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An active compound from *Kempferia parviflora* with inhibitory activity against GSK-3 kinase implicated in Type II Diabetes and Alzheimer's disease

by

Assist.Prof. Dr. Chulee Yompakdee

Assist.Prof. Dr. Warintorn Chavasiri

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บทคัดย่อ

วิถีการส่งสัญญาณของแคลเซียมมีบทบาทสำคัญในการควบคุมกระบวนการทางชีวภาพที่สำคัญๆ ในสิ่งมีชีวิตชั้นสูง ตั้งแต่ยีสต์ไปจนถึงมนุษย์ เนื่องจากยีนในยีสต์และมนุษย์มีการอนุรักษ์ที่สูง การค้นพบสารโมเลกุลขนาดเล็กที่ออกฤทธิ์ยับยั้งโปรตีนใดๆในยีสต์ จึงน่าที่จะออกฤทธิ์เช่นเดียวกันในมนุษย์ ตัวอย่างสารยับยั้งได้แก่ สารกดภูมิคุ้มกัน FK506 และ cyclosporine A ในงานวิจัยก่อนหน้าที่ได้ใช้ยีสต์สายพันธุ์พิเศษเป็นระบบคัดกรองหาสารออกฤทธิ์ยับยั้งวิถีการส่งสัญญาณของแคลเซียมในยีสต์สายพันธุ์กลายของ *Saccharomyces cerevisiae* จากสารสกัดพืชสมุนไพรไทย พบสารสกัดอย่างหยาบจากกระชายดำเป็นหนึ่งในตัวอย่างที่ให้ผลบวกแรง โดยการใช้ Δ*zds1* yeast-based assay ในการติดตามฤทธิ์จากส่วนย่อยจนกระทั่งได้สารบริสุทธิ์ 5-hydroxy-3,7-dimethoxyflavone (ในที่นี้ให้ชื่อว่า Cpd1) ซึ่งมีฤทธิ์ยับยั้งวิถีการส่งสัญญาณของแคลเซียมในยีสต์ ในงานวิจัยนี้มุ่งหมายที่จะค้นหาฤทธิ์ทางชีวภาพที่เกี่ยวข้องในวิถีการส่งสัญญาณแคลเซียมของสาร Cpd1 จากการใช้วิธีทางพันธุศาสตร์ของยีสต์ เพื่อค้นหาโปรตีนในวิถีการส่งสัญญาณของแคลเซียมที่ถูกยับยั้งการทำงานโดยสารออกฤทธิ์ Cpd 1 พบว่าเป็นโปรตีนไคเนส Mck1 ซึ่งยีน *MCK1* ในยีสต์มีความคล้ายคลึงกับยีนประมวลรหัสเอ็นไซม์ GSK3β ในสัตว์เลี้ยงลูกด้วยนม การมี GSK3β ในระดับที่สูงพบว่าเกี่ยวข้องกับการเกิดพยาธิสภาพหลายประการ ได้แก่ โรคเบาหวานประเภท 2 และโรคอัลไซเมอร์ เป็นต้น นอกจากนี้ยังพบว่าสาร Cpd1 ไม่แสดงความเป็นพิษต่อเซลล์ยีสต์อีกด้วย

Abstract

Calcium signaling pathways play pivotal roles in regulation of various important biological processes in eukaryotes ranging from yeast to human. Because of the high degree in gene conservation from yeast to human, the small molecule inhibitors discovered in the yeast based-drug screening system can be expected to exert their function in human as well. The immunosuppressive agents, FK506 and cyclosporine A, are an example. Our previous studies using a Δ*zds1* yeast-based assay to search for inhibitors in the calcium signaling pathway in *Saccharomyces cerevisae* mutant strain from the crude extract of Thai medicinal plants found the crude extract of *Kaempferia parviflora* as one of the strong positive candidates. Using Δ*zds1* proliferation assay to guide fractionation and purification, a compound 5-hydroxy-3,7-dimethoxyflavone (here designated as Cpd1) was obtained and showed inhibitory activity against the calcium signals in yeast. This study aimed to learn more on its biological activity related to the calcium signal inhibition. Genetic and biochemical analyses in yeast revealed that Mck1 kinase, a protein in the calcium signaling pathway was inhibited by Cpd1. Yeast *MCK1* is an ortholog gene coding for GSK3β. High level of GSK3β causes several pathogenesis including type II Diabetes and Alzheimer's disease. In addition, Cpd1 showed no cytotoxicity to the yeast cells.

Introduction

The search for novel drugs is still one of a major interest in the research especially in the health related area since infectious diseases are still a global problem. This is because of the development and spread of drug-resistant pathogens. (Espinel *et al.,* 2001). In addition the high worldwide mortality is one of the reasons on searching for novel anti-cancer drugs (Pisani et al., 1999).

Ca^{2+}-signaling pathway play roles in the regulation of diverse cellular processes, such as T-cell activation, secretion, muscle contraction, neurotransmitter release and probably many more (Clapham 1995). In the yeast, *Saccharomyces cerevisiae*, calcium signaling pathway regulates G2 cell cycle progression as shown in fig 1.

