รายงานฉบับสมบูรณ์

ทุนอุดหนุนโครงการวิจัย จากงบประมาณแผ่นดิน ประจำปี 2557

สารออกฤทธิ์กดภูมิคุ้มกันชนิดใหม่จากต้นส่องฟ้าดง

Clausena harmandiana (Pierre)

โดย

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บทคัดย่อ

วิถีการส่งสัญญาณแคลเซียมมีบทบาทสำคัญในการควบคุมกระบวนการทางชีวภาพมากมายในยูคาริโอต ตั้งแต่ยีสต์ จนถึงมนุษย์ สารออกฤทธิ์ยับยั้งวิถีการส่งสัญญาณดังกล่าวในมนุษย์ มีประโยชน์ต่อทางการแพทย์เป็นอันมาก สารยับยั้งเหล่านี้ได้แก่ FK506 และ cyclosporine A การทดสอบยาจากสารทดสอบได้พร้อมกันในปริมาณมากเพื่อหาสารออกฤทธิ์ยับยั้งวิถีการส่งสัญญาณแคลเซียมได้ถูกพัฒนาขึ้นมาก่อนนี้ โดยอาศัยหลักการที่สารออกฤทธิ์ยับยั้งสามารถฟื้นฟูเซลล์จากความผิดปกติของการเจริญในเซลล์ยีสต์ Saccharomyces cerevisiae ที่ขาดยีน ZDS1 (ΔADS1) ซึ่งมีสาเหตุมาจากการที่วิถีสัญญาณแคลเซียมถูกกระตุ้นในระดับที่สูง ในงานวิจัยก่อนหน้าคณะผู้วิจัยได้พบสารประกอบคูมารินชนิดหนึ่ง ให้ชื่อว่า CHA-01 จากส่วนสกัดจากใบของต้นส่องฟ้าดง Clausena harmandiana (Pierre) มีฤทธิ์ยับยั้งวิถีการส่งสัญญาณแคลเซียมในยีสต์ได้ ในงานวิจัยนี้คณะผู้วิจัยได้ค้นหาโมเลกุลเป้าหมายของ CHA-01 ในวิถีการส่งสัญญาณแคลเซียมในยีสต์และในเซลล์ไลน์ชนิดเจอร์แคท จากการใช้วิธีทางพันธุศาสตร์ของยีสต์ ในวิถีการส่งสัญญาณของแคลเซียมพบว่า แคลซินูริน น่าจะเป็นโมเลกุลเป้าหมายของ CHA-01 และเมื่อศึกษาในเซลล์ไลน์มนุษย์ชนิดเจอร์แคท พบว่า CHA-01 สามารถยับยั้งการแสดงออกของยีนประมวลรหัสอินเตอร์ลิวคิน 2 (IL-2) ยับยั้งการผลิน IL-2 ตลอดจนยับยั้งการตัดหมู่ฟอสเฟตออกจากโปรตีน NFAT ได้โดยผลการยับยั้งแปรตามความเข้มข้นของ CHA-01 ซึ่งผลการยับยั้งดังกล่าวของ CHA-01 คล้ายกับผลการยับยั้งโดย FK506 ยากดภูมิ ที่ออกฤทธิ์ยับยั้งการทำงานของแคลซินูริน ผลการทดลองดังกล่าวทำให้ทราบว่า CHA-01 เป็นสารออกฤทธิ์ยับยั้งการทำงานของแคลซินูรินนั่นคือ ยับยั้งแอคติวิตี้ของเอนไซม์ฟอสฟาเทส ด้วยเหตุนี้ CHA-01 จึงมีศักยภาพเป็นสารออกฤทธิ์กดภูมิ

Abstract

Calcium signal transduction pathways play important roles in the regulation of many biological processes in eukaryotes ranging from yeast to human. The small-molecule inhibitors of the pathways in humans are of great medical importance e.g. FK506 and cyclosporine A. A high throughput drug screening assay for the inhibitors was developed based on the ability of inhibitor that could recover the severe growth defect of a zds 1 null mutant Saccharomyces cerevisiae suffering from the hyperactivation of calcium signals. Our previous study found a coumarin compound, CHA-01, from leaf extract of Clausena harmandiana (Pierre) in the screens. In this study, we investigated the likely mechanism of CHA-01 action in both yeast and Jurkat T-cells. The results from using yeast genetics analyses suggested that calcineurin is a molecular target of CHA-01 in the calcium signaling pathway in yeast. In the human Jurkat T-cell line, CHA-01 showed a dose-dependent inhibition of IL-2 production and transcription, as well as an inhibition of NFAT dephosphorylation, similar to FK506. This study revealed that CHA-01 is an inhibitor of the calcineurin pathway, and that this is probably mediated via inhibition of calcineurin phosphatase activity. As such, CHA-01 is a potential immunosuppressant.

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Introduction

People with impaired immune system lead to autoimmune diseases or people with organ transplantation need immunosuppressive drugs. The patient needs long-term immunosuppressive drugs treatment. Hence, lead to drug toxicity to several organs especially kidney. In addition, patient has to pay for high cost of the long-term drug treatment. It is, therefore, an urgent need for novel immunosuppressive drug with higher efficiency and/or lower toxicity.

Most of the currently used drugs have been developed from natural products. Plants had been used for medicinal purposes long before recorded history. Therefore, the plant-derived drugs should be safer than chemically synthesized drugs.

T lymphocytes play a central role in adaptive immune response. For T cell activation, it needs to be activated by calcineurin, a calcium/calmodulin dependent protein phosphatase. Calcineurin activates nuclear factor of activated T cell, cytoplasmic (NFATc), a transcription factor, by dephosphorylating it. The activated NFATc is thentranslocated into the nucleus, where it up-regulates the expression ofpro-inflammatory cytokine genes (*e.g.* Interleukin-2 (IL-2), which, in turn, stimulates the growth and differentiation of T cell response.Calcineurin is the target of a class of drugs called calcineurin inhibitors such as FK506 (Tacrolimus) and Cyclosporin A. The calcineurin inhibitors are the potentially effective immunosuppressive drugs with the mechanism of action on calcineurin inhibition suppresses the transcription of Interleukin-2 by the prevents dephosphorylation of NFATp and its translocation to the nucleus (Wiederrecht *et al.*, 1993). However, the significant side effects include chronic nephrotoxicity, hyperkalemia and hyperuricemia of FK506 and Cyclosporin A became the serious problems in immunosuppressive therapy (Gummert *et al.*, 1999). Therefore, a number of new compounds have been developed for use as immunosuppressive drugs to treat the autoimmune diseases and prevent the host's rejection in solid organ transplantation with the advantage of low toxicity.

Calcineurin is a protein phosphatase with highly conservation from yeast to humans. Its function is calcium dependent and involved in diverse cellular responses such as cell proliferation, T-cell activation and secretion in higher eukaryotes.

Calcium ion (Ca^{2+}) is a universal second messenger important in the regulation of diverse biological processes. When intracellular Ca^{2+} level increased, Calmodulin (CaM) binds Ca^{2+} and consequently undergoes a conformational changes that allows the Ca^{2+}/ CaMcomplex to bind to and activate various target proteins (Cyert, 2001). Calcineurin is one

of its targets. Due to the evolutionary conservation of calcineurin, the results of investigation of the calcineurin-dependent signaling mechanism in yeast are to be expected to have valuable implication for the mechanisms operating in higher eukaryotes.

In *Saccharomyces cerevisiae*, the calcium signals are implicated in the regulation of the G2/M cell cycle progression (Miyakawa & Mizunuma, 2007). The novel yeast-based screening procedure was developed to detect new bioactive compounds that specifically inhibit the calcium signals in *S. cerevisiae.* Hyperactivation of calcium signals of a null mutant in *zds1*, a negative regulator of Swe1 kinase, (Δ*zds1*) showed severe growth defect as well as abnormal budding morphology. An inhibitor of this pathway could lead to recovery of the growth defect as well as abnormal budding morphology (Shitamukai *et al.*, 2000).

Clausena harmandiana (Pierre) is an evergreen shrub with 1 to 1.5 m tall. The plant is native to Asia. In Thailand, it is locally called “Song-fa” The young leaves and leaves are used as fodder for cattle and buffalo. Its young leaves, fruit and young shoots are also used as human food. For traditional uses, its roots, young leaves, bark and flowers are often mixed with other herbs and used to reduce intestinal gas and food poisoning. The roots also help to relieve eye-pain, headaches and fever (Arbab *et al*. 2012).

