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New Eremophilane Sesquiterpenoid Compounds, Eremoxylarins A and B Directly Inhibit Calcineurin in a Manner Independent of Immunophilin

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Abstract In the course of our screening program for a new Ca^{2+}-signal transduction inhibitor using the hypersensitive mutant strain of *Saccharomyces cerevisiae* (*zds1*Δ *erg3*Δ *pdr1*Δ *pdr3*Δ), new eremophilane sesquiterpenoid compounds eremoxylarins A and B were found to restore the growth inhibition caused by the hyperactivated Ca^{2+}-signal. These compounds showed lethal activity against the *mpk1*Δ strain, specifically, compared to the *cnb1*Δ strain, and ion-sensitive activity against the wild-type strain in the presence of LiCl, indicating that their molecular target might be the calcineurin pathway. They inhibited calcineurin directly without immunophilins at IC_{50}=2.7 and 1.4 μM with competitive inhibition *in vitro*. The eremophilane sesquiterpenoid structure in eremoxylarins could be a good leading compound for immunosuppressants and anti-allergy drugs.

Keywords eremophilane sesquiterpene, eremoxylarin, *Saccharomyces cerevisiae*, calcium signaling, calcineurin (PP2B)

Introduction

The Ca^{2+}-signal transduction pathways have important roles in the regulation of such diverse cellular processes as T-cell activation, muscle contraction, neurotransmitter release, and secretion [1]. The only cause for a growth defect in the G2 phase of the *zds1*Δ cells of *Saccharomyces cerevisiae* in medium with $CaCl_2$ depends on the hyperactivation of cellular Ca^{2+}-signal [2]. The inhibitors of Ca^{2+}-signal transduction are detected by their ability to stimulate the growth of the cells as a growth zone around a paper disc containing the active compound (Fig. 1) [3, 4]. The screening using *S. cerevisiae* is very convenient for the discovery of new drug candidates, because it has cellular processes that are highly conserved to human cells. In addition, technical advantages of this organism, such as simple growth conditions, rapid cell division, and the availability of genetic tools, have expanded the application of yeast as a screening tool in the field of drug discovery. Thus the drugs isolated by the yeast screening system may be applicable to mammalian cell systems, especially to humans. However, the drug permeability through the cell membrane of *S. cerevisiae* is not especially good. Thus, we used a drug-sensitive strain with disrupted genes of *erg3*, *pdr1*, and *pdr3* except *zds1* [5].

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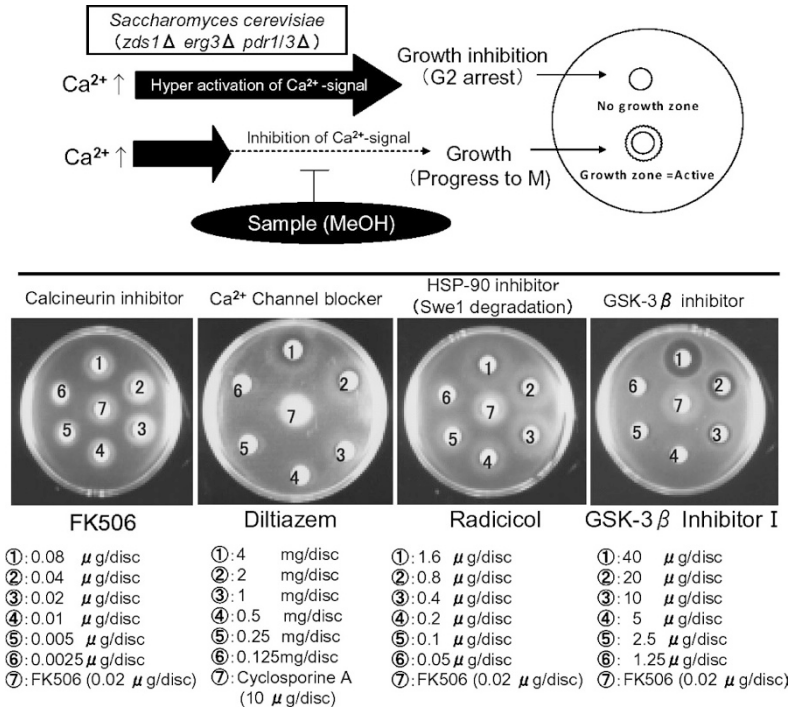

Fig. 1 Illustration of the screening method for Ca^{2+}-signal transduction inhibitor using a mutant strain of *Saccharomyces cerevisiae* (*zds1*Δ *erg3*Δ *pdr1*Δ *pdr3*Δ) and the restored growth activity of various compounds against it.