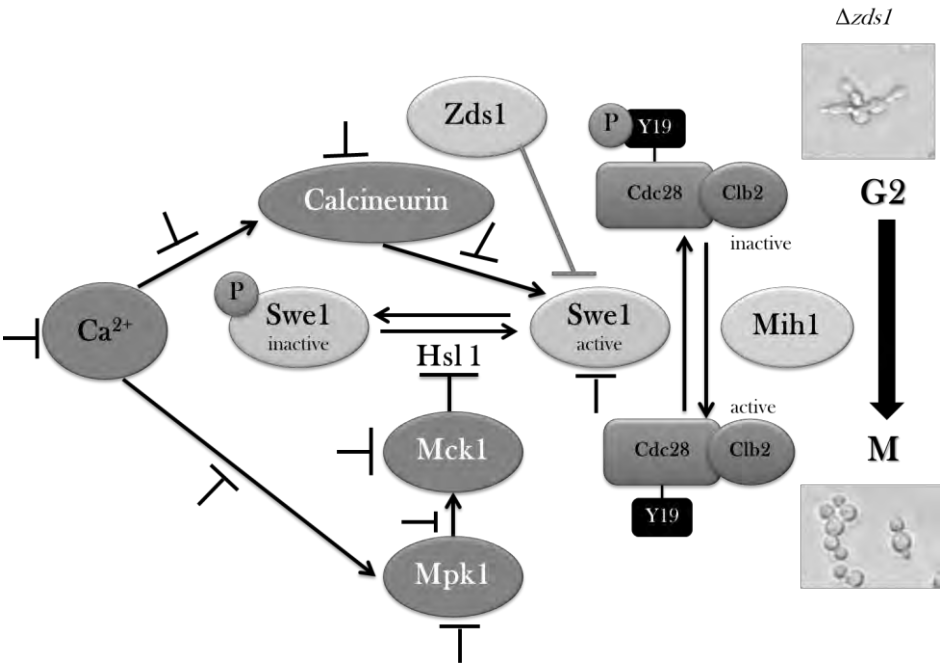


Figure 1 Model for the Ca^{2+}-activated signaling pathways that regulate G2-cell cycle progression in *S. cerevisiae*. ⊤ represents potential target molecules of the drugs that are expected to be isolated by this screening system. (Modified from Shitamukai *et al*. 2000).

The pathways have been extensively studied in the yeast *S. 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 (Mizunuma et al., 1998). 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 Δ*zds1* yeast strain as indicator cells has been developed (Shitamukai et al., 2000). The Δ*zds1*yeast- based 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 (Mizunuma et al., 1998; Foskett et al., 2007). Generally, small molecule inhibitors of Ca^{2+}-dependent signaling pathways exert their physiological effects by an evolutionary conserved manner throughout eukaryotes (Mager and Winderickx, 2005). Consequentially, inhibitors found to be functional in yeast may well be active in relevance to humans.

Our previous extensive screening study using this Δ*zds1* mutant yeast-based assay revealed that the crude extract of *Kaempferia parviflora* Wall. Ex. Baker contained a potent inhibitory activity (Boonkerd et al., 2011). *K. parviflora* is a member of Zingiberaceae (ginger) family, locally known in Thailand as Black Galingale or Kra-chai-dam. Members of this family, including *K. parviflora*, are traditionally very popular for health promotion (Putiyanan et al., 2004). The rhizomes of *K. parviflora,* locally known as Thai ginseng, have been used as a traditional medicine for various purposes, including the treatment of leucorrhea, oral diseases (Chomchalow et al., 2003, Sudwan et al., 2006), stomach ache, flatulence, digestive disorders, gastric ulcer as well as diuresis and tonic (Wattanapitayakul et al., 2007). Boonkerd et al (2014). reported that a flavonoid 5-hydroxy-3,7-dimethoxyflavone or designated as Cpd1 from *K. parviflora* exhibited an inhibitory activity against the Ca^{2+}-induced G2 cell-cycle arrest, but a detailed mechanism for the action of Cpd1 in yeast still needs to be investigated.

Glycogen synthase kinase 3 (GSK-3) was first identified in 1980 as an enzyme that phosphorylated and inactivated glycogen synthase (GS) (Embi et al., 1980). There are two isoforms of GSK3, which are semi-redundant and ubiquitously expressed in tissues. The α-isoform encodes a 51 kDa polypeptide and the β-isoform a 47 kDa polypeptide (Woodgett, 1990). GSK-3 functions by phosphorylating a serine or threonine residue on its target substrate. GSK-3β has recently been the subject of much research because it has been implicated in a number of diseases, including Type II diabetes (Diabetes mellitus type 2), Alzheimer's Disease, inflammation, cancer, and bipolar disorder.

GSK-3β was originally discovered in the context of its involvement in regulating glycogen synthase. After being primed by casein kinase 2 (CK2), glycogen synthase gets phosphorylated at a cluster of three C-terminal serine residues, reducing its activity (Rayasam et al., 2009). In addition to its role in regulating glycogen synthase, GSK-3β has been implicated in other aspects of glucose homeostasis, including the phosphorylation of insulin receptor IRS1 (Liberman et al., 2005) and of the gluconeogenic enzymes phospho-enolpyruvate carboxykinase and glucose 6 phosphatase (Lochhead et al., 2001). Moreover, the activity of GSK-3β has been associated with both pathological features of Alzheimer's disease, namely the buildup of Amyloid-β (Aβ) deposits and the formation of neurofibrillary tangles. GSK-3β is thought to directly promote Aβ production and to be tied to the process of the hyperphosphorylation of tau proteins, which leads to the tangles (Jope et al., 2007).