We previously found that the crude leave extract of C. harmandiana showed a potent positive in the Δ*zds1* yeast-based assay. The same assay was used to guide fractionation and purification until a pure compound designated, CHA-01, was obtained (our unpublished result). In this study, we aimed to learn more on the biological activity of CHA-01 that involved in the calcium signaling pathway in yeast and in mammalian cells.

Materials and Methods

Plant materials

The fresh leaves of *C. harmandiana* (Pierre) were harvested from Khoa Hin Sorn Royal Development Study Center, Chachoengsao province in March, 2010. Fresh leaves were air-dried in open air for three to four weeks and powdered with electrical blender. The voucher specimen number BKF171577 was deposited at the Bangkok Forest Herbarium (BKF), Royal Forest Department, Bangkok, Thailand.

Extraction and fractionation

The dried powdered leaves (1.37 kg) were extracted with n-Hexane. The Hexane extract (39.26 g) was fractional extracted to four fractions: fraction A, B, C and D, respectively. The Δ*zds1*yeast-based assay (Wangkangwan *et al.*, 2009) was used as the preliminarily assay to guided fractionation and purification until a coumarin compound, CHA-01, was obtained (1.50 g).

Yeast strains and cultivation

The *Saccharomyces cerevisiae*, Δ*zds1* strain, YNS17 (Chanklan *et al.*, 2008) was used as an indicator cell for preliminary screening. The mutant cells were cultivated on YPAUD (yeast extract peptone dextrose with adenine and uracil) agar at 30° C for 2 days. All strains of *S. cerevisiae* that used in this study were shown in Table 1.

Table 1 *S. cerevisiae* strains used in this study

Strain	Genotype	Source of reference
W303-A1	*MATa trp1 leu2 ade2 ura3 his3 can1-1*	Miyakawa, T., Hiroshima University
Δ*zds1* (YNS17)	Same as W303-A1 except *zds1::TRP1 syr1::HIS3 pdr1::hisG-URA3-hisG pdr3::hisG-URA3-hisG*	Chanklan, *et al.* 2008
YRC1	*MATa GAL-CMP2ΔC::URA3 zds1::TRP1 erg3::HIS3 pdr1::hisG pdr3::hisG*	Chanklan, *et al.* 2008
Δ*cnb1*	Same as W303-A1except *cnb1*	Miyakawa, T., Hiroshima University
Δ*mpk1*	Same as W303-A1 except *mpk1*	Miyakawa, T., Hiroshima University

Yeast-based assay procedures for calcium signaling inhibitors

The yeast-based assay was modified from that described in Shitamukai *et al.*, 2000 (Wangkangwan *et al.* 2009). In brief, the indicator cells (Δ*zds1*) were cultured on YPAUD plate at 30°C for 2 days. Then inoculated in 5 ml YPAUD broth and incubated with shaking at 200 rpm at 30°C until cell density reached early log phase (approximately 0.5-1 x 10^7 cell/ml). For growth effect of CHA-01 were examined using liquid culture assay. The mutant strain (Δ*zds1*) approximately 5 x 10^6 cell/ml was grown in YPAUD broth containing with various concentrations of CHA-01 (0, 125 and 250 μM, respectively) in final concentration of 0.5% DMSO. The cell suspensions were treated with either 0.5% DMSO as a negative control or with 250 nM FK506 as a positive control, respectively. Then the treatments were incubated at 30^{o}C, for 30 min. After that, $CaCl_2$ was added to the final concentration of 100 mM except the treatment of control without $CaCl_2$. The assays were incubated at 30 ^{o}C, the growth of yeast cells were monitored by counting the cells with haemacytometer.

Measuring β-galactosidase activity

The assay for detecting of free Ca^{2+} in yeast cells under the hyperactivation was modified from that in Tutulan-cunita *et al.*, 2005 for determination of the levels of β-galactosidase activity from the lacZ reporter. The wild-type yeast strain (W303-A1) was transformed with a plasmid of pKC190, carrying *PMR2A*-lacZ reporter under the activation of four tandem copies of the calcineurin-dependent responsive element (CDRE). Cells were incubated in YPAUD medium containing 75 mM $CaCl_2$ with various concentrations of CHA-01 (0.5, 1 and 2 mM, respectively), 500 nM FK506, 0.5% DMSO solvent and 1 mM $MgCl_2$were used as the controls. The tested cells were incubated at 30 ^oC for 4 hours. β-galactosidase activity was measured using ONPG assay (Miller, 1972).

Flow cytometric analysis and Hoechst staining

Flow cytometric analysis was performed by FACSCalibur (Becton Dickinson, Franklin Lakes, NJ), as described previously (Mizunuma *et al.* 1998). The yeast cell suspensions were fixed and permeabilised as reported in Mizunuma *et al.* 1998 prior to staining with 5 μM Hoechst 33342 (Sigma, St. Louis, MO).

Jurkat Human T Cell Line and its Cultivation

The Jurkat T cell line (Human acute T cell leukemia), ATCC number CRL-2063, was obtained from the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Thailand. The cell line was cultivated at 37 °C in a 5% (v/v) CO_2 atmosphere in CM (RPMI-1640 medium with L-Glutamine (HyClone, USA) supplemented with 10% (v/v) fetal bovine serum (HyClone), 100 U/mL penicillin (General Drugs House Co. Ltd., Bangkok, Thailand), 0.4 mg/mL streptomycin (M & H Manufacturing Co. Ltd, Samut Prakan, Thailand) and 1% (w/v) sodium pyruvate (HyClone)) .

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The expression of IL-2 mRNA was assessed by two-stage semi-quantitative reverse transcription-polymerase chain reaction (sqRT-PCR). The Jurkat T-cell line was inoculated

into CM at a density of 0.5 x 10^6 cells/mL. The cells were pre-treated with various concentrations of CHA-01for 30 min, and then stimulated with 25 ng/mL of phorbol 12-myristate 13-acetate (PMA) (Merck, Darmstadt, Germany) and 1 μg/mL of ionomycin (Io) (Merck, Darmstadt, Germany) (PMA/Io) at 37 °C in 5% (v/v) CO_2 for 24 h. Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. In brief the cell suspension (1 mL) was harvested by centrifugation at 8000 x g for 5 min, and the cell pellet was suspended in 1 mL Trizol reagent. The DNA and protein were separated by chloroform-phase separation and the RNA in the aqueous phase was precipitated with isopropanol and then suspended in diethylpyrocarbonate (DEPC)-treated water. In the first stage, cDNA was synthesized from the obtained total RNA using random hexamer primers (Fermentas, Lithuania) and reverse transcriptase (Thermoscientific, Lithuania). In the second stage gene-specific sqPCR was performed with Taq polymerase using the gene-specific primers for a 380 bp fragment of β-actin (sense, 5'-ACCAACTGGGACGACATGGAGAA-3' and antisense 5'-GTGGTGGTGAAGCTGTAGCC-3' (Huang et al, 1995), and for a 458 bp fragment of IL-2 (5'-ATGTACAGGATGCAACTCCTGTCTT-3' and antisense 5'-GTTAGTGTTGAGATG ATGCTTTGAC-3'(Nakamura et al, 1996). The PCR reaction comprised an initial denaturation step at 94˚C for 5 min, followed by 30 cycles of 94˚C for 1 min, 55˚C for 1 min, 72˚C for 1 min. Extension were continued at 72˚C for 10 min. The PCR products were separated by 2% (w/v) agarose-TAE gel electrophoresis, and the gels were stained with 10 μg/ml Ethidium Bromide for 5-10 min and viewed by UV transillumination.

Assay for IL-2

Jurkat cells were treated as described in the RT-PCR section and then the IL-2 levels in the obtained culture supernatants were measured using the human IL-2 ELISA Ready-SET-Go! kit (eBioscience, San Diego, CA) according to the manufacturer's instructions.

