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Oroidin Inhibits the Activity of the Multidrug Resistance Target Pdr5p from Yeast Plasma Membranes

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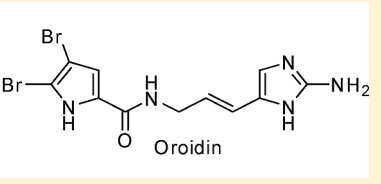
ABSTRACT: Oroidin was isolated from the marine sponge *Agelas sventres* and inhibited the activity and function of Pdr5p, an enzyme responsible for the multidrug resistance phenotype in *Saccharomyces cerevisiae*. This compound may help in the development of new drugs that reverse this dangerous phenotype of pathogenic yeast and fungi.

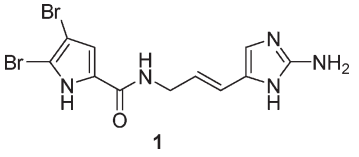


Multidrug resistance (MDR), which confers cross-resistance to several functionally and structurally diverse antitumor agents, is one of the most serious obstacles in the treatment of cancer. It also contributes to the failure of chemotherapy against diseases such as cystic fibrosis, malaria, and fungal infections.[1] Tumor cells with an MDR phenotype overexpress membrane proteins that act as energy-dependent drug efflux pumps, such as P-glycoprotein (P-gp) (ABCB1) and MRP.[2,3] In *Saccharomyces cerevisiae*, the major multidrug exporters are the ABC transporters Pdr5p and Snq2p. These proteins show limited sequence similarity and share many substrates and inhibitors with mammalian P-gp. Thus, yeast Pdr5p can be used to screen for compounds that may be effective inhibitors of P-gp.[4]

Different compounds have been investigated for their ability to reverse MDR mediated by these proteins in cancer patients. These include verapamil, phenothiazines, quinidine, quinacrine, amiodarone, tamoxifen, progesterone, cyclosporine A, dexniguldipine, GF-902128, PSC-833, and VX710. However, these compounds show moderate to severe toxicity.[2,3] Efforts to identify natural products as inhibitors of MDR exporters have the potential to provide a large number of novel drug leads.

Marine sponges are an abundant source of structurally unique and biologically active substances.[5] In particular, sponges belonging to the order Agelasida are well-known sources of bromopyrrole-derived alkaloids and modified peptides.[6] Recent examples of bromopyrrole alkaloids include nagelamides K−R isolated from *Agelas* sp., which exhibited moderate antibacterial activity against various pathogenic bacterial strains,[7] sceptrin from *Agelas conifera*, which binds to the actin equivalent MreB of *Escherichia coli*,[8] and agelasine D from *Agelas* sp., which displayed antifouling activity against larvae of the barnacle *Balanus improvisus*.[9] Also reported were agelasines J, K, and L from *Agelas cf. mauritiana*, which showed moderate antimalarial activity,[10] and dispyrin from *Agelas dispar*, which is a potent ligand for therapeutically important G-protein coupled receptors.[11]

We investigated an extract of the sponge *Agelas sventres* that displayed inhibitory activity against Pdr5p from yeast plasma membranes. A bioassay-guided fractionation led to the isolation of the known metabolite oroidin (**1**) as the only active compound in this assay. Oroidin has shown fish-deterrent activity[12] and inhibited bacterial attachment on surface cells.[13] Oroidin-free base potently inhibited the malaria etiological agent *Plasmodium falciparum*[14] and interfered with membrane depolarization and calcium metabolism.[15] However, the compound has yet to be shown to modulate multidrug resistance.



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The effects of **1** as an inhibitor of Pdr5p-dependent ATP hydrolysis were investigated. The ATPase activity of the plasma membrane fractions (PM) from yeast (strain AD124567) that overexpresses Pdr5p was measured as oligomycin-sensitive release of inorganic phosphate. The result of Figure 1 shows that **1** almost completely inhibited Pdr5p ATPase activity with an IC_{50} value of 20 μM. The Lineweaver−Burk plot (Figure 2), which shows the effects of different concentrations of ATP and oroidin on Pdr5p ATPase activity, indicates that both the inhibitor and ATP compete for the same site.