The aim of this study was to investigate the molecular target of the flavonoid, 5-hydroxy-3,7-dimethoxyflavone, from *K. parviflora* in Ca^{2+}-signaling pathways in yeast *S. cerevisiae* and study the biological activity of Cpd1 *in vitro* inhibition.

Material and methods

Source of 5-hydroxy-3,7-dimethoxyflavone

5-hydroxy-3,7-dimethoxyflavone or Cpd1 from *K. parviflora* was previously isolated, identified and reported in Boonkerd et al. 2014.

Yeast strains and cultivation

All mutant strains of *S. cerevisiae* except YCY1 were obtained from Prof. Tokichi Miyakawa, Hiroshima University. Details of the strains were listed in Table 1.

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

Strain	**Genotype**	**Source of reference**
W303-1A	*MAT a trp1 leu2 ade2 ura3 his3 can1-1*	Rothstein R, 1983
YNS17	*MAT a trp1 leu2 ade2 ura3 his3 can1-1 zds1::TRP1 syr1::HIS3 pdr1::hisG-URA3-hisG pdr3::hisG-URA3-hisG*	Miyakawa T, Hiroshima University
Δ*mpk1*	*MAT a trp1 leu2 ade2 ura3 his3 can1-1 mpk1::HIS3 syr1::HIS3 pdr1::hisG-URA3-hisG pdr3::hisG-URA3-hisG*	
Δ*cnb1*	*MAT a trp1 leu2 ade2 ura3 his3 can1-1 cnb1::HIS syr1::HIS3 pdr1::hisG-URA3-hisG pdr3::hisG-URA3-hisG*	
YRC1	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 zds1::TRP erg3::HIS3 pdr1::hisG pdr3::hisG GAL1-CMP2ΔC::URA3*	
YRC2	*MAT a trp1 leu2 ade2 ura3 his3 can1-1 swe1::GALp-SWE1-HA::LEU2 syr1::HIS3 pdr1::hisG-ura3-hisG pdr3::hisG-URA3-hisG*	

YCY1	same as YNS17 except Δ*ura3* (*MAT a trp1 leu2 ade2 ura3 his3 can1-1 zds1::TRP1 syr1::HIS3 pdr1::hisG pdr3::hisG*)	this study
NSC1	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 zds1::TRP erg3::HIS3 pdr1::hisG pdr3::hisG pYES2::GAL1p-MPK1*	Wangkangwan, 2007
NSC2	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 zds1::TRP erg3::HIS3 pdr1::hisG pdr3::hisG pYES2::GAL1p-MCK1*	

S. cerevisiae mutant cells were cultivated on YPAUD (yeast extract peptone dextrose supplemented with adenine and uracil) or YPAUR (yeast extract peptone raffinose supplemented with adenine and uracil) medium at 30° C with shaking at 200 rpm for 2 days. For yeast transformants bearing plasmid with *URA3* marker, the cells were cultivated in Synthetic Complete Uracil drop out medium with 2%Raffinose (SR-Ura). Under induction condition, SG-Ura was used in which 1% galactose was added in SR-Ura. The cells were cultivated with shaking at 200 rpm, 30°C.

Sources of plasmids

Plasmids, pKC190, pYES2::*GAL1p-CMP2*ΔC and pYES2::*GAL1p-MPK1*, were obtained from Prof. Tokichi Miyakawa, Hiroshima University, Japan. Plasmid pYES2::*GAL1p-MPK1* was obtained from Wangkangwan, 2008.

β-galactosidase activity assay

Cells of the yeast transformant, the wild-type yeast strain (W303-1A) transformed with a plasmid of pKC190, carrying *PMR2A*-lacZ reporter under the activation of four tandem copies of the calcineurin-dependent response element (CDRE) were incubated in SC-Ura medium containing 150 mM $CaCl_2$ with 500 μM Cpd1 or 1 mM $MgCl_2$ as a control. The

tested cells were incubated at 30 ^{o}C for 4 h. β-galactosidase activity was measured using ONPG assay as described in Miller, 1972.

Yeast transformation

The mutant yeast cells transformed with indicated plasmid using the method according to Gietz and Woods, 2002.

Results and Discussion

Previously Boonkerd et al. (2014) demonstrated that Cpd1 from *K. parviflora* exhibited Ca^{2+}-signaling inhibitory activity in *S. cerevisiae*. To further determine the molecular target of Cpd1 that acts on the pathways, the following assays were performed.

Effect of Cpd1 on free intracellular Ca^{2+}-levels

In order to evaluate free intracellular Ca^{2+}, the genetic method was carried out using the method modified from Tutulan-Cunita *et al.* (2005). The wild-type yeast strain (W303-A1) was transformed with a plasmid of pKC190, carrying *PMR2Ap*-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 $CaCl_2$ (150 mM) Cunningham and Fink, 1996). If free intracellular Ca^{2+} is high, the free Ca^{2+} will bind to CDRE of the *PMR2A promoter* and will result in induction of the *lacZ* reporter which will then lead to detectable β-galactosidase activity. Therefore, the higher free intracellular Ca^{2+}, the higher β-galactosidase activity could be measured.