Western Blotting Analysis

The phosphorylation/dephosphorylation levels of NFAT in the PMA/Io co-stimulated cells were determined as follows. Jurkat cells were pre-treated with various concentrations of CHA-01(0 to 100 μM) or with 100 nM FK506 for 30 min at 37 °C in CM and 5% (v/v) CO_2 and then activated by culturing with 25 ng/mL PMA and 1 μg/mL Io in CM at 37 °C and 5% (v/v) CO_2 for 24 h. The treated cells were then harvested, lysed and the total protein content extracted as previously described (Palaga et al, 2008). The protein concentration was measured using the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The proteins were separated by 10% SDS-PAGE at a constant voltage of 50 V for 3 h and then transferred onto PVDF membrane (BIO-RAD, USA) by Trans-Blot SD Semi-dry transfer cell (BIO-RAD, USA). For Western blotting, the PVDF membrane was blocked in 5%skim milk for 30 min and then incubated for overnight in the respective primary antibody, rabbit anti-NFAT1 antibody (Cell Signaling, USA) or mouse anti-β-actin (Cell Signaling, USA), at a 1:10000 dilution in 5%skim milk. After washing, blots were incubated for 1 h in the appropriate secondary antibody, donkey anti-rabbit IgG or sheep anti-mouse IgG, conjugated to horseradish peroxidase (Amersham Biosciences, UK), washed as above and then developed by chemiluminescence (Amersham Biosciences, UK).

Results

CHA-01 exhibited dose-dependent effect on growth defect restoration in Ca^{2+}-hyperactivated Δzds1 cells

Since we isolated CHA-01 from the crude extract of *C. harmandiana* (Piere) that exhibited potent effect on Ca^{2+}-signal mediated cell cycle arrest/delay, to see whether CHA-01 contained such activity found in the crude extract, the growth of Δ*zds1* mutant yeast cells were cultivated in either presence or absence of CHA-01 followed by activation with $CaCl_2$. The results showed that in the absence of $CaCl_2$ activation, the Δ*zds1* cells treated with 250 μM CHA-01 could grow at the same rate as those without CHA-01. This indicated that CHA-01 at 250 μM contained no cytotoxic effect to the yeast cells. For the $CaCl_2$ activation condition, the Δ*zds1* cells treated with 0.5% DMSO (as negative control) showed growth defect. However, when the cells were treated with either 250 μM CHA-01 or 250 nM FK506 (as a positive control) prior to cultivation in high calcium containing medium, the growth of the cells was restored. Likewise, when the lower dosage of CHA-01(at 125 μM) was used to treat Δ*zds1* cells prior to $CaCl_2$ activation, the growth defect could be restored though in lesser extent (Fig.1). The results suggested that CHA-01 inhibits Ca^{2+}-signal mediated cell cycle arrest/delay in yeast *S. cerevisiae*.

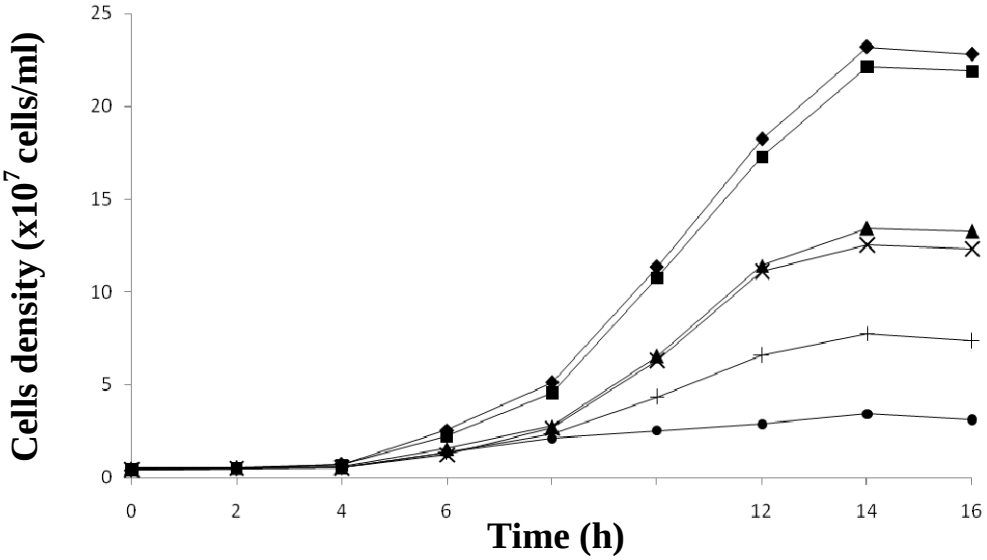

Figure 1.Dose dependent effect of CHA-01 on growth of Δzds1 in high-calcium containing medium. Symbols are as follow:
(close circle) YPAUD medium+0.5%DMSO+ 100mM $CaCl_2$; (plus) YPAUD+125μM CHA-01 +100mM CaCl;(close triangle) YPAUD+250nM FK506+100mM $CaCl_2$; (Cross) YPAUD+250μM CHA-01 +100mM $CaCl_2$; (close square)YPAUD+250μM CHA-01 and (close diamond) YPAUD medium

Effect on free intracellular Ca^{2+}-levels in yeast cells (β-galactosidase assay)

To learn more on the action mechanism of CHA-01, a molecular target of CHA-01 in the Ca^{2+}-signaling pathway in yeast was determined. The wild-type yeast strain (W303-A1) was transformed with a plasmid of pKC190, carrying *PMR2A-lacZ* reporter under the activation of four tandem copies of the calcineurin-dependent responsive element (CDRE). The CDRE-driven *LacZ* reporter gene was activated in response to the external $CaCl_2$ (75 mM) (Cunningham & Fink, 1996). In this experiment the yeast strain was cultured in YPAUD containing 75 mM $CaCl_2$, then incubated with and without CHA-01 for 4 hr and the β-galactosidase activity was determined (Miller, 1972). In the absence of $CaCl_2$, β-galactosidase activity was barely detectable while in the presence of $CaCl_2$, the activity was high (Fig. 2, treatment 2 and 3). In high $CaCl_2$ condition, the treatment with FK506, a known calcineurin inhibitor, showed relatively low level of β-galactosidase activity (Fig. 2, treatment 1). Varying amount of CHA-01 ranging from 0.5-2 mM cause decreased in β-galactosidase activity in dose-dependent manner (Fig. 2, treatment 5-7). The results

demonstrated indirectly that CHA-01 might act by decrease the free intracellular Ca^{2+} or might bind to calcineurin.

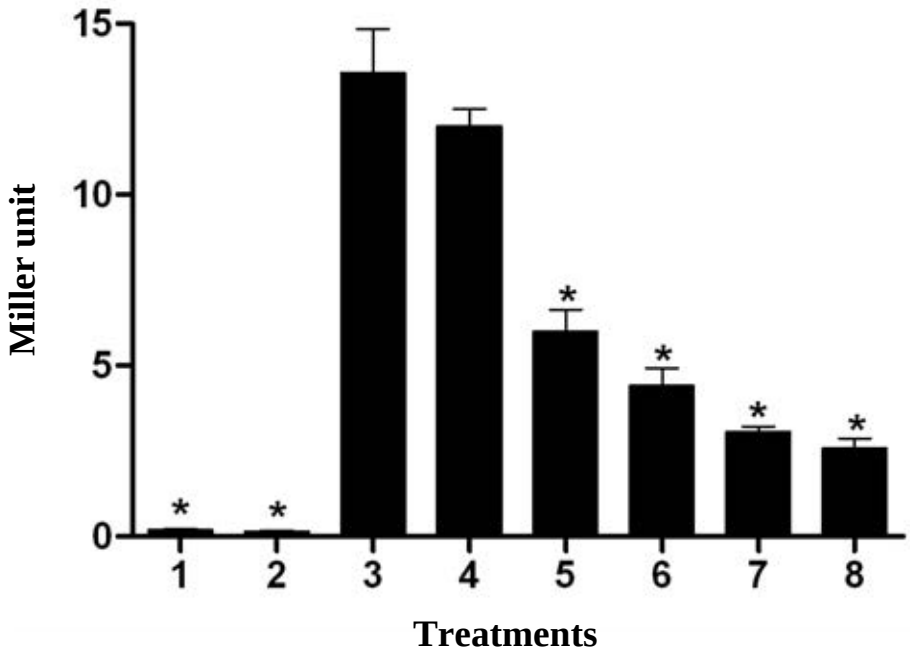

Figure 2. Dose dependent effect of CHA-01on β-galactosidase levels in yeast. Treatments are as follow: 1) YPAUD medium + 500nM FK506 + 75mM $CaCl_2$, 2) YPAUD medium, 3) YPAUD medium + 75mM $CaCl_2$, 4) YPAUD medium + 0.5% DMSO + 75mM, 5) YPAUD medium + 0.5mM CHA-01+ 75mM $CaCl_2$, 6) YPAUD medium + 1mM CHA-01+ 75mM $CaCl_2$, 7) YPAUD medium + 2mM CHA-01+ 75mM $CaCl_2$ and 8) YPAUD medium+ Mg^{2+} 75mM $CaCl_2$

