological activities including antiinflammatory, antiulcer, local anaesthetic, hepatoprotective, antitumor and immunostimulating properties (Sforzin et al., 2000; Kujumgiev & Popov, 1996; Banskota et al., 2002; Awale et al., 2008; Marcucci, 1995; Burdok, 1998; Popova et al., 2005). For this reason propolis is widely used as a popular remedy in folk medicine, as a constituent of biocosmetics, health food and today represents an important raw material for many health products and constitute a new interdisciplinary area for research (Matsuda, 1994).

Propolis chemical composition is qualitatively and quantitatively variable, depending on the season, the species of bee, vegetation and the area of collection; in tropical regions, for example, because of the difference in vegetation, the chemical composition of propolis is very different (Lotti et al., 2010). Its colour also varies depending on its botanical source, the most common being dark brown, but red propolis has also been observed in tropical countries such as Brazil and Cuba (Park et al., 2002; Cuesta-Rubio et al., 2007). The major components isolated from methanolic extract of red Brazilian propolis (Alagoas, Northeast of Brazil) are isoflavonoids (including pterocarpan, isoflavans, isoflavones), flavanones and polyprenylated benzophenones (Trusheva, 2006; Awale et al., 2008). This type of Brazilian Red Propolis (BRP) demonstrated a notable antimicrobial activity

against *Staphylococcus aureus* and *mutans* (Alencar et al., 2007) and cytotoxicity against human pancreatic PANC-1 cancer cell line (Awale et al., 2008). In this work we show that BRP is also able to inhibit the enzymatic activity and the rhodamine 6G transport of Pdr5p from *Saccharomyces cerevisiae*. Thus BRP could be a new tool for the study of the mechanism of multidrug resistance and work as a natural compound to reverse this phenotype in yeast.

Materials and Methods

Plant material

Samples of raw red propolis produced by *Apis mellifera* bees were collected in a Northeast region of Brazil (Alagoas State) in the mangrove area. Methanol extract of BRP was obtained by maceration of ground propolis sample with methanol and stored at 5 °C in the dark.

Standard and samples

Compounds BRP-1-5 were isolated from Brazilian red propolis sample and identified by spectroscopic methods.

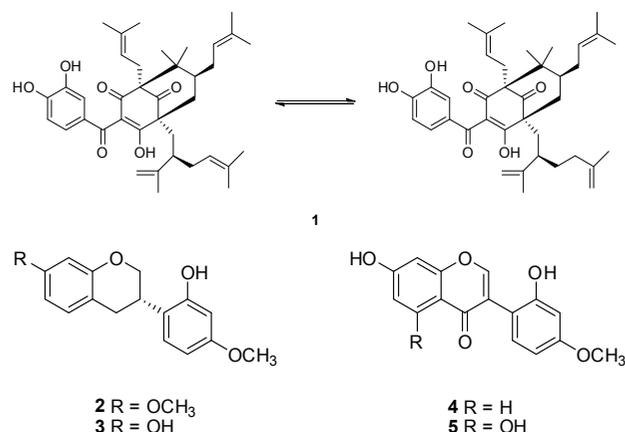
General experimental procedure

A Bruker DRX-600 NMR spectrometer, operating at 599.19 MHz for ¹H and at 150.86 MHz for ¹³C, using the UXMNMR software package was used for NMR experiments; chemical shifts are expressed in δ (parts per million) referring to the solvent peaks δ H 3.34 and δ C 49.0 for CD₃OD, δ H 7.27 and δ C 77.0 for CDCl₃; coupling constants, J, are in hertz. Preparative HPLC was performed on a Agilent 1100 series system consisting of a G-1312 binary pump, a G-1328A Rheodyne injector, a G-1322A degasser and a G-1315A photodiode array detector (PDA). TLC analyses were performed with Macherey-Nagel precoated silica gel 60 F₂₅₄ plates.