The Ca^{2+}-signaling pathways for growth regulation are composed of several signaling molecules such as the Ca^{2+} channel (target of anti-hypertension drugs) [6], calcineurin (target of immunosuppressants) [7], Pkc1 protein kinase C (target of anti-cancer drugs) [8], Mpk1 MAPK (target of anti-cancer drugs) [9], and Mck1 GSK-3 (target of anti-diabetes and Alzheimer's disease drugs) [10]. In fact, calcineurin inhibitors FK506 and cyclosporine A [11, 12], which are important clinical medicines as immunosuppressants, showed a growth zone of the cells in this screening (Fig. 1) [3, 6]. The inhibition mechanism of FK506 and cyclosporine A to calcineurin is remarkable, because both compounds are bound by their respective binding proteins (immunophilins), named FKBP12 and cyclophilin A, and their complexes inhibit calcineurin, leading to suppressed T cell activation [11, 12]. In addition, we have already found that the Ca^{2+} channel blocker diltiazem [13], the inhibitor of HSP90 called radicicol [5], and the GSK-3β inhibitor GSK-3β inhibitor I [14] also inhibit each molecular target in the Ca^{2+}-signal transduction of *S. cerevisiae* and showed the growth zone only and/or the growth zone with an inhibition zone on the plate (Fig. 1).

In many molecular targets, calcineurin is a Ca^{2+}/CaM-dependent serine/threonine protein phosphatase and is a fascinating drug target for immunosuppressants and anti-inflammation drugs. To find a new type of calcineurin inhibitor different from FK506 and cyclosporine A, we used a unique screening system and identified two new eremophilane sesquiterpenoid antibiotics, elemoxylarins A and B [15] (Fig. 2). These compounds showed a growth zone on the plate in a dose-dependent manner and inhibition of calcineurin without any immunophilin. This report describes the biological properties of these compounds.

Materials and Methods

Chemicals and Strains

Eremoxylarins A and B were isolated using a previously reported protocol [15]. All of the yeast strains were derivatives of strain W303-1A. The strains used in this study were the following: YNS17 (*MATa zds1::TRP1 erg3::HIS3 pdr1::hisG URA3 hisG pdr3::hisG*) [6]. DHT14 (*cnb1*Δ strain, *cnb1::His3*) and TNP46 (*mpk1*Δ strain, *mpk1::HIS3*) [16]. *zds1*Δ *erg3*Δ *fkb1*Δ strain (*zds1::TRP1 erg3::HIS3 fkb1::His3*) and *zds1*Δ *erg3*Δ *cph1*Δ strain (*zds1::TRP1 erg3::HIS3 cph1::Leu2*). Difco® YPD broth and YPD agar were purchased from Becton Dickinson (Franklin Lakes, NJ). Diltiazem were purchased from Wako Pure Chemical Industries (Osaka, Japan).