Effect of CHA-01 on calcineurin or Mpk1 kinase

The calcineurin mediated and Mpk1 mediated Ca^{2+}-signaling pathway in yeast act in parallel to regulate functionally redundant cellular events important for cell growth and morphogenesis (Nakamura et al. 1996). To determine whether calcineurin or Mpk1 kinase is a molecular target of CHA-01 in the Ca^{2+}-signaling pathway in yeast, a synthetic lethality test was performed. Single gene disruption in yeast either gene coding for regulatory subunit of calcineurin (Δ*cnb1*) or Mpk1 kinase (Δ*mpk1*) does not affect cell viability. However, the double disruptant (Δ*cnb1*Δ*mpk1*) showed lethal phenotype. If CHA-01 can inhibit one of the two genes, the treatment of CHA-01 in the single disruptant should result in lethal phenotype. Either Δ*cnb1* or Δ*mpk1* was cultivated in YPAUD medium in the absence of presence of 250 μM CHA-01 and incubated at 30 °C with shaking. The cell growth was monitored every 2 hrs

for 16 hrs. In Δ*cnb1* culture treated with CHA-01, the cells could growth normally at the same growth rate as that with no CHA-01 treatment (Fig. 3A). However, in the Δ*mpk1*culture treated with 250 μM CHA-01 or 250 nM FK506 showed growth defect compared to that without any treatment (Fig. 3B). This result suggested that calcineurin might be a molecular target of CHA-01.

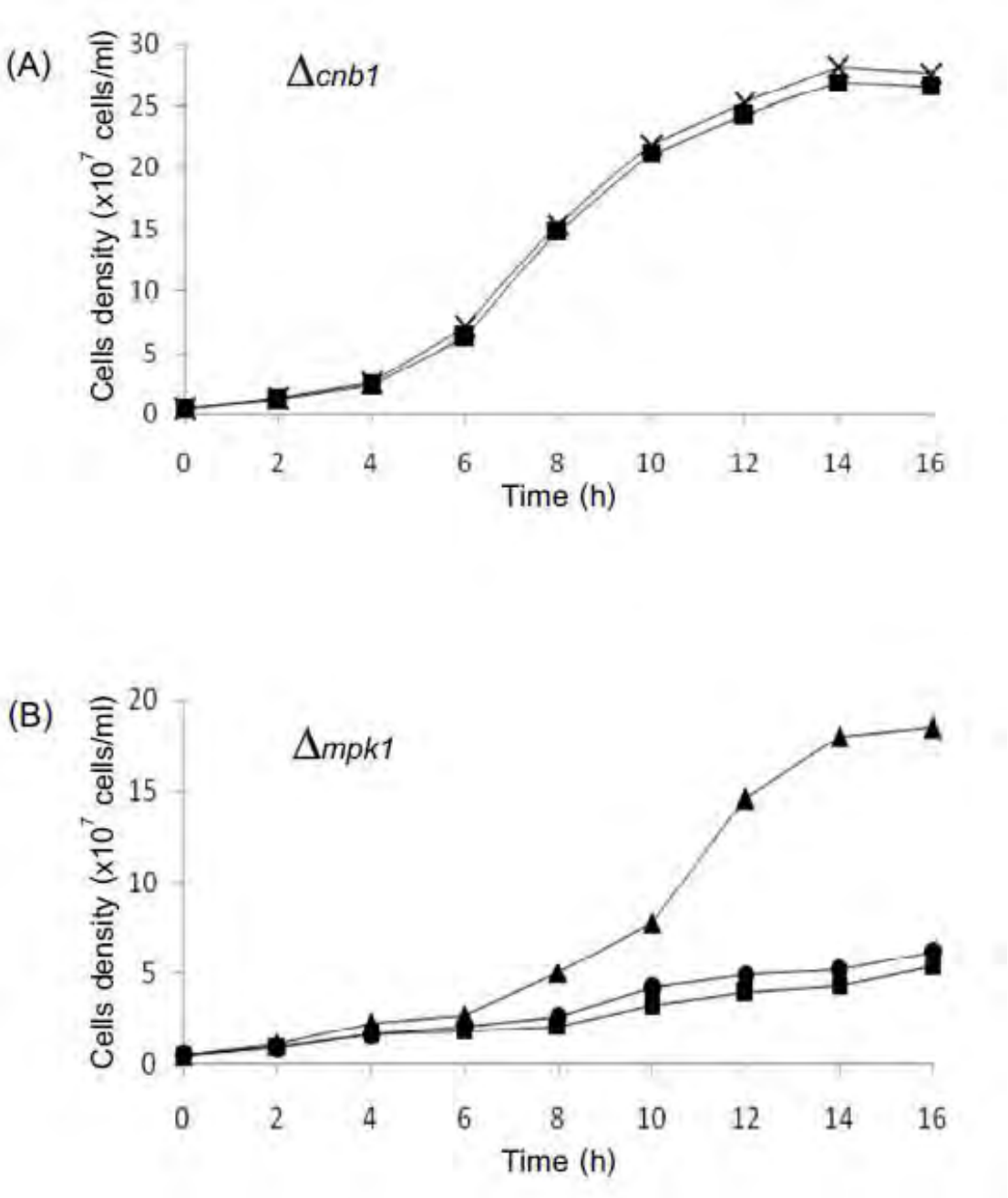

Figure 3. Effect of CHA-01 on the growth of mutant yeast cells. The Δcnb1strain (A) or Δmpk1 strain (B) was cultivated in YPAUD medium either with (close square) or without close triangle) addition of 250 μM CHA-01 or addition of 250 nM FK506 (close circle). The

cultures were incubated at 30 °C with shaking and the cell suspension was sampled every 2 hrs for cell density determination.

Effect of CHA-01 on the yeast strain overexpressed calcineurin (YRC1 strain)

To confirm whether calcineurin is a molecular target of CHA-01, the YRC1 strain (Δ*zds1 GAL1p-CMP*Δ2C) which overexpressed calcineurin under galactose induction was treated with either absence or presence of 250 μM CHA-01 for 30 minutes prior to add galactose to final concentration of 2% to induce the catalytic subunit of calcineurin gene expression. We found that the strain with overexpressed calcineurin showed growth defect while the cells treated with either 250mM CHA-01 or 250 nM FK506 prior to addition of 2% galactose to induce expression of calcineurin showed growth defect recovery (Fig. 4). The results suggested that CHA-01 directly inhibits the activity of calcineurin exhibiting growth defect.