Isolation of compounds

A portion of dry extract was fractionated over Sephadex LH-20 column (100 cm x 5.0 cm) using methanol as elution solvent. Major fractions were purified by HPLC-RI (Waters 590 series pumping system equipped with a Waters R401 refractive index detector) in isocratic elution mode using Luna C8 column (250 x 10 mm, 10 μ m) (methanol-water 55:45 v/v), both from Phenomenex (Torrance, CA, USA), to yield pure compounds, which were identified as isomeric mixture of guttiferone E and xanthocymol (1) (Gustafson et al.,

1992), 7-*O*-methylvestitol (**2**) (Piccinelli et al., 2005), vestitol (**3**) (Piccinelli, 2005), biochanin A (**4**) (Chang et al., 1994), and formononetin (Chang et al., 1994) (**5**), respectively.



Preparation of plasma membrane

Plasma membrane fractions were obtained from yeast *S. cerevisiae* mutants AD124567 and AD 1234567 (Decottignies et al., 1998) as previously reported by Rangel et al. (2008). The plasma membranes highly enriched in Pdr5p were stored in liquid nitrogen.

ATPase activity assay

ATP hydrolysis was determined by incubating Pdr5p enriched membranes (0.013 mg/mL final concentration) in 96-well plate at 37 °C for 60 min in a reaction medium containing 100 mM Tris-HCl pH 7.5, 4 mM MgCl₂, 75 mM KNO₃, 7.5 mM NaN₃ and 0.3 mM ammonium molybdate and 3 mM ATP). The compounds BRP-1-5 were added from stock solution in dimethylsulfoxide up to 5% v/v final concentration. After the incubation period the reaction was stopped with 1 % SDS as described previously (Dulley, 1965). The released inorganic phosphate (Pi) was measured as described previously (Fiske & Subbarow, 1925). As control plasma membranes prepared from the PDR5 deleted strain AD1234567 were used. The difference between the ATPase activity of AD124567 membranes and the activity of AD1234567 corresponds to the Pdr5p mediated ATPase activity.

R6G efflux by intact cells

Rhodamine efflux assays were performed as described previously by Niimi et al. (2004) with minor changes. Briefly, log-phase AD124567 or AD1234567 cells were washed four times with distilled water. The pellet was resuspended in water and incubated

for 15 min at 4 °C. Soon after, the cell suspension was centrifuged and the pellet was resuspended in 50 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES)-NaOH, pH 7.0. To deplete intracellular energy, the cells were incubated for 30 min at 30 °C with 2-deoxyglucose (final concentration 5 mM). Then R6G was added at a final concentration of 15 μM and incubated for 30 min at 30 °C. R6G equilibrated cells were then washed twice with distilled water and resuspended with HEPES buffer to an OD of 10. Approximately 1x10⁸ cells were incubated at 30 °C with or without BRP-1 and glucose at a final concentration of 0.2% was added to start the reaction. Glucose free controls were included in all experiments. After 13 min the cells were pelleted and the R6G present in the supernatant was spectrophotometrically quantified using a spectrophotometer (P 582-Micronal- Brazil) at a wavelength of 527 nm.

BRP-1 MIC as a measure of toxicity against *S. cerevisiae*

The MIC of BRP-1 for Pdr5p-hyperexpressing strain AD124567 and isogenic null mutant AD1234567 was determined by a microdilution method according to Clinical and Laboratory Standards Institute (CLSI; former NCCLS M27-A) with slight modifications using 96-well microtiter plates. Cells in YPD medium were inoculated at 1x10⁴ cells per well and incubated at 30 °C for 48 h with shaking (150 rpm) in the presence of 50 μM of BRP-1. Cell growth was monitored at 600 nm with a microplate reader (Fluostar Optima, BMG Labtech, Offenburg, Germany).