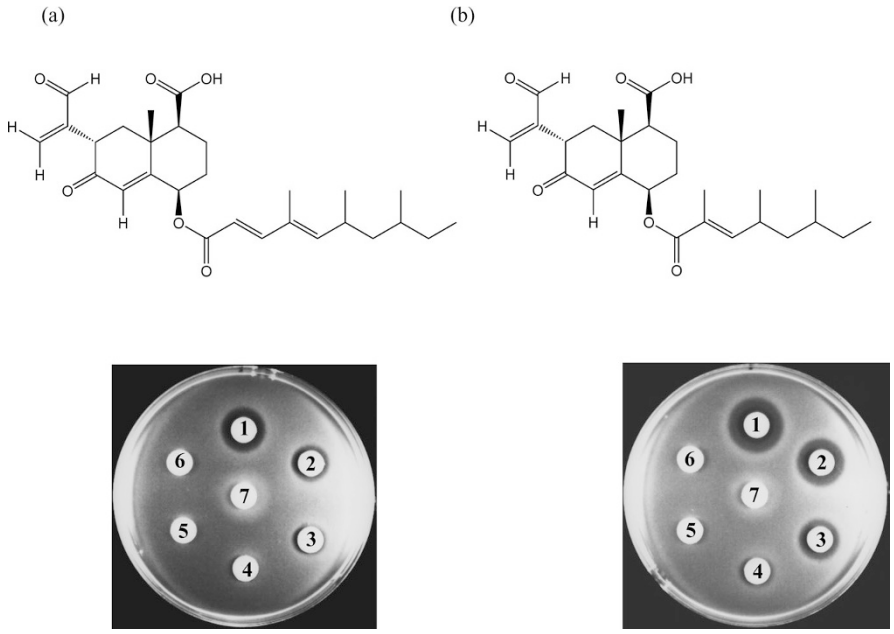

Fig. 2 Restored growth activity of eremoxylarins A (a) and B (b) against a mutant strain of *Saccharomyces cerevisiae* (*zds1*Δ *erg3*Δ *pdr1*Δ *pdr3*Δ).

Assays were carried out as described in Materials and Methods. 1: 40 μg/disc, 2: 20 μg/disc, 3: 10 μg/disc, 4: 5.0 μg/disc, 5: 2.5 μg/disc, 6: 1.25 μg/disc, 7: 0.02 μg/disc (FK506).

Radicicol and calcineurin colorimetric drug discovery kit (AK-804) were purchased from BIOMOL® International, LP (PA, USA). FK506 was kindly given by Fujisawa Pharmaceutical Co., Ltd. (the present Astellas Pharma Inc., Tokyo, Japan). GSK-3β inhibitor I was purchased from Calbiochem (CA, USA). Cyclosporine A and trifluoperazine dihydrochloride were purchased from SIGMA-ALDRICH (St. Louis, MO). Paper discs (8-mm) were purchased from Toyo Roshi (Tokyo, Japan). Unless otherwise stated, chemicals used in this study were of the best grade commercially available.

Screening of Ca^{2+}-signal Transduction Inhibitors

The screening was done using a hypersensitive mutant strain of *S. cerevisiae* (*zds1*Δ *erg3*Δ *pdr1*Δ *pdr3*Δ) and 8-mm paper discs containing each compound. A 6.0-ml aliquot of pre-cultured cells (A_{590}=1.0) and 3.0 ml of 5 M $CaCl_2$ were suspended in 41 ml of YPD-agar medium at 50°C and this suspension was spread over each of four plates. Various concentrations of the sample in MeOH were applied to an 8-mm paper disc (40 μl/disc) and the disc was placed on the surface of the agar. The plates were incubated at 28°C for 3 days. The inhibitory activity of the Ca^{2+}-signal transduction was determined by the diameter of the growth zone and/or inhibition zone of the cells using various compounds against their molecular targets. The immunosuppressive drugs, FK506 (0.02 μg/disc) or cyclosporine A (10 μg/disc) were used as a positive control (Fig. 1).

Presumption of the Molecular Target

For the determination of a preliminary molecular target pathway, we used the synthetic lethal character of *cnb1*Δ and *mpk1*Δ in *S. cerevisiae* [16]. A 6.0-ml aliquot of the pre-cultured cells (A_{590}=1.0) was suspended in 44 ml of YPD-agar medium at 50°C and this suspension was spread over each of four plates. A 5.0-μl aliquot of eremoxylarins was spotted on the plate of each *cnb1*Δ and *mpk1*Δ strain at various concentrations and the strains were grown on YPD plates at 28°C for 2 days. The sensitivity of the compound was measured by its inhibition zone. The immunosuppressive drug FK506 (0.1 μg/spot) was used as a positive control.

Ion Sensitivity

Ion sensitivity of eremoxylarin was measured using the wild-type strain (W303-1A). A 6.0-ml aliquot of pre-cultured cells (A_{590}=1.0) was suspended in 41 ml of YPD-agar medium at 50°C with or without 0.16 M LiCl and this suspension was spread over each of four plates. A 5.0-μl aliquot of various concentrations of the sample was spotted on the surface of the agar. The plates were incubated at 28°C for 2 days. The inhibitory activity of calcineurin was

judged by the inhibition zone with or without LiCl. The immunosuppressive drug FK506 (0.1 μg/spot) was used as a positive control.