The yeast transformants W303-1A [pKC190] were cultured in SC-Ura containing 150 mM $CaCl_2$ either in the presence or absence of 500 μM Cpd1 at 30 ^{o}C for 4 h before β-galactosidase measurement. It was found that under low Ca^{2+} condition, the *PMR2A* promoter was not activated, hence bearly detectable on β-galactosidase activity (treatment 1) while in the presence of high Ca^{2+} condition, the cells showed high β-galactosidase level (treatment 2). In the presence of FK506, a known calcineurin inhibitor, almost no β-galactosidase activity could be measused (treatment 3). In the presence of 500 μM Cpd1, the β-galactosidase activity was as high as in the treatment 2 (Fig. 2). The results suggested that the cytosolic Ca^{2+}-level was not influenced by the Cpd1 and that Cpd1 inhibited the Ca^{2+}-signal mediated cell-cycle regulation at a step following elevation of the cytosolic Ca^{2+} concentration.

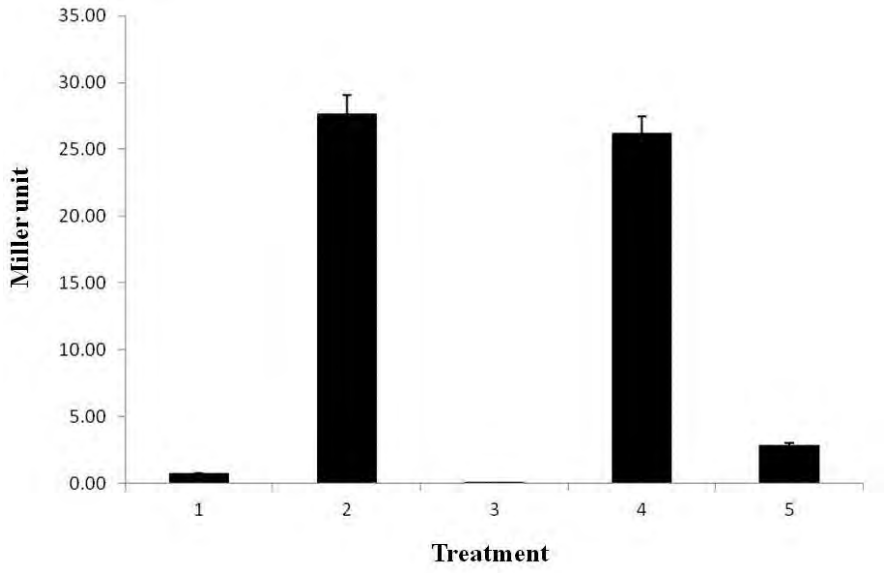


Figure 2 The effect of Cpd1 from *K. parviflora* on intracellular Ca^{2+}- levels. Wild-type yeast (strain W303-1A) was transformed with plasmid pKC190 carrying *PMR2A–lacZ,* grown in Sc-Ura medium at 30 ^{o}C for 4 h: **1**, 0 mM $CaCl_2$; **2**, 150 mM $CaCl_2$; **3**, 150 mM $CaCl_2$ + 500 nM FK506; **4,** 150 mM $CaCl_2$ + 500 μM Cpd1; **5**, 150 mM $CaCl_2$ + 1 mM $MgCl_2$.

Effect of Cpd1 on Cnb1 and Mpk1

The Ca^{2+}-signals in yeast cells were transduced in two parallel pathways: calcineurin or Mpk1 MAP kinase cascade (Nakamura et al. 1996). To see whether either calcineurin or Mpk1 MAP kinase was a molecular target of Cpd1, a synthetic lethality test was performed. The growth of either Δc*nb1* (the mutant yeast cells lacking regulatory subunit of calcineurin) or Δ*mpk1* (the MAP kinase mutant yeast) cultivated in YPAUD medium either in the presence or absence of Cpd1 or FK506, a potent calcineurin inhibitor, was monitored. The results showed that the Δc*nb1* or Δ*mpk1* cells could grow normally in the presence of 200 μM Cpd1 while the Δc*nb1* cells could not grow in the presence of 250 nM FK506 (Fig. 3A and Fig. 3B). These results suggested that neither the Mpk1 nor calcineurin is the molecular target molecule of Cpd1.