Intracellular accumulation of R6G

This assay was performed as previously described by Sharma et al. (2009) with slight modifications. Yeast cells in the logarithmic phase of growth were washed three times with phosphate-buffered saline (PBS) pH 7.0. The pellet was resuspended in PBS and then de-energized by incubating for 45 min at 30 °C with 2-deoxyglucose (final concentration 5mM) and dinitrophenol (final concentration 5 mM). The de-energized cells were pelleted, washed twice (9000 rpm 2 min) with PBS without glucose, and then resuspended in PBS (with glucose 2%). The cell suspension was incubated with de BRP-1 (final concentration 100μM) at 30 °C by 5 min. Then R6G was added at a final concentration of 2.2 μM and incubated for 30 min at 30 °C. After washing three times with PBS without glucose (9000 rpm, 2 min) the cells were resuspended with the same buffer and their fluorescence visualized using a Nikon Eclipse E400 (Nikon, Japan).

Statistical analysis

All experiments were carried out at least three times and the results are expressed as mean±SD. Standard deviation and IC₅₀ values were calculated using the computer program Sigma Plot version 10 (SPSS Science Marketing).

Results

ATPase activity assay

The effects of BRP-1-5 on the Pdr5p ATPase activity are shown in (Figure 1). It was observed that only BRP-1 was able to inhibit the enzyme activity in a final concentration of 100 μM. In contrast, BRP-2, 3 and 4 stimulate the enzyme activity while BRP-5 was ineffective. BRP-1 was chosen for the following experiments. The determination of the IC₅₀, value of the Pdr5p ATPase activity for BRP-1 is shown in (Figure 2). The results show that BRP-1 is a very strong inhibitor of the Pdr5p ATPase activity with an apparent IC₅₀ of 1.35 μM.

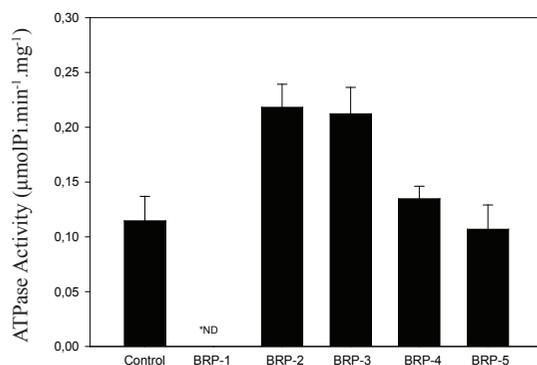


Figure 1. Modulatory effects of BRP compounds on the Pdr5p ATPase activity. Pdr5p-enriched plasma membranes were incubated in the presence of 100 μM of BRP-1, BRP-2, BRP-3, BRP-4 and BRP-5. The ATPase activity was measured as described in Materials and Methods. The control bar represents the enzymatic activity in the absence compounds. The data presents means±SD of three independent experiments. *ND=not detected.

Determination of BRP-1 toxicity for *S. cerevisiae*

It is known that propolis can be very toxic for bacterial (Alencar et al., 2007) and human cells (Awale et al., 2008). We therefore investigated the toxic effects BRP-1 against *S. cerevisiae* cells. Figure 3 shows that even in the presence of high concentrations of BRP-1 (almost 50 times more than the IC₅₀ value), no toxicity of BRP-1 against yeast cells could be detected (without compound).

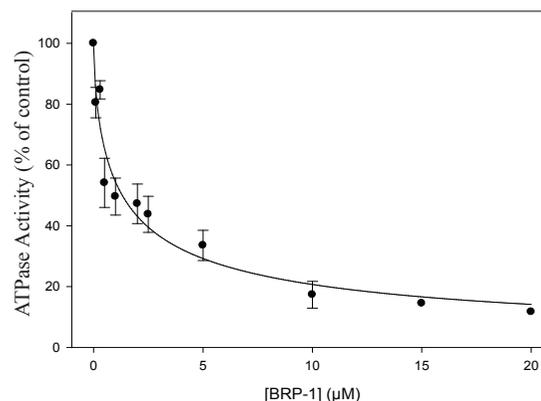


Figure 2. Inhibition of Pdr5p ATPase activity by BRP-1. The enzymatic activity was measured as described in Materials and Methods in the presence of different concentrations of BRP-1 (0.1 to 20 μM). Data represent means±SD of three independent experiments. The control represents 100% of enzymatic activity in the absence of compound.