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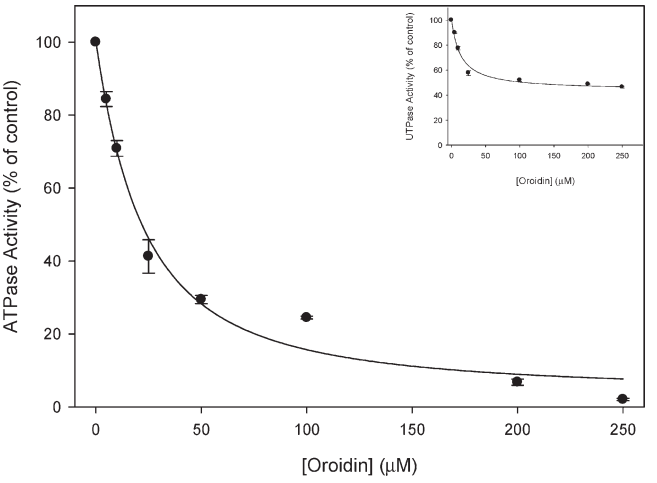

Figure 1. Inhibition of oligomycin-sensitive ATPase activity by oroidin from *Agelas sventres*. The ATPase reaction was initiated by addition of plasma membranes to 13 μg/mL in the standard assay medium containing ATP as described in the Experimental Section. Data are means ± SD of at least three independent experiments. The oligomycin-sensitive ATPase activity of control (without oroidin) corresponds to 0.174 μmol of $Pi \cdot mg^{-1} \cdot min^{-1}$. Inset: Oroidin inhibition of UTPase activity at pH 7.5.

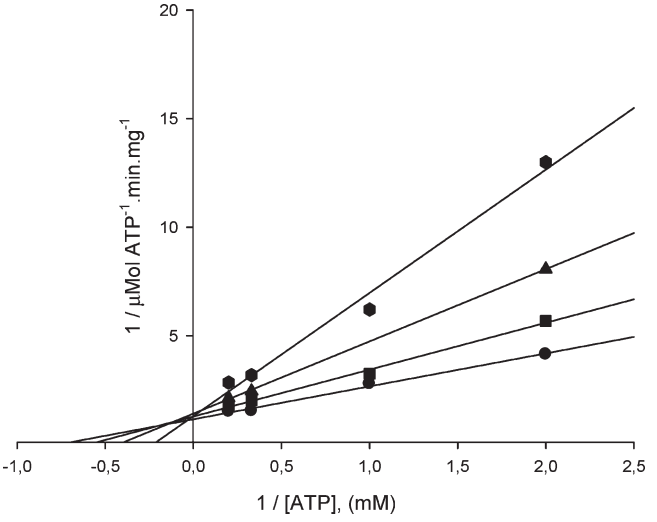

Figure 2. Lineweaver−Burk plot. The competitive inhibition experiment was performed without (●) or in the presence of (■) 5 μM, (▲) 20 μM, or (⬢) 50 μM oroidin. The assay medium was as described in the Experimental Section using 0.5, 1.0, 3.0, or 5.0 mM ATP. The double-reciprocal plot presents triplicate assays from a single representative experiment.

ABC transporters such as the yeast Pdr5p have the capacity to hydrolyze other substrates including uridine triphosphate (UTP).[4] Oroidin also inhibited Pdr5p UTPase activity (Figure. 1 inset), but only a maximum of 50% inhibition (compared to control) could be achieved in the presence of 200 μM **1**. An IC_{50} value for this observed inhibition of 13 μM was close to the value for inhibition of ATPase activity. As proposed in previous studies,[16,17] some inhibitors that block the ATP hydrolysis of ABC transporters may compete with a triphosphate substrate at the catalytic site or may bind at sites that impair ATP access.

In addition to inhibition of enzyme activity, we investigated the effect of **1** on xenobiotic transport mediated by Pdr5p. The

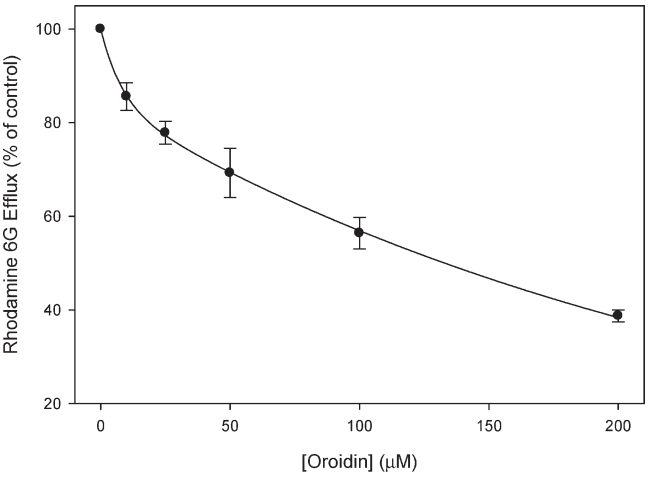

Figure 3. Oroidin inhibition of R6G efflux in strain AD124567. The efflux of R6G in the supernatant fraction was measured after incubation of the cells in the presence of different concentrations (0−200 μM) of oroidin as described in the Experimental Section. The data present means ± SD of three independent experiments.

blockade of xenobiotic transport has already been demonstrated for the well-known Pdr5p inhibitor FK506.[18] The ability of oroidin to interfere with ATP-dependent transport catalyzed by Pdr5p was tested by measuring the compound's effect on the glucose-dependent efflux of the fluorescent substrate rhodamine 6G (R6G) of strain AD124567. Oroidin caused dose-dependent inhibition of glucose-dependent R6G efflux, with 60% inhibition achieved at 200 μM **1** (Figure 3).