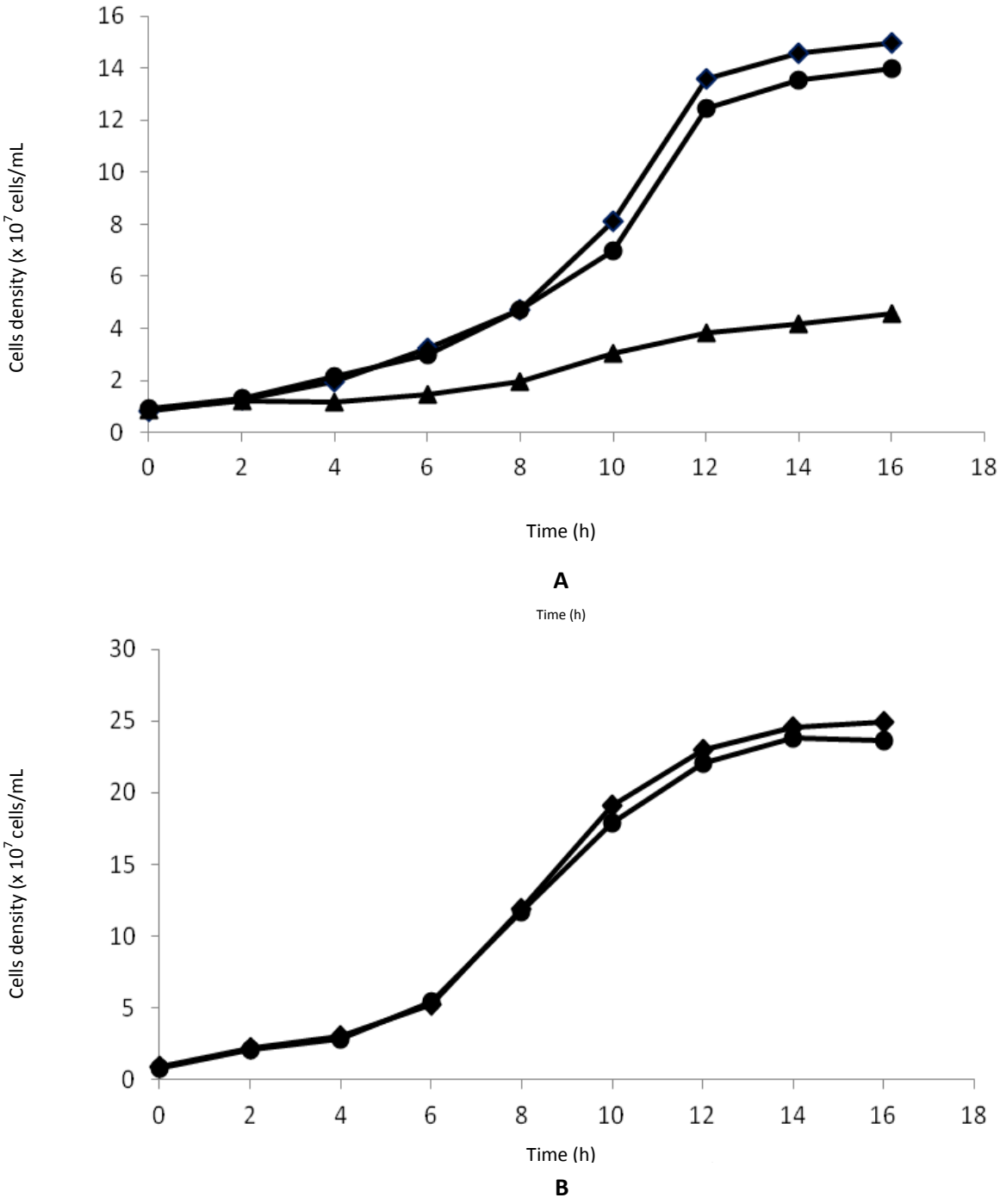


Figure 3 Synthetic lethality test for effect of Cpd1 on Calcineurin and Mpk1 MAP kinase. The Δ*cnb1* (A) *or* Δ*mpk1* (B) cells of 5x10^6 cells/mL were cultivated in YPAUD broth at 30 °C with shaking at 120 rpm. The cells density was measured every 2 h until 14 h of incubation times. The symbols are as follow: ◆ , YPAUD medium; ● , YPAUD medium + 200 μM Cpd1**;** ▲ , YPAUD medium + 250 nM FK506.

Effect of Cpd1 on growth of Δzds1 cells

To see whether Cpd1 inhibited the Ca^{2+}-signals or not, phenotype of Δ*zds1* cells was observed. The calcium hyperactivation caused growth defect (G2/M delay) in Δ*zds1* cells (Mizunuma et al. 1998). If Cpd1 could inhibit the Ca^{2+}-signals, then the growth defect phenotypes should be alleviated. In the experiment, yeast Δ*zds1* cells were grown in YPAUD broth either in the presence or absence of 500, 250 and 125 μM Cpd1 at 30 °C, 120 rpm. The growth of Δ*zds1* cells was monitored. It was found that the growth of the yeast Δ*zds1* cells was severely defected when cultivated under induced condition (100 mM $CaCl_2$). However, in the presence of 500, 250 and 125 μM Cpd1 even cultivated in the induced condition, the growth defect could be restored to the similar extent as that of control (uninduced condition) and in the presence of 250 nM FK506 (Fig. 4 and Table 2). The results confirmed that Cpd1 have an effect on inhibitory activity against the Ca^{2+}-induced G2 cell-cycle arrest.