Dependence of Calcineurin Inhibition Activity on Immunophilins

The examination of immunophilin independence was done using the two mutant strains (*zds1*Δ *erg3*Δ *fkb1*Δ and *zds1*Δ *erg3*Δ *cph1*Δ) and 8-mm paper discs containing each compound. A 6.0-ml aliquot of the pre-cultured cells (A_{590}=1.0) and 3.0 ml of 5.0 M $CaCl_2$ were suspended in 41 ml of YPD-agar medium at 50°C and this suspension was spread over each of four plates. Various concentrations of the sample in MeOH were applied to an 8-mm paper disc (40 μl/disc) and the disc was placed on the surface of the agar. The plates were incubated at 28°C for 3 days. Calcineurin inhibitory activity was determined by a growth zone. The immunosuppressive drugs FK506 (0.02 μg/disc) and cyclosporine A (40 μg/disc) were used as positive controls.

Calcineurin Assay

The calcineurin activity was measured using a commercial kit with a small modification, in which free phosphate ion released from a substrate phosphopeptide (DLDVPIPGRFDRRVpSVAAE) was quantified by colorimetric analysis (650 nm) using the malachite green method. Briefly, the substrate phosphopeptide (37.5 μM) was incubated with human calcineurin (20 U) and calmodulin (0.0625 μM) at 37°C for 6 hours in 25 mM Tris-HCl (pH 7.5), 50 mM NaCl, 3.0 mM $MgCl_2$, 0.25 mM DTT, 0.0125% NP-40, and 0.25 mM $CaCl_2$ in the absence or presence of the sample (in 2.0 μl of MeOH, 2.0% of total volume). The calmodulin antagonist trifluoperazine was used as a positive control.

Results

Restored Growth Activity of Eremoxylarins against the *zds1*Δ Mutant Yeast

The Ca^{2+} channel blocker diltiazem showed a growth zone at high concentration (4.0 mg/disc) and the HSP90 inhibitor radicicol showed a growth zone at low concentration (1.6 μg/disc) [5]. The synthetic GSK-3β inhibitor GSK-3β inhibitor I showed a growth zone around a clear inhibitory zone. Conversely, calcineurin inhibitors of clinical immunosuppressants FK506 and cyclosporine A showed only a growth zone without an inhibitory zone against the mutant strain at very low concentration (Fig. 1). The phenotype of a growth zone has a different character depending on each molecular target and/or inhibitor. Under the same conditions, eremoxylarins A and B showed restored growth activity against the sensitive mutant strain (*zds1*Δ *erg3*Δ *pdr1*Δ *pdr3*Δ) in a dose-dependent manner with a small inhibition zone (Fig. 2). Although the phenotype of ermoxylarins are similar to those of diltiazem and GSK-3β inhibitor I (Figs. 1 and 2), there are differences in the growth restored specificity with or without Ca^{2+} (data not shown). Eremoxylarin A has less potent activity than that of eremoxylarin B.