Phase contrast (Figure 4, A−D) and fluorescence (E−H) microscopy of rhodamine 6G show oroidin blocked Pdr5p-dependent efflux of R6G. The absence of the Pdr5p efflux pump in strain AD1234567 (Figure 4E) and either oroidin or FK506 treatment (Figure 4G,H) caused retention of the fluorescent dye in the cytoplasm of the *S. cerevisiae* resistant strain AD124567, compared with complete efflux in the untreated positive control (Figure 4F). The inhibition of efflux by **1** and FK506 was comparable (Figure 4G,H). The inhibition of Pdr5p ATPase activity and the inhibition of R6G efflux indicate that oroidin chemically reverses the multidrug resistance phenotype.

In order to verify that **1** specifically affected the transporter but not other processes, the sensitive (AD1234567) and resistant (AD124567) strains were maintained in YPD medium containing **1** for 48 h. Both strains showed a growth yield reduction of approximately 30% (Figure 5), but only at the highest concentration (200 μM) of **1**. These results confirmed that inhibition of Pdr5p was due to the transporter inhibition and not to reduced growth capacity.

Our results identify oroidin as a new inhibitor of ABC Pdr5p from the yeast plasma membrane. Further studies will be required to determine its specificity and ascertain whether it can be used as a potential drug lead for MDR-associated human health problems.

■ EXPERIMENTAL SECTION

Sponge Material. Samples of *Agelas sventres* were collected by trawling at 62−75 m depth from the *R.V. Astro Garoupa* by the Project of Environmental Characterization and Monitoring of Potiguar Basin and by scuba diving in Potiguar Basin in March 2007.[19] The Potiguar Basin is

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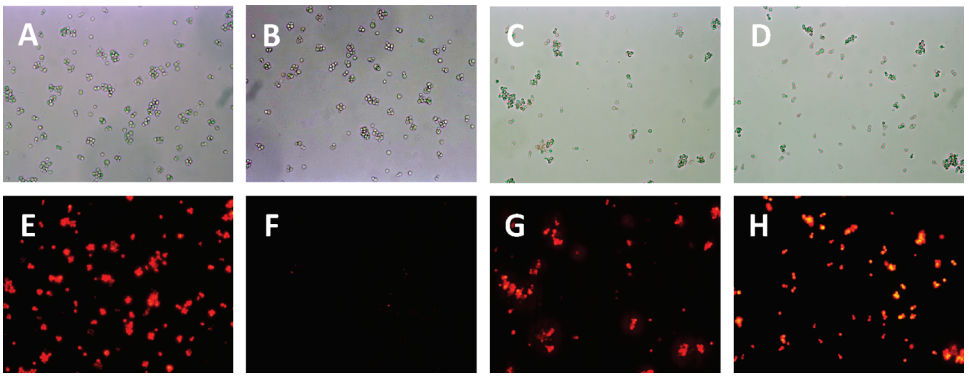

Figure 4. Fluorescence microscopy of R6G intracellular accumulation induced by oroidin. Upper panels (A−D) show the phase contrast microscopy, while the lower panels (E−H) depict fluorescence of the cells. A and E show the Pdr5p null strain (AD1234567, negative control); B and F, the Pdr5p-overexpressing strain (AD124567, untreated positive control). C and G show strain AD124567 after incubation with 100 μM oroidin for 2 h; D and H show strain AD124567 after incubation with 20 μM FK506 for 2 h (inhibitor control).

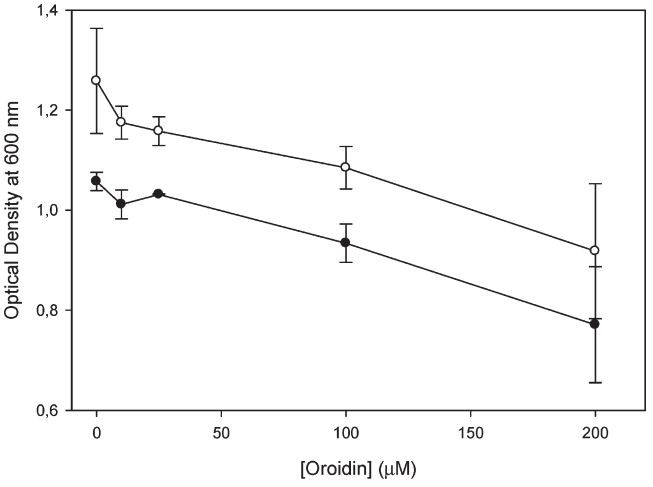

Figure 5. Cell yield of AD1234567 and AD124567 strains in the presence of oroidin. The AD1234567 (open circles) and AD124567 (solid circles) strains were grown in YPD medium for 48 h in the presence of different concentrations (0−200 μM) of oroidin as described in the Experimental Section. The data present means ± SD of three independent experiments.