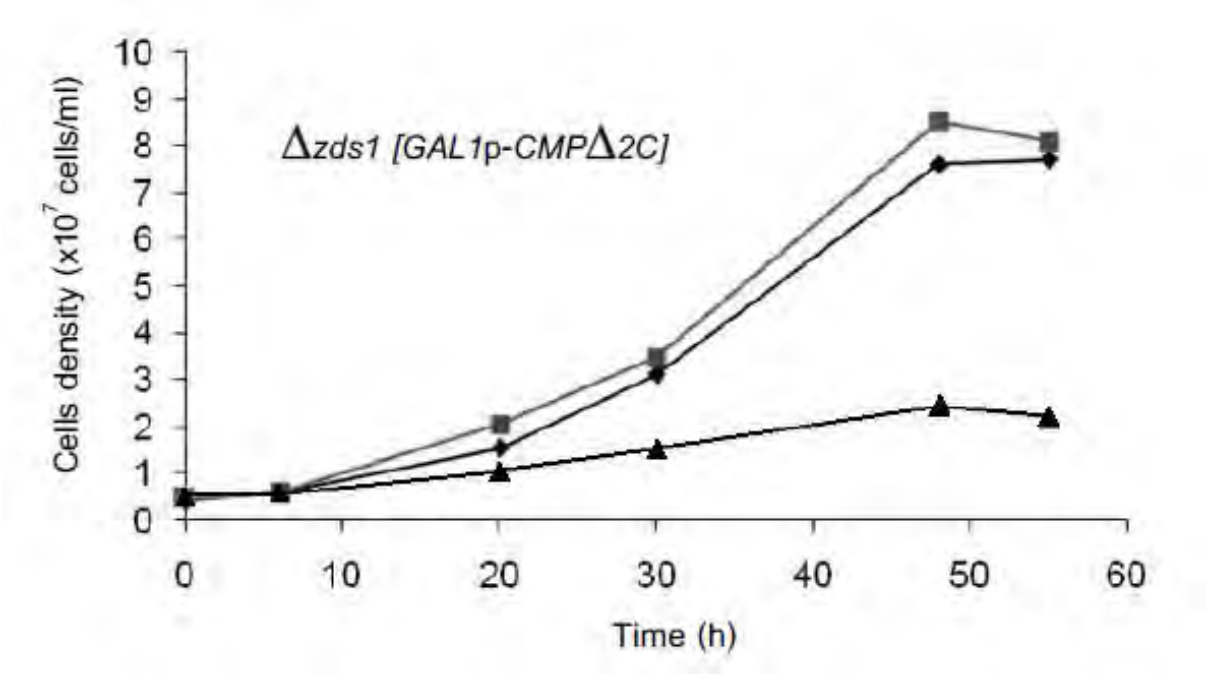

Figure 4. Effect of CHA-01 on the growth of Δzds1 cells overexpressed calcineurin. The YRC1 (Δzds1GAL1p-CMPΔ2C) strain was grown in SC medium containing 2% galactose 1% raffinose that was prior added with either 0.5% DMSO (close triangle), or 250 nM FK506 (close square) or 250 μM CHA-01 (close diamond) 30 minutes before induction of GAL1 promoter. The cultures were incubated with shaking at 30 °C. The cell density was monitored using haemacytometer.

The Ca^{2+} hyperactivation not only affect the growth of *Δzds1* cells but also affect its cell morphology (Mizunuma et al. 1998). Overexpression of each one of signaling molecules in the Ca^{2+}-signaling pathway will lead to the deleterious effects like the effects of Ca^{2+} hyperactivation. To further confirm the inhibitory activity of calcineurin by CHA-01, we also observed the other phenotypes resulting from hyperactivation in Ca^{2+}-signaling pathway in yeast, the abnormal budding morphology and unequal nuclear division between mother and daughter cells. Cells of YRC1strain (Δ*zds1 GAL1p-CMP2*ΔC) were treated with either 250 μM CHA-01 (Fig. 5B) or 0.5%DMSO (Fig. 5A) prior to addition of $CaCl_2$ and the cell suspension was sampling for morphology observation at 12 h. The results showed that DMSO treated cells displayed an abnormal morphology, with elongated buds (Fig. 5A, left), and Hoechst 33342 nuclear stained cells revealed unequal nuclear division between mother and daughter cells (Fig. 5A, middle). In contrast, the cells treated with 250 μM CHA-01 prior to overexpress calcineurin showed normal morphology (Fig. 5B, left) with an equal distribution of nuclei in the mother and daughter cells (Fig. 5B, middle). The results clearly demonstrated that CHA-01 treatment could alleviate the effects of abnormal cell morphology and abnormal nuclear division as a result of overexpression of calcineurin.

The growth defect caused by hyperaction of Ca^{2+}-signaling pathway was a result from G2 cell cycle arrest/delay (Mizunuma et al. 1998). The results from Fig.1 revealed that CHA-01 could alleviate the growth defect caused by Ca^{2+}-hyperactivation. To confirm the inhibitory activity of CHA-01 for the growth defect caused by the Δ*zds1*yeast strain overexpressed calcineurin, cell cycle analysis was performed on the propidium iodide-stained *Δzds1 GAL1p-CMP2*ΔC cells treated with or without CHA-01 prior to overexpression of calcineurin. For YRC1 strain without CHA-01 treatment, the cells showed a flow cytometric profile with a 3.3-fold higher in 2C DNA content than those with a 1C DNA content, indicating that a G2 phase delay was induced by overexpression of calcineurin (Fig. 5A, right panel). In contrast, in the 200 μM CHA-01 treated YRC1 strain prior to overexpression of calcineurin, the proportion of cells with a 2C DNA content decreased to about 2.4-fold than those with a 1C DNA content (Fig. 5B, right panel). The results demonstrated that CHA-01 treatment could alleviated the G2/M cell-cycle delay caused by calcineurin overexpression.

Taken together, the results from Fig. 5 suggested that CHA-01 inhibits activity of overexpressed calcineurin *in vivo* (in the mutant yeast cells).

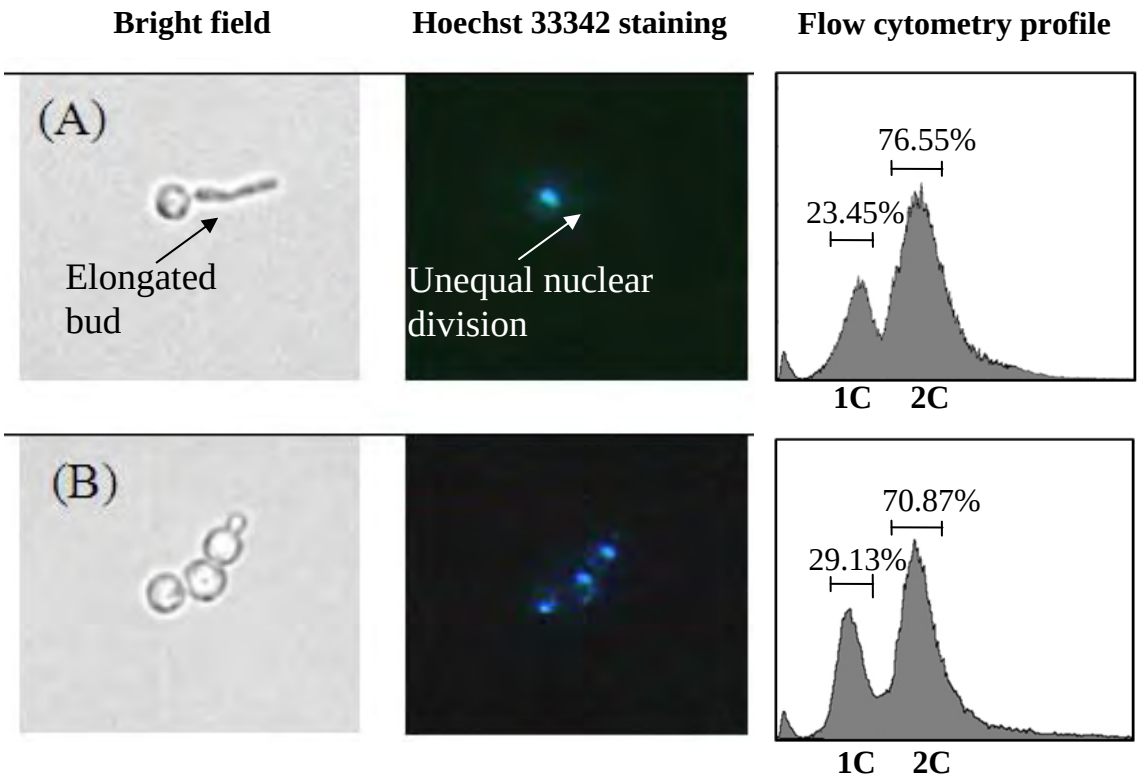

Figure 5. Effect of CHA-01 on cell morphology and nuclear division of Δzds1 mutant cells. DMSO solvent (0.5%) treated cells (A) or 250 μM CHA-01 treated cells (B) grown in high $CaCl_2$ containing medium. Cell morphology was examined at 12 h later. Left: the cells under light microscope, Right: Hoechst 33342 stained cells under fluorescence microscope. Flow cytometric profile was analysed at 12 h after cultivation.

Calcineurin is a Ca^{2+}/Calmodulin-dependent serine/threonine protein phosphatase. It is important in coupling of Ca^{2+}-signals to various cellular response. It is a heterodimer enzyme composed of a catalytic A subunit and a regulatory B subunit. The phosphatase activity is dependent on both binding of Ca^{2+} to the B subunit (regulatory subunit) and the Ca^{2+}-dependent binding of calmodulin to the A subunit (catalytic subunit). In yeast *S. cerevisiae*, calcineurin catalytic subunit (A subunit) is encoded by two homologous, functionally redundant genes, *CMP1/CNA1* and *CMP2/CNA2* (Cyert *et al*., 1991; Liu *et al*., 1991). The B subunit is encoded by a single gene, *CNB1*. The primary sequence of both subunits and heterodimeric quaternary structure is highly conserved from yeast to mammals. In mammalian cells, calcineurin regulates various cellular processes, including T-cell activation, cardiac development and hypertrophy, memory and angiogenesis (Miyakawa and Mizunuma, 2007).