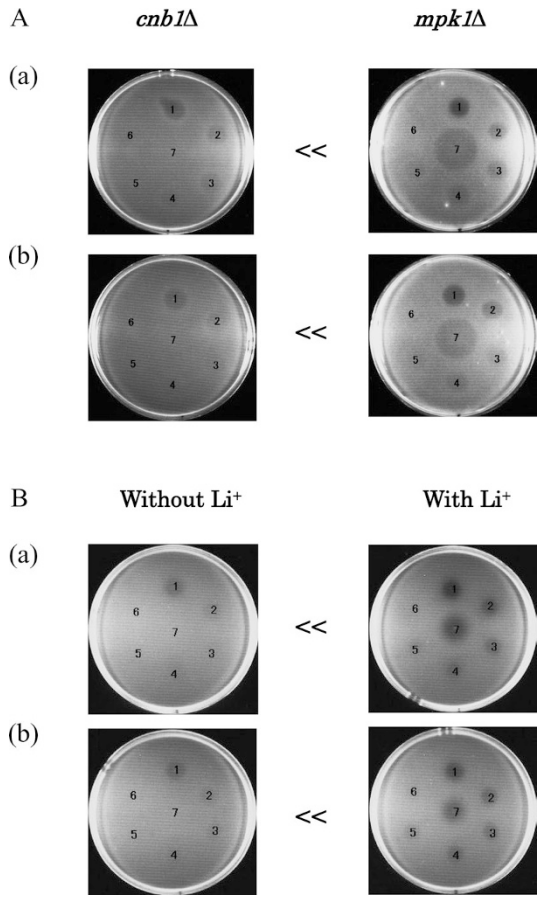

Fig. 3 Presumption of the molecular target of eremoxylarins A and B.

A: Synthetic lethal activity of eremoxylarins A (a) and B (b) against the deletion mutant of *cnb1*Δ and *mpk1*Δ in *Saccharomyces cerevisiae*. Assays were carried out as described in Materials and Methods. 1: 10 μg/spot, 2: 5.0 μg/spot, 3: 2.5 μg/spot, 4: 1.25 μg/spot, 5: 0.63 μg/spot, 6: 0.31 μg/spot, 7: 0.1 μg/spot (FK506). B: Inhibition activity of eremoxylarins A (a) and B (b) against the wild type *Saccharomyces cerevisisae* under high salt (Li^+) stress conditions. Assays were carried out as described in Materials and Methods. 1: 10 μg/spot, 2: 5.0 μg/spot, 3: 2.5 μg/spot, 4: 1.25 μg/spot, 5: 0.63 μg/spot, 6: 0.31 μg/spot, 7: 0.1 μg/spot (FK506).

Lethal Activity of Eremoxylarins against the *cnb1*Δ or *mpk1*Δ Mutant Strain

The cell-cycle regulation by Ca^{2+} is executed through the activation of two parallel pathways, calcineurin and the Mpk1 MAP kinase cascade, and the deletion of both genes showed a lethal phenotype [16]. Isogenic strains differing only in the presence or absence of functional calcineurin or MAP kinase were tested for sensitivity to eremoxylarins in growth inhibition assays. Eremoxylarins A and B showed the growth inhibition zone against the *mpk1*Δ strain specifically having the same character as FK506. It was shown that both compounds might be acted on the pathway of calcineurin (Fig. 3A).

Salt Tolerance Activity of Eremoxylarins

Mutations in the catalytic or regulatory subunits of calcineurin caused a defect of tolerance to salt such as NaCl and LiCl [17]. Using this character, calcineurin could be confirmed as the sample target. As we expected, eremoxylarins showed an inhibition zone to the wild type of *S. cerevisiae* with 0.16 M LiCl that is similar in character to FK506 (Fig. 3B). The wild-type strain exposed to eremoxylarins and 0.16 M LiCl lost the salt tolerance activity, therefore, it was shown that both compounds might be acted on calcineurin.

Independence of Calcineurin Inhibition Activity on Immunophilins

The immunosuppressants FK506 and cyclosporine A bind to specific immunophilins and their complexes selectively inhibit calcineurin, leading to the suppression of T-cell proliferation [11, 12]. Thus, using the deletion mutant strain of each immunophilin, *fkb1*Δ and *cph1*Δ with the combined character of *zds1*Δ *erg3*Δ, eremoxylarins which can inhibit calcineurin directly without its immunophilins such as FKB1 and CPH1 in *S. cerevisiae* could be examined. The immunosuppressant FK506 (0.02 μg/disc) showed a clearly restored growth zone only to the strain of *zds1*Δ *erg3*Δ *cph1*Δ [Fig. 4A(b)-No. 3] and the immunosuppressant cyclosporine A (40 μg/disc) showed a clearly restored growth zone only to the strain of *zds1*Δ *erg3*Δ *fkb1*Δ [Fig. 4A(a)-No. 4]. Eremoxylarins A and B showed faint growth zones compared to those of FK506 and cyclosporine A on both plates, because this mutant strain did not have disrupted pdr1 and pdr3 genes [Fig. 4A(a), (b)-No. 1 and No. 2]. These results also support the idea that eremoxylarins inhibit calcineurin directly without known immunophilins FKB1 and CPH1 in *S. cerevisiae*.