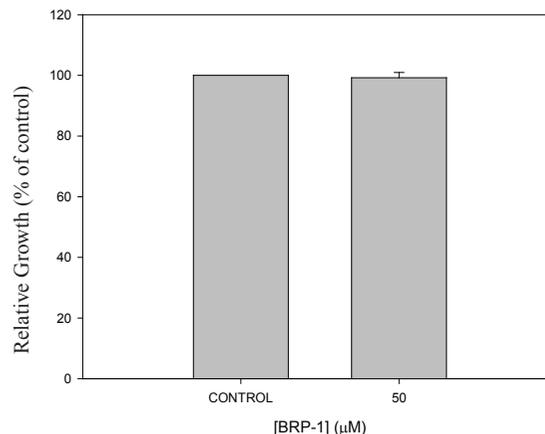


Figure 3. Toxicity of the BRP-1 against AD 124567 strain. The cells were treated with BRP-1 at a final concentration of 50 μM, as described in Materials and Methods using a 96-wells microtiter plate. The control bar corresponds to the 100% of growth without compound.

Effect of BRP-1 on the intracellular accumulation and the efflux of R6G

The effects of BRP-1 on the accumulation and retention of R6G was first evaluated by fluorescence microscopy (Figure 4). The Pdr5p-hyperexpressing strain AD124567 treated with BRP-1 (final concentration 100 μM) showed a strong fluorescence, indicating an intracellular accumulation of R6G (Figure 4H), compared to the control in the absence of BRP-1 (Figure 4G). In the presence of 100 μM BRP-1, AD124567 cells accumulated R6G at levels comparable with those of cells treated with FK-506 (Figure 4F), a known inhibitor of Pdr5p, similar to AD1234567 cells that do not express Pdr5p at all (Figure 4E).

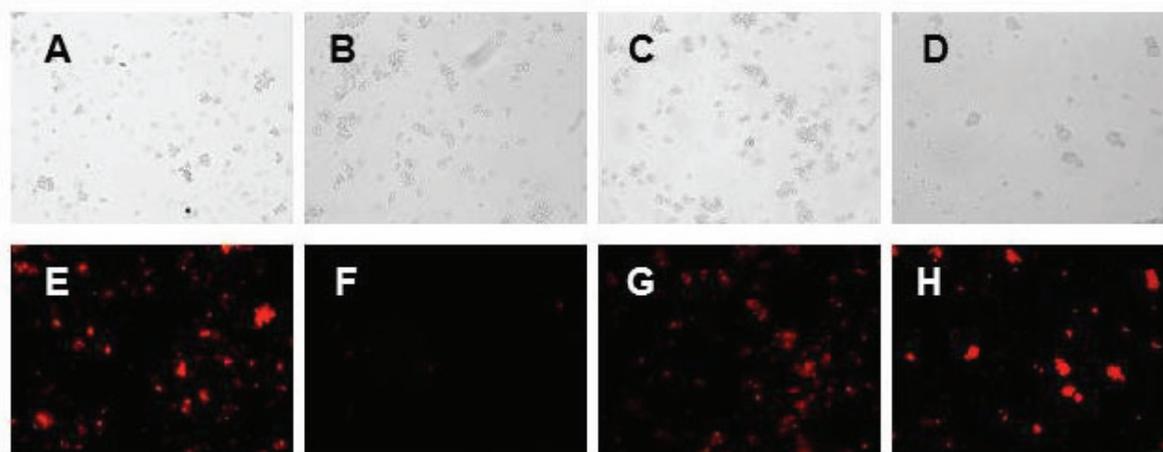


Figure 4. Fluorescence Microscopy of R6G intracellular accumulation of R6G induced by BRP-1. Upper panels (A-D) show the phase contrast microscopy and lower panels (E-H) depict fluorescence of the cells. (A) and (E) show the strain AD1234567 (negative control); (B) and (F) show the Pdr5p-overexpressing strain (AD124567); (C) and (G) show the strain AD124567 incubated 2 h with FK506 (10 μ M); (D) and (H) AD124567 incubated 2 h with 100 μ M BRP-1.

