Note

Pinostrobin from *Boesenbergia pandurata* Is an Inhibitor of Ca\(^{2+}\)-Signal-Mediated Cell-Cycle Regulation in the Yeast *Saccharomyces cerevisiae*

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Upon searching plant extracts for inhibitors of the Ca\(^{2+}\) signaling pathway using the zds1Δ-yeast proliferation based assay, a crude rhizome extract of *Boesenbergia pandurata* was found to be strongly positive, and from this extract pinostrobin, alpinetin, and pinocembrin chalcone were isolated as active components. Further biochemical experiments confirmed that pinostrobin possesses inhibitory activity on the Ca\(^{2+}\) signals involved in the control of G2/M phase cell cycle progression in *Saccharomyces cerevisiae*.

Key words: yeast based assay; *Boesenbergia pandurata*; pinostrobin; Ca\(^{2+}\) signal; inhibitor

Calcium ion (Ca\(^{2+}\)) signals play fairly diverse but important roles in the regulation of diverse cellular processes in higher eukaryotes.1,3 The pathways have been extensively studied in the yeast *Saccharomyces cerevisiae*, and they are implicated in the regulation of G2/M cell cycle progression. Inappropriate activation of the Ca\(^{2+}\)-signaling pathway in *S. cerevisiae* causes a deleterious physiological effect and various defects, including growth defects in the zds1-null mutant yeast.2

Based on these observed phenotypes, and in particular the fact that the delayed G2 progression caused a loss of proliferation in the presence of calcium ions, a unique positive screening system utilizing the zds1Δ yeast strain as indicator cells has been developed.3 The yeast-based screening system is based on the premise that compromised cell growth in the zds1Δ mutant yeast can be rescued by small molecule chemicals on solid medium containing CaCl\(_2\). Thus the assay is likely to detect inhibitors of the calcium dependent pathway targets. Small-molecule inhibitors of the Ca\(^{2+}\) signaling pathways in humans are of great medical importance, since Ca\(^{2+}\) signaling in mammalian cells plays pivotal roles in the regulation of diverse cellular processes, including T-cell activation, secretion, motility, and apoptosis.2,4 Calcineurin is a calcium-dependent protein phosphatase that functions in T cell activation, in which the known inhibitors, FK506 and cyclosporine A, are widely used as potent immunsuppressants.5 In addition, several cell-cycle inhibitors have potential as anticancer agents.6

Herein, we report on the use of the above yeast-based assay to guide the fractionation and purification of the active components in a positive crude plant extract, *Boesenbergia pandurata*, and further biochemical experiments that confirmed that pinostrobin, one of the compounds in the crude extract, possesses inhibitory activity on the Ca\(^{2+}\) signals involved in control of G2/M phase cell cycle progression in *S. cerevisiae*.

The *S. cerevisiae* mutant zds1Δ strain, YNS17, (MATa zds1::TRP1 erg3::HIS3 pdr1::hisG URA3 hisG) was used for indicator cells in a zds1Δ proliferation based screening assay modified from Shitamukai *et al.*3 Assay plates, comprised of YPD containing 150 mM CaCl\(_2\) and 0.7% (w/v) soft agar, were seeded with 6 × 10\(^5\) cells (zds1Δ cells)/ml. Five microlitres of the test sample, dissolved in ethanol at a concentration of zero (solvent control) or 5 mg/ml, and 5 μl of 100 mM FK506 (positive control) were dotted onto the assay plates and then incubated at 30°C for 2 d. A positive assay showed a growth zone at and around the application spot. *B. pandurata* was collected from Nakhon Pathom Province, in the central part of Thailand. A voucher specimen (BKF 152279) has been deposited at the Bangkok Forest Herbarium (BKF), Royal Forest Department, Chatuchak, Bangkok 10900, Thailand. The rhizomes of *B. pandurata* were harvested, sliced, dried in a shed, and ground into powder. The dried rhizome powder (2.4 kg) was successively macerated with CH\(_2\)Cl\(_2\), EtOAc, and CH\(_3\)OH for 5 d, and the extraction was repeated 3 times. The CH\(_2\)Cl\(_2\) fraction