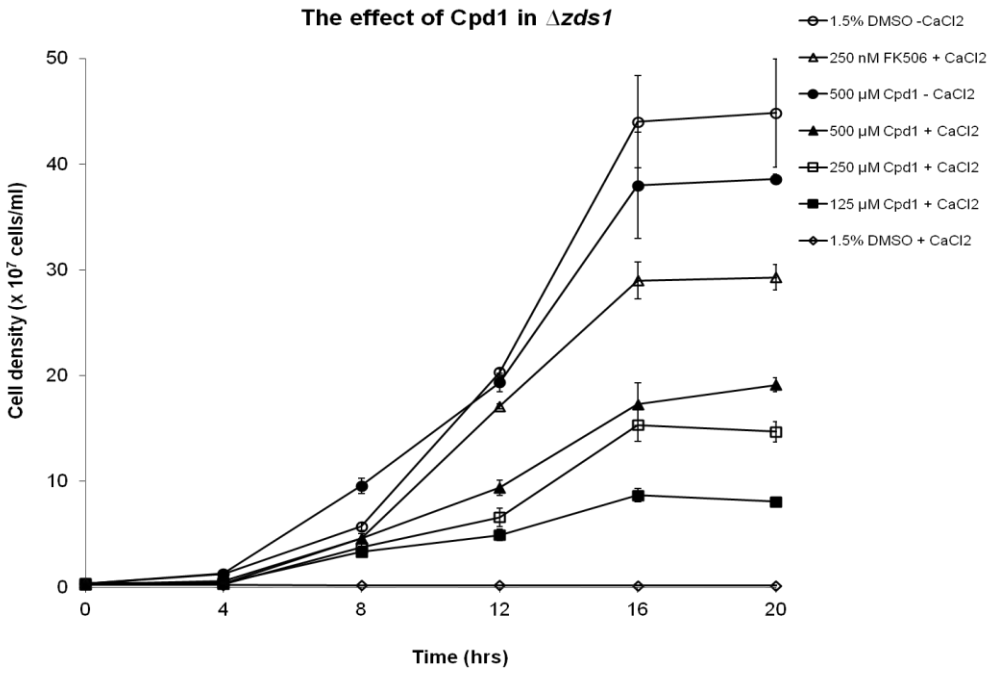
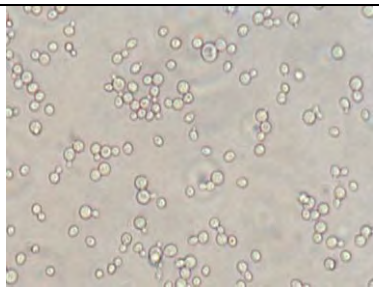
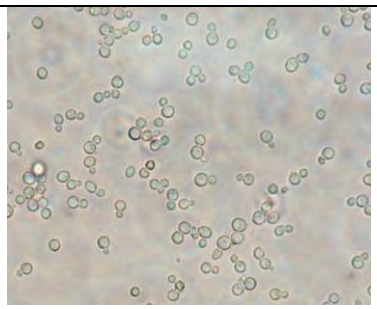
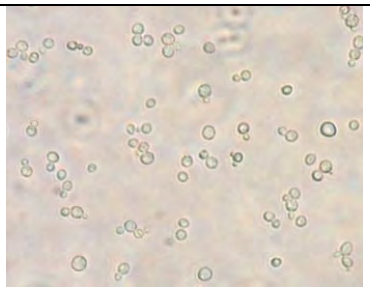
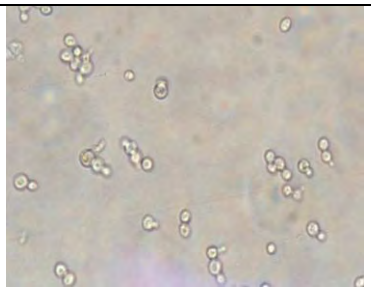
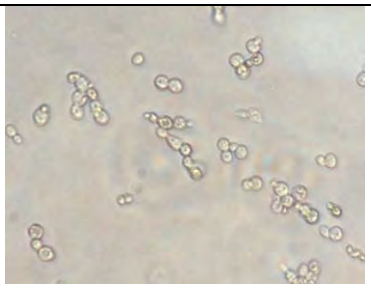
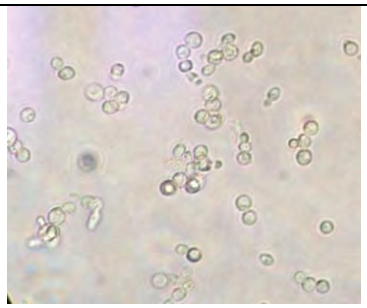
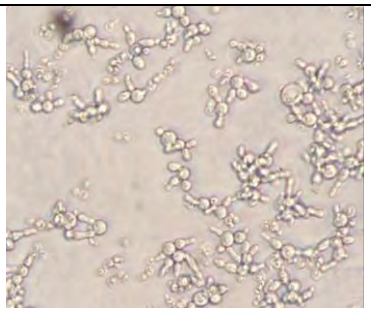


Figure 4 The effect of Cpd1 on the growth of Δ*zds1* mutant yeast cells. The cells were cultivated in YPAUD medium at 30 °C with shaking at 120 rpm. The cell density was measured every 4 h until 20 h of incubation times. The symbols are as follow: ○, 1.5% DMSO - $CaCl_2$; ●, 500 μM Cpd1 - $CaCl_2$; △, 250 nM FK506 + $CaCl_2$; ▲, 500 μM Cpd1 + $CaCl_2$; □, 250 μM Cpd1 + $CaCl_2$; ■, 125 μM Cpd1 + $CaCl_2$; ◇, 1.5% DMSO + $CaCl_2$**.**

Table 2 The effect of Cpd1 on the growth of Δ*zds1* mutant yeast cells under induced condition (100 mM $CaCl_2$).

Treatment	Phenotype	% elongation buds after cultivated 20 h.
1.5%DMSO (normal control)		0%
250 nM FK506 (positive control)		0%
500 μM Cpd1 (toxicity control)		0%
500 μM Cpd1		1%
250 μM Cpd1		1%

125 μM Cpd1		2%
1.5%DMSO (negative control)		95%

Effect of Cpd1 on growth of Δzds1 cells overexpressed MPK1 and Δzds1 cells overexpressed Calcineurin

The Ca^{2+}-signals in yeast cells were transduced in two parallel pathways: calcineurin or Mpk1 MAP kinase cascade (Nakamura et al. 1996). To see the effect of Cpd1 on calcineurin or Mpk1 MAP kinase, the growth of cells overexpressed *MPK*1 (the NSC1 strain) and overexpressed *Calcineurin* (the YRC1 strain) was observed in either presence or absence of Cpd1. To induce expression of *MPK*1 and *Calcineurin* under control of *GAL1* promoter, the mutant yeast strain was cultivated in YPAUR medium containing 2% galactose either in the presence or absence of 500, 250 and 125 μM Cpd1 and used FK506, a potent calcineurin inhibitor. The growths of the cultures were monitored for 48 h. The results showed that in the presence of 250 μM FK506, the growth defect could be partially alleviated. However, either in the absence or presence of Cpd1, the mutant cells overexpressed *Calcineurin* (the YRC1 strain) showed severely growth defect (Fig. 5 and Table 3). In the other hand the mutant cells overexpressed *MPK*1 (the NSC1 strain) showed the growth defect could be restored to the similar extent as that of control (uninduced condition) (Fig. 6 and Table 4). This suggested that Mpk1 MAP kinase cascade was the molecular target of Cpd1 in the Ca2+ -signaling pathways in yeast.