located on the north continental shelf of Rio Grande do Norte and Ceará states (Northeastern Brazil). The sampling area ranged between 04°30′00″ to 05°10′00″ S and 36°10′00″ to 36°50′00″ W, off Guamaré. Vouchers of *A. sventres* are deposited in the Porifera collections of the Departamento de Zoologia of Universidade Federal de Pernambuco (UFPEPOR-307, -376, and -408).

Extraction and Isolation of Oroidin. *A. sventres* (100 g) was exhaustively extracted with EtOH. After evaporation, the aqueous suspension was sequentially partitioned against hexane, CH_2Cl_2, EtOAc, and *n*-BuOH. After evaporation to dryness, 271 mg of the hexane extract, 140 mg of the CH_2Cl_2 extract, 162 mg of the EtOAc extract, and 302 mg of the *n*-BuOH extract were obtained. The *n*-BuOH extract was the only active material and was fractionated by chromatography on a C_{18} reversed-phase silica gel column (10 g) eluted with a gradient of MeOH in H_2O to give seven fractions (AgsBu-1 to AgsBu-7). The second fraction of this separation (AgsBu-2, 82.5 mg) was further separated by C_{18} reversed-phase HPLC (Synergi Fusion RP80 C18, 250 × 4.6 mm, 4 μm pore) using 40:60 MeOH/H_2O as eluent. Oroidin (22.3 mg) was isolated as the single active compound and identified by comparison of its spectroscopic data with literature values.[20,21]

Preparation of Plasma Membranes. The plasma membranes containing the overexpressed Pdr5p protein were prepared from the yeast *S. cerevisiae* mutant strain AD124567 as previously reported.[22]

NTPase Assays. The enzymatic activities using ATP or UTP as substrates were assayed in a standard medium (50 μL final volume) containing 100 mM Tris-HCl pH 7.5, 4 mM $MgCl_2$, 75 mM KNO_3, 7.5 mM NaN_3, and 0.3 mM ammonium molybdate, in the presence of 3 mM ATP or UTP. The reaction was started by the addition of plasma membrane to 13 μg/mL, maintained at 37 °C for 60 min, and stopped by addition of 1% SDS, as previously described.[23] The released inorganic phosphate (Pi) was measured as described.[24] Oroidin was added from stock solutions in DMSO up to a 5% v/v final concentration. The difference in NTPase activity in the presence or absence of 3 μM oligomycin corresponded to a Pdr5p-mediated ATPase activity.

Rhodamine 6G Efflux by Intact Cells. Rhodamine efflux assays were performed as previously reported.[25] Briefly, log-phase AD124567 cells were washed four times with distilled H_2O, harvested by centrifugation, resuspended in 50 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH (pH 7.0) buffer, and incubated overnight at 4 °C. To deplete intracellular energy, cells were incubated for 30 min with 2-deoxyglucose and then with 15 μM rhodamine 6G for 30 min. The cells were washed twice with distilled H_2O and resuspended in HEPES buffer at an optical density of 10. Cell samples (400 μL) were incubated at 30 °C in the presence or absence of oroidin. Glucose at a 0.2% final concentration was added to start the reaction. After 13 min, cells were pelleted, and rhodamine 6G present in the supernatant was fluorimetrically quantified using a microplate reader (Fluostar Optima, BMG Labtech, Offenburg, Germany) with an excitation wavelength of 529 nm and an emission wavelength of 553 nm.

Intracellular Accumulation of Rhodamine 6G. Yeast cells (2×10^6 cells/mL) in the log-phase of growth at 30 °C in YPD medium were incubated with and without **1** (100 μM) or FK506 (20 μM) for 2 h at 30 °C. Rhodamine 6G (2 μg/mL) was added, and the cells were incubated for 30 min at 30 °C. After washing, cells were resuspended with 50 mM HEPES buffer at pH 7.0, and their fluorescence was visualized using a Nikon Eclipse E300 microscope.

Growth Yield Assay. The growth yield of both strains of *S. cerevisiae* used in this work, the resistant Pdr5p overexpressing strain (AD124567) and the hypersensitive Pdr5p null strain (AD1234567), was determined using 96-well microtiter plates as previously described.[25] Briefly, 4×10^3 cells were inoculated into wells in the absence or in the presence of different concentrations of **1** and cultivated at 30 °C for 48 h with constant agitation. Cell growth was evaluated at 600 nm in a microplate reader (FLUOstar OPTIMA, BMG LABTECH).

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