Abbreviations: YPD, yeast extract peptone dextrose; TLC, thin layer chromatography; PI, propidium iodide

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that showed the strongest biological activity in the yeast-based assay was selected for further fractionation and characterization. The full purification details are available upon request, but in brief, the concentrated CH$_2$Cl$_2$ extract (100 g) was separated by silica gel quick column using a gradient solvent starting from hexane, and was increased in polarity by mixing with EtOAc and MeOH. Thin layer chromatography (TLC) using CH$_2$Cl$_2$ as the mobile phase (resolving) solvent and activity in the yeast-based assay were followed to guide the fractionation and purification processes. The positive fractions were further purified using a chromatographic technique and precipitation until suitable crystals were obtained. NMR (Varian Mercury Plus 400, Palo Alto, CA) and mass spectrometry (Micromass UK Limited, Manchester, UK) were employed to elucidate the structure of the pure compounds as compared with those reported in the literature.\(^8,9\) Flow cytometric analysis was performed by FACSCalibur (Becton Dickinson, Franklin Lakes, NJ), as described previously,\(^1\) and the yeast cell suspensions were fixed and permeabilised as reported elsewhere\(^2\) prior to staining with 5 μM Hoechst 33342 (Sigma, St. Louis, MO).

A crude extract of _B. pandurata_ was one of the positive crude extracts found from the screens (our unpublished data, Fig. 1A). Clear yeast colony growth was seen with the positive control, FK506, and not with the solvent only control, while the _B. pandurata_ crude ethanolic extract showed a doughnut-like halo with a clear zone in the center. _B. pandurata_, the finger root plant, is an herb that belongs to the Zingiberaceae family. The fresh rhizomes are commonly used in traditional Thai medicine in several applications.\(^8\)

To search for and to purify the active compounds, the same procedure for fractionation monitoring of the fractions and further purification as that described above was performed. Spectroscopic techniques were used to identify pure compounds. Three pure compounds were obtained: compound 1 as a bright pale-yellow crystal, compound 2 as a white amorphous powder, and compound 3 as a red crystal, with yields of 10.85, 1.73 and 1.20% respectively w/w relative to the amount of starting rhizome material (data not shown). NMR and mass spectroscopic analysis (data available upon request) confirmed their purification to apparent homogeneity and, by comparison to those reported in the literature, suggested that the identities of compounds 1 to 3 were pinostrobin or 5-hydroxy-7-methoxyflavane none\(^8,10\) (C$_{16}$H$_{14}$O$_4$), alpinetin or 7-hydroxy-5-methoxyflavanone\(^10\) (C$_{16}$H$_{14}$O$_4$), and pinocembrin chalcone or 2',4',6-trihydroxychalcone\(^11\) (C$_{15}$H$_{12}$O$_3$) respectively (Fig. 1B).

To compare biological activities, these purified compounds were separately tested on _zdsΔ_ yeast proliferation assay plates at varying concentrations (Fig. 1B). The positive (FK506) and solvent (ethanol) controls exhibited good and no growth respectively, while the three pure compounds exhibited different but significant dose-dependent growth of the yeast. Pinostrobin potentially showed the highest biological activity among the three. The minimal effective concentrations of pinostrobin, alpinetin, and pinocembrin chalcone were <0.5, 1, and 0.5 mM respectively.

Next we evaluated the effects of the pure compounds on the growth of _zdsΔ_ cells in liquid YPD broth, and found that in YPD broth with 75 mM CaCl$_2$, 1 mM pinostrobin had no detectable toxic effect upon cell growth (Fig. 1C), while alpinetin and pinocembrin chalcone at 1 mM and lower concentrations were cytotoxic to the cells (data not shown). Treatment of the cells with 1 mM pinostrobin or 500 nM FK506 (positive control) at 30 °C for 30 min followed by the addition of 75 mM CaCl$_2$ (final concentration) in YPD rescued the proliferation of the Ca$^{2+}$ sensitive _zdsΔ_ yeast cells to the same extent (Fig. 1C). Since pinostrobin had the lowest cytotoxic effect upon the mutant yeast cells and the yield obtained from the crude extract was the highest among the three positive compounds, only pinostrobin was selected for further characterization.