Dose-dependent in vitro inhibition of calcineurin activity by CHA-01

The calcineurin inhibitors, cyclosporine and tacrolimus, are used as primary immunosuppressive drugs in transplant patients. The results from our studies suggested that CHA-01 is a calcineurin inhibitor.

To see whether CHA-01 could also inhibit the activity of calcineurin *in vitro*, the calcineurin assay kit was used. Various concentrations of CHA-01 (62.5, 125, 250 and 500 μM, respectively) were added to the reaction buffer with calmodulin and calcineurin. Phosphatase activity was measured by the amount of free phosphate (P_i) release from substrate and detected free Pi released in the unit of nanomoles of P_i released per minute per milligram of protein from the substrate. It was found that in treatment 1 and 2 where H_2O and DMSO were tested, high level of free P_i release were detected. While treatment 3 where 150 nM FK506, a known calcineurin inhibitor, was tested, very level of free P_i could be detected. Treatment 4-7 where 500, 250, 125 and 62.5 μM were tested, levels of free P_i released were decreased in dosage dependent manner (Fig. 6).

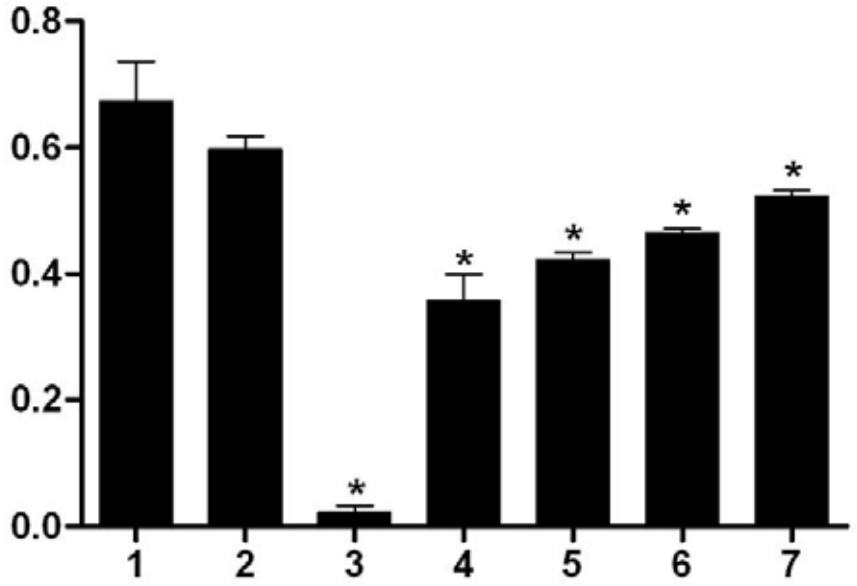

*Figure 6. Dose dependent effect of CHA-01 on calcineurin activity in vitro. Treatments are as follow: 1) H_2O, 2) 0.5% DMSO, 3) 150nM FK506, 4) 500μM CHA-01 I, 5) 250μM CHA-01, 6) 125μM CHA-01 and 7) 62.5μM CHA-01 were tested with calcineurin/calmodulin and detected the free Pi release. The experiments were repeated in triplicates. *Statistically significant from that of control.*

CHA-01 showed no acute cytotoxicity to Jurkat T cells

Because of the highly conservation of calcineurin found among eukaryotes, we further explore the biological activity of CHA-01 in human cell line. The calcineurin inhibition activity found in yeast *Saccharomyces cerevisiae* led us to investigate the role of CHA-01 in human T cell leukemia, Jurkat cells. We first asked whether CHA-01 toxic to the Jurkat T cells or not. The cells were treated either in the absence (0.5% DMSO) or presence of CHA-01 at 0.1, 1, 10 and 100 μM and incubated at 37 °C under 5% CO_2 atmosphere for 24 h. The cells viability was measured by MTT proliferation assay (Fig.7).

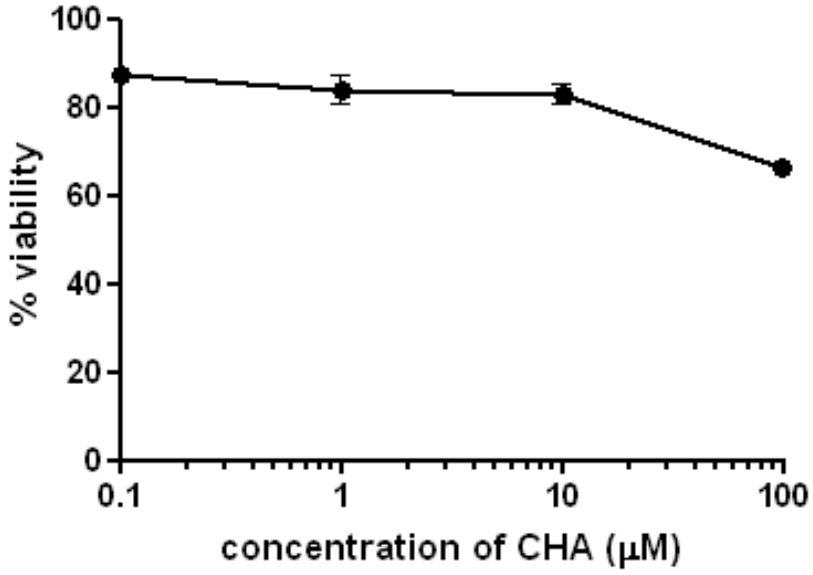

Figure 7. Cytotoxicity of CHA-01 on Jurkat T cells. Varying concentrations of CHA-01 were added to Jurkat T cells in 96 well plates. The plates were incubated at 37°C 5% CO_2 for 24 h. Cell viability was determined by staining with MTT.

The results showed no acute cytotoxicity of CHA-01 was found at concentration ranging from 0, 0.1, 1 and 10 μM. For dosage at 100 μM, CHA-01 displayed slightly toxic to the Jurkat T cells (Fig. 7).

Effect of CHA-01 on Interleukin-2 (IL-2) production in human T cell leukemia, Jurkat cells

The classical calcineurin inhibitors, cyclosporine and tacrolimus (FK506), inhibits the phosphatase activity of calcineurin and prevents the nuclear translocation of a nuclear factor activated T cells (NFAT), a crutial transcriptional activator of T cells, thus leads to suppression of immune system (Liu et al. 1991). To see the effect of CHA-01 treatment on IL-2 production from Jurkat T cell leukemia, Western blot analysis was performed. Jurkat T cells were stimulated with PMA/Io followed by treating with either one of the following: 0.5% DMSO (as a negative control), 100 nM FK606 (as a positive control) or CHA-01 (dosage ranging from 0.25 – 25 μM). The supernatants were collected and subjected to IL-2 determination by indirect ELISA (XX). It was found that CHA-01 treated cells produced IL-2 in significantly lower level than those of the untreated and 0.5% DMSO treated cells. Furthermore, the inhibitory effect of CHA-01 on IL-2 production in Jurkat T-cells was dose dependent manner (Fig. 8).