Calcineurin Inhibition Activity of Eremoxylarins

To determine the molecular target, the inhibition activity of

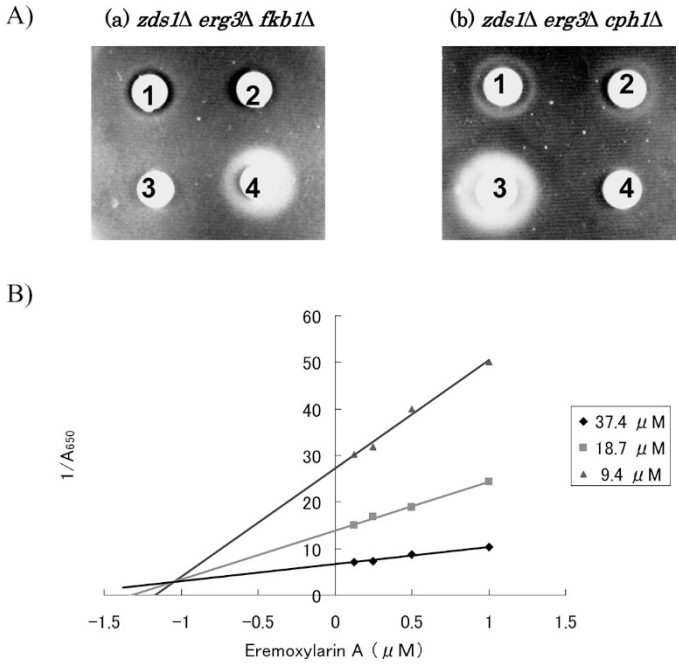

Fig. 4 Eremoxylarins A and B inhibit calcineurin without any immunophilin.

A: Restored growth activities of eremoxylarins A, B, FK506, and cyclosporine A against the deletion mutant of immunophilins FKB1 (a) and CPH1 (b). Assays were carried out as described in Materials and Methods. 1: Eremoxylarin A (40 μg/disc), 2: Eremoxylarin B (80 μg/disc), 3: FK506 (0.02 μg/disc), 4: Cyclosporine A (40 μg/disc). B: Dixon Plot of eremoxylarin A to calcineurin. Assays were carried out as described in Materials and Methods. 1/*v* was defined as 1/Δ650 nm.

eremoxylarins A and B on calcineurin *in vitro* was directly examined. Eremoxylarins A and B inhibited calcineurin in a dose-dependent manner and showed IC_{50}=2.7 and 1.4 μM, whereas the control compound trifluoperazine showed IC_{50}=20.8 μM. The inhibition mechanisms of eremoxylarins A and B were both competitive inhibition against calcineurin with a synthetic substrate in the Dixon plot (Ki=1.1 and 0.7 μM, respectively) as shown in Fig. 4B. Although an increase in calmodulin could affect the inhibition activity of the calmodulin antagonist trifluoperazine, it did not affect the inhibition activities of eremoxylarins A and B (data not shown).

Discussion

Here, a new calcineurin inhibitor using a phenotypic screening system of mutant yeast has been discovered. Although FK506 and cyclosporine are excellent therapeutic immunosuppressants, those compounds alone can not inhibit calcineurin and each needs an immunophilin that is a binding protein for this inhibition. Because immunophilins have been shown to be involved in various other biological processes [18], some of the side effects of FK506 and cyclosporine might be caused by the inhibition of these processes. However, a direct and selective inhibitor has not been reported so far [19]. Eremophilane sesquiterpenoid compounds are a different type from the known compounds FK506 (macrolide), cyclosporine A (peptide), and cantharidin derivatives. Eremoxylarins A and B can inhibit calcineurin without any immunophilin and showed potent inhibition activity to human calcineurin. Although faint growth zone on both mutant strains, *zds1*Δ *erg3*Δ *fkb1*Δ and *zds1*Δ *rg3*Δ *cph1*Δ by eremoxylarins (Fig. 4A) might suggest that they have other biological activities such as HIV-1 integrase inhibition, phospholipase D inhibition and anti-tumor activities [20～22] which have already reported in related compounds, the eremophilane sesquiterpenoid structure of eremoxylarins could be a good leading compound for immunosuppressants and anti-allergy drugs.

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