Hyperactivation of the Ca$^{2+}$ signals in the _zdsΔ_ yeast strain caused the cells to arrest or delay at the G2 phase of the cell cycle.\(^2\) To determine whether the purified compound would rescue Ca$^{2+}$ hyperactivated cells from cell cycle arrest or delay at the G2 phase, flow cytometric analysis of propidium iodide stained _zdsΔ_ cells was performed. The control reference _zdsΔ_ cells grown in YPD media without CaCl$_2$ condition revealed about 2.5× more cells with 2C DNA content than with 1C DNA content (71.9 and 28.1% respectively) (Fig. 2A, 1). The addition of 100 mM CaCl$_2$ caused accumulation of a population of cells with 2C DNA content (74.3%) and decreased in that with 1C DNA content (25.8%) (Fig. 2A, 1). On the other hand, treatment of _zdsΔ_ cells with 150 mM CaCl$_2$ caused an accumulation of dead cells (a population of cells with less than 1C DNA content; Fig. 2A, 1). Treatment of cells with the calcineurin inhibitor, FK506 (as a positive control) prior to the addition of 100 mM CaCl$_2$ to the medium decreased the proportional number of cells with a 2C DNA content and increased those with a 1C DNA content (66.0 and 34.0% respectively), compared to those in the negative control experiment (Fig. 2A, 2). Treatment with 1 mM pinostrobin followed by the addition of 100 mM CaCl$_2$ into the medium showed the same pattern of flow cytometric profile as that of the FK506 treated cells (64.9 and 35.1% respectively) (Fig. 2A, 3). However, treatment with 1 mM pinostrobin or 500 nM FK506 immediately after CaCl$_2$ exposure did not rescue the cells from the deleterious effect on the G2 phase delay caused by Ca$^{2+}$ hyperactivation (data not shown). This result indicates that treatment of cells with 1 mM pinostrobin prior to the addition of CaCl$_2$ prevented the yeast cells from G2 delay as a consequence of Ca$^{2+}$ hyperactivation.

Besides causing _zdsΔ_ cells to arrest in the G2 phase, the hyperactivation of Ca$^{2+}$ signals also induces abnormal bud emergence.\(^2\) Hoechst 33342 nuclear staining revealed that 54% of DMSO (solvent control) treated Ca$^{2+}$ hyperactivated cells displayed an abnormal morphology, with elongated buds, and unequal nuclear budding (the nuclei were only in the mother cells and were not found in the daughter cells) (Fig. 2B, 1). In contrast, the cells treated with 500 nM FK506 (positive control) or 1 mM pinostrobin prior to exposure to 100 mM CaCl$_2$ showed a normal nuclear morphology with an equal distribution of nuclei in the mother and daughter cells (Fig. 2B and 3 respectively) and only 7
and 11% of the cells displayed elongated buds respectively (Fig. 2B).

Taken together, the flow cytometric profiles, cell morphology, and nuclear staining experiments indicate that pinostrobin can relieve the hyperactivation of Ca\(^{2+}\) signals in yeast responsible for the abnormal morphology and growth arrest associated with G2 arrest in S. cerevisiae. We conclude that pinostrobin from B. pandurata has an inhibitory effect on the relevant Ca\(^{2+}\) signaling pathway in the yeast S. cerevisiae, but its targets were not addressed, and this awaits further work. Nevertheless, this study provides support for the usefulness of positive screening systems in drug discovery. Pinostrobin can be isolated from many natural sources, such as Polygonum lapathifolium L. ssp. Nodosum with an anti-leukemic activity against Jurkat and HL-60 cell lines,\(^{12}\) and Piper lanceaeolatum with anti-Candida albicans bioactivity.\(^{13}\) Pinostrobin and pinocembrin chalcone from B. pandurata also showed potent anti-mutagenic activity against Trp-P-1 on an Ames test.\(^{14}\) It will be of interest to focus further on the molecular targets of pinostrobin.

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