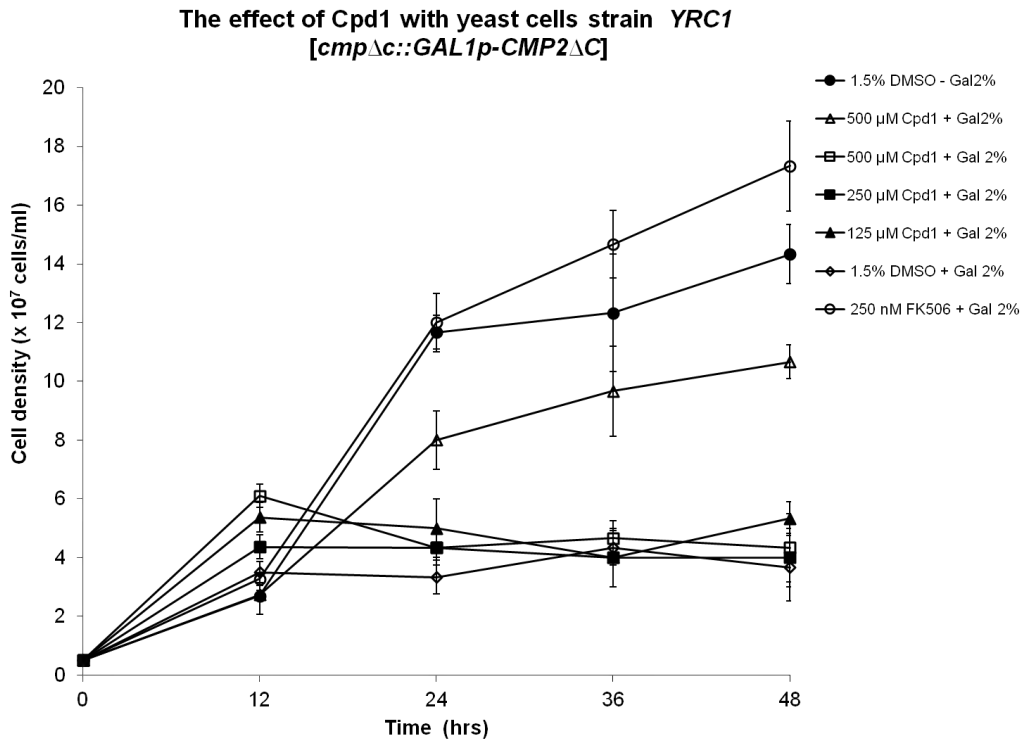
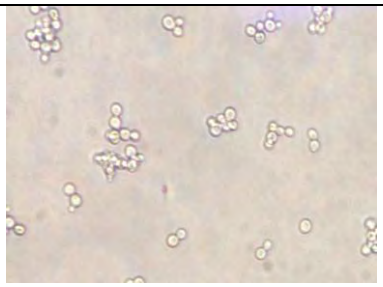
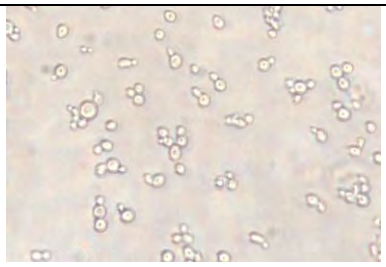
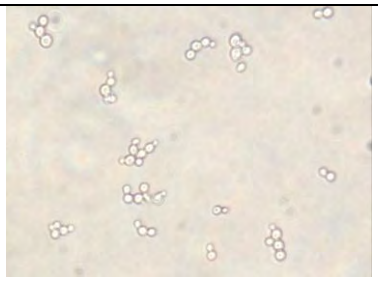
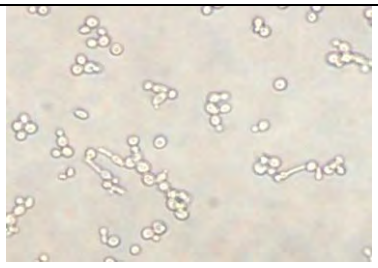
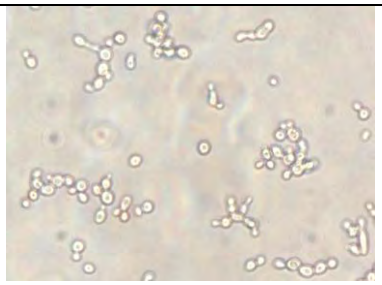
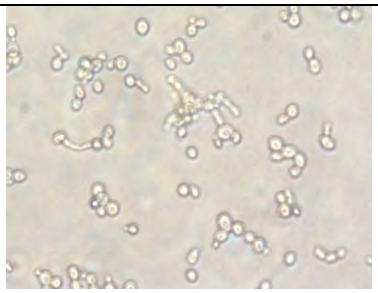
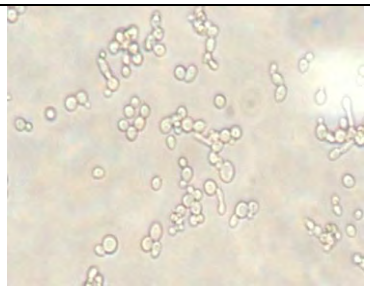