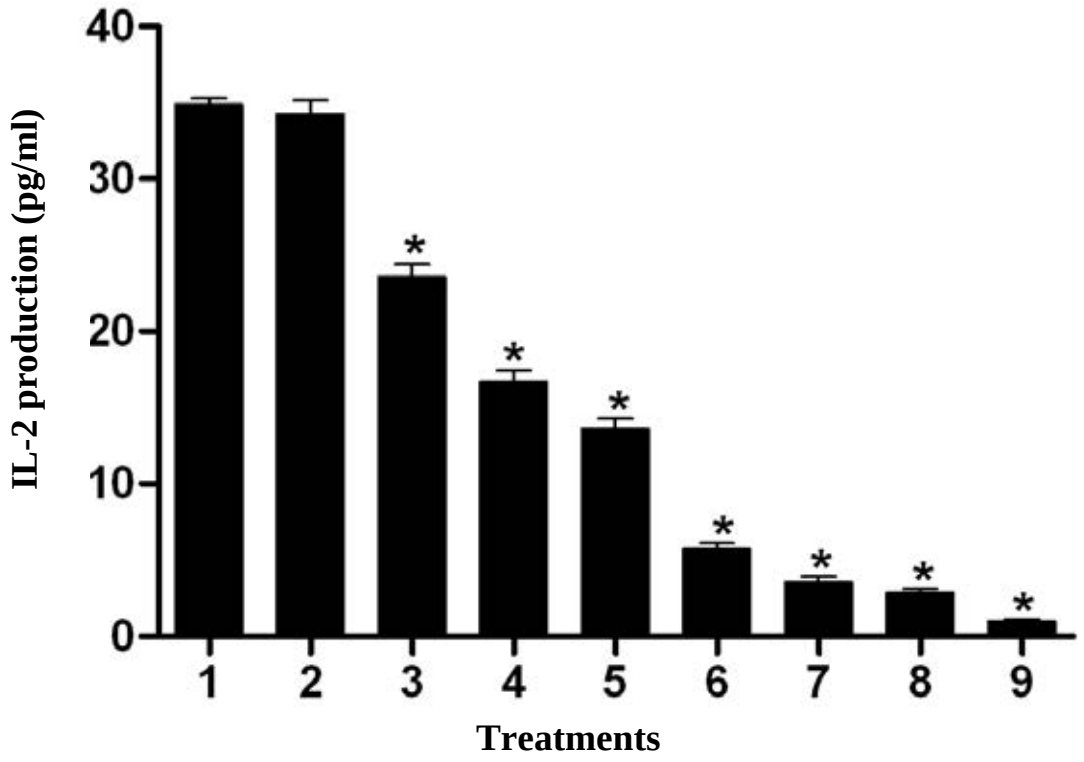

*Figure 8. Dose dependent effect of CHA-01 on IL-2 production in Jurkat T cells. The Jurkat cells were stimulated with PMA/Io and treatments are as follows: 1) untreated cells, 2) 0.5% DMSO treated cells, 3) 0.25μM CHA-01-treated cells, 4) 1μM CHA-01-treated cells, 5) 2.5μM CHA-01-treated cells, 6) 5μM CHA-01-treated cells, 7) 25μM CHA-01-treated cells, 8) 100 nM FK506-treated cells and 9) unstimulated cells. The experiments were performed in triplicates. *Statistically significant*

Effect of CHA-01 on IL-2 gene expression in Jurkat T cell leukemia cell line

To determine the level of IL-2 gene expression in the CHA-01 treated Jurkat cells, RT-PCR was performed. The Jurkat T cells were stimulated with PMA/Io prior to being treated with CHA-01 (dosage ranging from 5-50 μM) or 100 nM FK506. RNA from the treated cells were extracted and reversed to cDNA prior to be used as a template for PCR reaction using primers specific to IL-2 gene and β-actin gene (as a loading control), respectively. A 458 bp PCR product could be clearly detected in the untreated Jurkat cells (Fig. 9, lane 1) while no such band could be detected in 100 nM FK 506 treated Jurkat cells (Fig. 9, lane 5) nor in unstimulated Jurkat cells (Fig. 9, lane 6). CHA-01 treated Jurkat cells showed rather faint band (Fig. 9, lane 2, 3) and barely detectable band of IL-2 gene (Fig. 9, lane 4). The results clearly demonstrated that CHA-01 inhibits IL-2 gene expression in dose-

dependent manner. The IL-2 gene expression of 50 μM CHA-01 treated Jurkat cells almost as low as that in 100 nM FK506 treated cells or the unstimultated cells (Fig. 9A). The level of IL-2 gene expression in CHA-01 treated cells or FK506 treated cells were significantly lower than those of untreated cells (Fig. 9B).

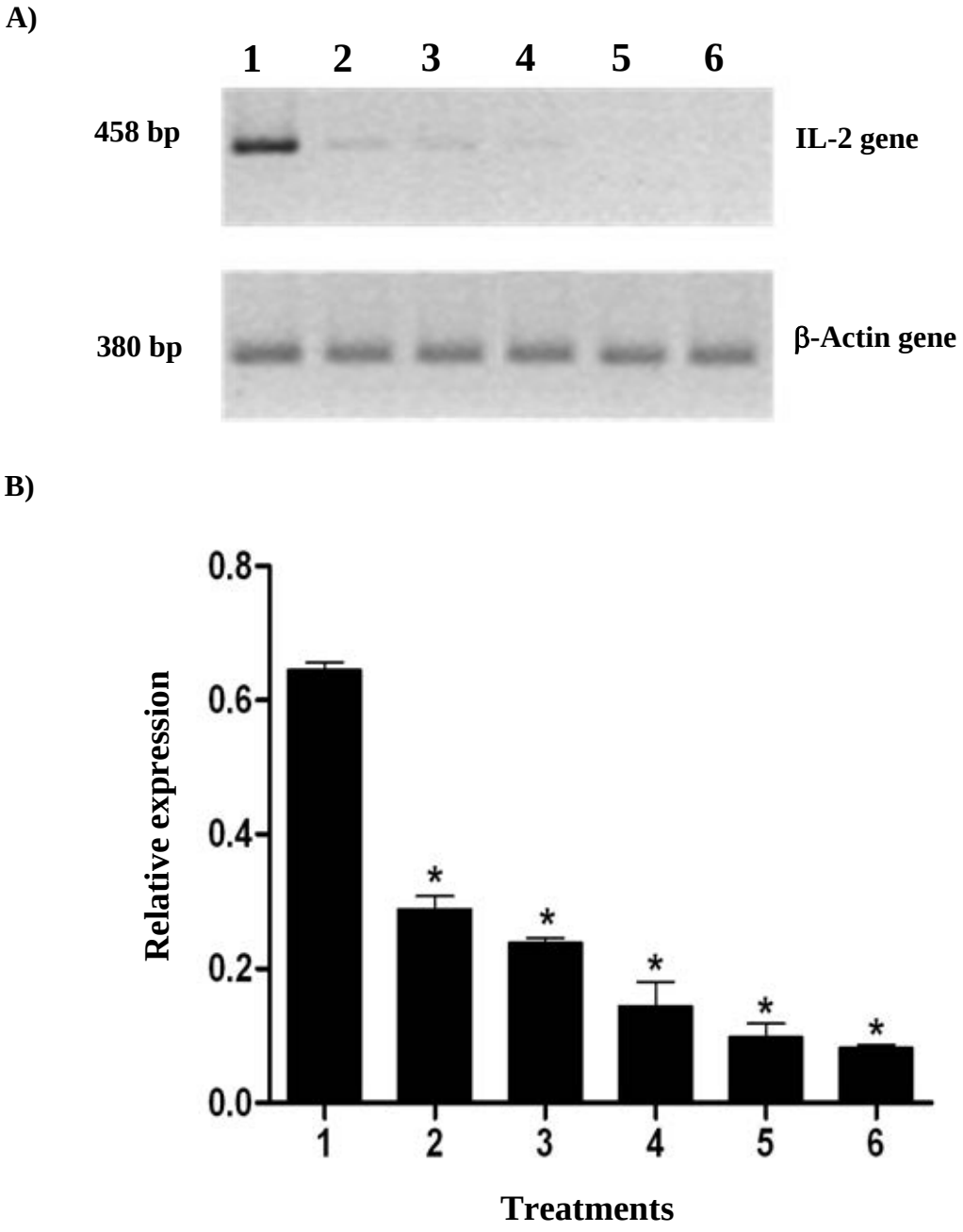

*Figure 9. Dose dependent effect of CHA-01 on IL-2 mRNA expression. A) Jurkat cells were stimulated with PMA/Io and treatments are as follow: 1) untreated cells, 2) 5μM CHA-01-treated cells, 3) 25μM CHA-01-treated cells, 4) 50μM CHA-01-treated cells, 5) 100nM FK506-treated cells and 6) unstimulated cells. The RNA of the treated cells were extracted and converted to cDNA before PCR reaction using IL-2 and β-actin specific primers, respectively. B) Relative expression of IL-2 from each treatment was compared to that of the untreated one. The experiments were perfomed in triplicates. *Significantly different from that of untreated one.*

Effect of CHA-01 on NFAT phosphorylation in Jurkat T cells

Due to the results that we found CHA-01 inhibits IL-2 gene expression, hence reduction in IL-2 production. We asked further whether it could be as a result of inhibition of calcineurin activity on NFAT dephosphorylation. To evaluate this, the Jurkat T cells were stimulated with Io prior to treatment with either 0.5% DMSO or CHA-01 (dosage ranging from 12.5 -100 μM) or 100 nM FK506. Cell extracts were prepared and subjected to Western blot analysis using anti-NFAT1 and anti-β-actin antibodies. It was found that in the unstimulated Jurkat cells only NFATp, but not NFAT, could be detected (Fig. 10, lane 1). This was due to no activation on calcineurin. In the absence of CHA-01 (Fig. 10, lane 2), the Io stimulated cells showed both bands of NFATp and NFAT (Fig. 10, lane 3). While the 100 nM FK506 treated cells, only NFATp could be detected (lane 8). In CHA-01 treated cells, the higher the dose of CHA-01, the lower the band of NFAT (lane 3-7). At dosage of 100 μM CHA-01 (lane 7), dephosphorylation of NFATp was totally inhibited as same as that found in 100 nM FK506 treated cells.