In order to quantify the inhibition of energy-dependent R6G efflux by BRP-1 the R6G efflux of the strain AD124567 was measured at different concentrations of BRP-1 (Figure 5). BRP-1 caused a dose-dependent inhibition of the glucose-dependent R6G efflux, with 80% of inhibition achieved at 100 μ M. This inhibition by BRP-1 was very strong with an IC₅₀ value similar to the IC₅₀ value measured for the inhibition of the (8.14 μ M).

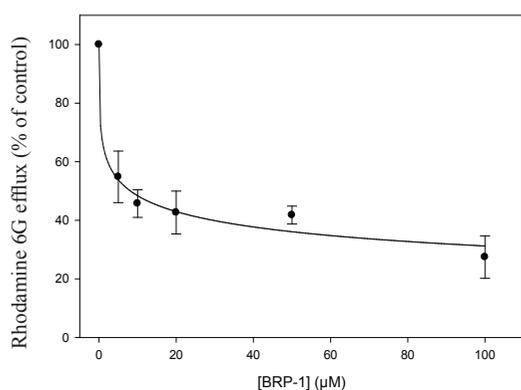


Figure 5. Inhibition of R6G efflux in strain AD124567 by BRP-1. The efflux of R6G was measured after incubation of the cells in the presence of different concentrations of BRP-1 as described in the Material and Methods. Data represent means \pm SD of three independent experiments.

Discussion

Resistance to chemotherapy is a clinical problem in patients with infectious diseases caused

by prokaryotic or eukaryotic cells. This problem is currently of growing significance with increasing numbers of HIV patients, who are highly susceptible to opportunistic fungal infections due to their severely compromised immune system. MDR caused by the overexpression of multidrug efflux pumps frequently develops in such patients due to their long-lasting prophylactic treatment regimes, but similar MDR can also be observed in some plant and animal pathogens and even in human tumour cells. Therefore, it is necessary to develop strategies that can reverse this drug resistance and natural compounds could be a valuable source material to find potent inhibitors of these multidrug efflux pumps.

In this work, we investigated five different compounds isolated from Brazilian red propolis type, which were purified, identified and named as: guttiferone E/xanthocymol (1); 7-O-methylvestitol (2); vestitol (3); biochanin A (4) and formononetin (5). From those five compounds 1 showed a strong inhibition (Figure 1) of the enzymatic activity of the multidrug efflux transporter Pdr5p of *S. cerevisiae*. Its IC₅₀ value was lower than those reported for other natural compounds such as enniatin (Ivnitski-Steele et al., 2009) or curcumin (Sharma et al., 2009) two well-known inhibitors of multidrug efflux transporters. BRP-1 was not toxic to the yeast cells used in this work even at high concentrations (50 μ M). Purified BRP-1 (100 μ M), effectively inhibited the efflux of R6G of Pdr5p overexpressing cells and was able to inhibit the Pdr5p ATPase activity at similar concentrations *in vitro* (Figure 4H). These results suggest that BRP-1 acts as an inhibitor of the Pdr5p ATPase activity by

blocking its transport mechanism as the R6G efflux of whole cells was effectively inhibited by BRP-1 at low concentrations.

Thus BRP-1 is a very promising natural compound that can effectively inhibit multidrug efflux transporters such as Pdr5p and can be used to reverse MDR of cells overexpressing these efflux pumps function. We expect these results to aid the understanding of Pdr5p and possibly many other multidrug efflux pumps opening new perspectives to improve the treatment of infectious diseases in which these transporters cause treatment failure of traditional synthetic drugs.

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