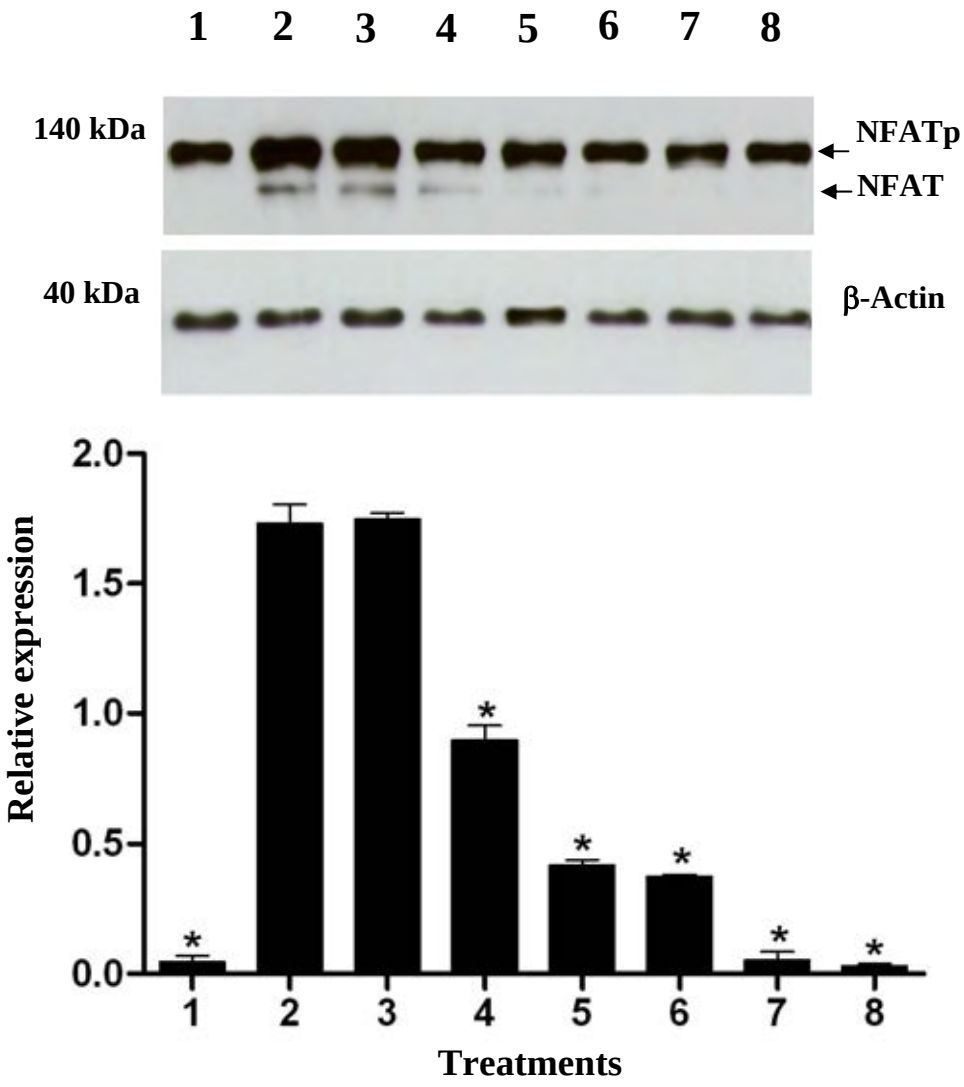

Figure 10. Effect of CHA-01 on NFAT phosphorylation in Jurkat cells. Jurkat cells were stimulated with Io prior to treatments as follows: 1) unstimulated cells, 2) untreated cells, 3) 12.5μM CHA-01-treated cells, 4) 25μM CHA-01-treated cells, 5) 50μM CHA-01-treated cells, 6) 75μM CHA-01-treated cells 7) 100 μM CHA-01-treated cells and 8) 100nM FK506-treated cells (A). *The relative expression of NFATp from three replicate experiments (B). *Significant difference at $p \leq 0.001$.*

Discussion and Conclusion

Using the Δ*zds1* yeast-based screening assay, it was previously found that CHA-01, a coumarin isolated from *C. harmandiana* had an apparent Ca^{2+}-signaling inhibitory activity. This compound has previously been reported as a component of this plant and to induce a spasmolytic activity on isolated guinea-pig ileum preparations, but its function at the molecular level was unknown (Patnaik and Dhawan, 1982).

In this study, the calcineurin-mediated pathway in yeast was identified as the potential target of CHA-01. Given that calcineurin is highly conserved from yeast to human, the effect of CHA-01 on human cells was evaluated using the human Jurkat leukemic T-cell line, in which the function of calcineurin can be measured by the activation of the IL-2 gene expression in the activated cells, including after PMA/Io stimulation. CHA-01 was found to inhibit in a dose-dependent manner the IL-2 production and mRNA level in PMA/Io-stimulated Jurkat cells. Moreover, the dephosphorylation of phosphorylated NFAT, a critical step in the activation of IL-2 gene expression, was inhibited by CHA-01. Overall, the data obtained in the yeast and human Jurkat cell systems indicated that CHA-01 is an inhibitor of the calcineurin-mediated pathway. Indeed, the behaviors of CHA-01 and FK506 in the yeast and Jurkat cell systems were broadly similar in all tested assays.

That the level of direct *in vitro* inhibition of calcineurin phosphatase activity by CHA-01 was fairly low (~40%) (Fig. 6) could be due to the lack of an as yet unidentified immunophilin-like protein in the assay system that may be required for the inhibition of calcineurin by CHA-01. An approach to identify the protein(s) that bind to CHA-01 is currently under way.

Although the toxicity of CHA-01 has not been formally addressed, it is found in a fairly significant proportion, at 0.11% (w/w), of the crude extract of *C. harmandiana* leaves (Yingyongnarongkul, unpublished data) yet the young leaves, fruit and young shoots are used as human food, and along with its roots, bark and flowers are also often mixed with other herbs and are used in traditional medicine to reduce intestinal gas and food poisoning. The roots also help to relieve eye-pain, headaches and fever (Arbab et al, 2012). Furthermore, the young leaves and leaves are also used as fodder for cattle and buffalo (Arbab et al, 2012). Taken together with the finding that CHA-01 showed no acute cytotoxicity to Jurkat T cells

up to dosage of 100 μM, this emphasized the potential for further development of CHA-01 as an immunosuppressive drug, albeit subject to proper toxicity evaluation.

Our studies clearly demonstrated that the yeast-based system can offer powerful means for the discovery of medicinally interesting compounds and the elucidation of drug-action mechanisms, owing to the power of molecular genetic techniques available in this organism. The beneficial features of using yeast also derive from the fact that many small molecule inhibitors act on common target molecules in an evolutionally conserved manner throughout eukaryotes. As shown in this study, investigating the related pathways in yeast and human cells in parallel could provide an effective methodology for discovering and studying the mechanism of action of medicinally interesting small-molecule compounds.

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Output

1. Suauam P, Yingyongnarongkul B, Palaga T, Miyakawa T and **Yompakdee C**. A potential immunosuppressant from *Clausena harmandiana* (Pierre) as discovered by a yeast-based assay. The 4th Life Science Symposium "Advances in Medical Biotechnology" Chulalongkorn University – Peking University. March 26-27, 2014. Chulalongkorn University, Thailand. (International, Oral presentation).

2. Suauam P, Yingyongnarongkul B, Palaga T, Miyakawa T, **Yompakdee C.** CHA-01, a Potential Immunosuppressant Revealed by the Ca^{2+}-Sensitive Δ*zds1* Yeast Growth and Jurkat T-cell Interleukin-2 Production Assays. Manuscript in preparation.