Figure 5 The effect of Cpd1 on the growth of Δ*zds1* overexpressed *Calcineurin* mutant yeast cells, YRC1 strain. The YRC1 strain (*cmp2*Δ*c::GAL1p-CMP2*Δ*C*) of 5 x10^6 cells/mL was cultivated in YPAUR medium (YP medium with adinine, uracil and 2% raffinose at 30 °C with shaking at 200 rpm. The cells density was measured every 12 h until 48 h of incubation times. The symbols are as follow: ○, 250 nM FK506 + 2% galactose ; ●, 1.5% DMSO - 2% galactose ; △, 500 µM Cpd1 - 2% galactose ; ▲, 125 µM Cpd1 + 2% galactose; □, 500 µM Cpd1 + 2% galactose ; ■, 250 µM Cpd1 + 2% galactose ; ◇, 1.5% DMSO + 2% galactose**.**

Table 3 The effect of Cpd1 on the growth of Δ *zds1* overexpressed *Calcineurin* mutant yeast cells under induced condition (2% galactose).

Treatment	Phenotype	% elongation buds after cultivated 48 h.
1.5%DMSO (normal control)		0%
250 nM FK506 (positive control)		0%
500 µM Cpd1 (toxicity control)		0%
500 µM Cpd1		50%
250 µM Cpd1		68%

125 µM Cpd1		63%
1.5%DMSO (negative control)		87%

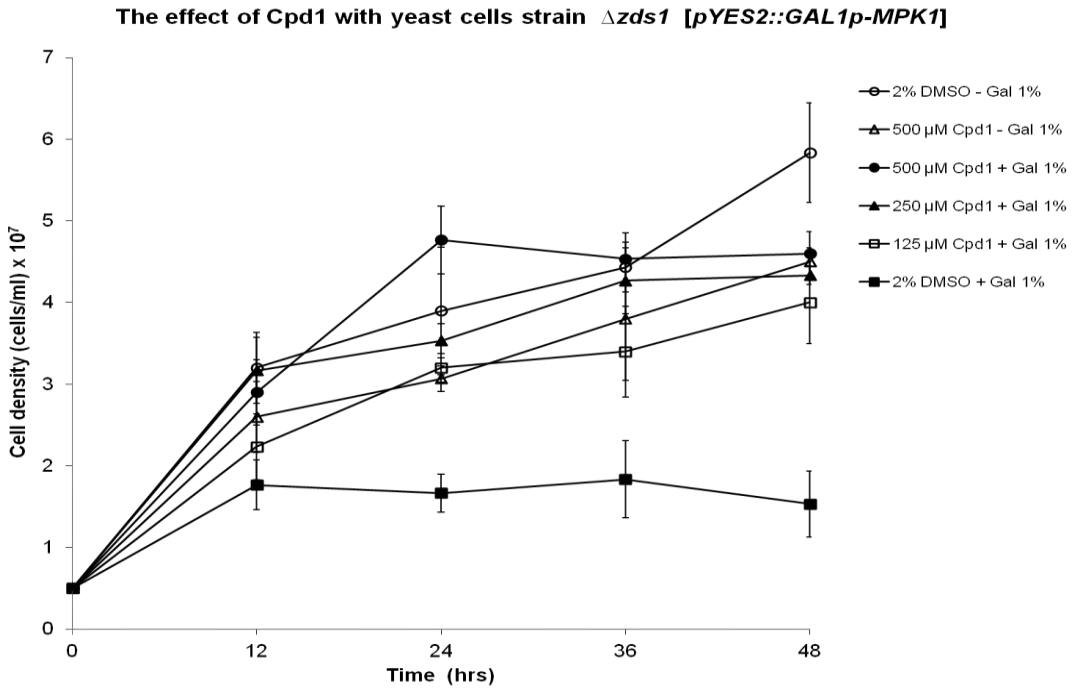
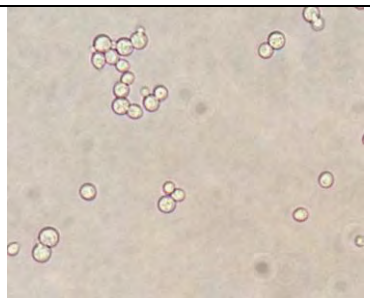
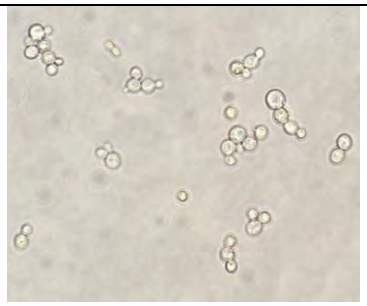
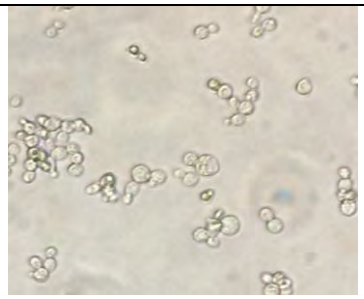
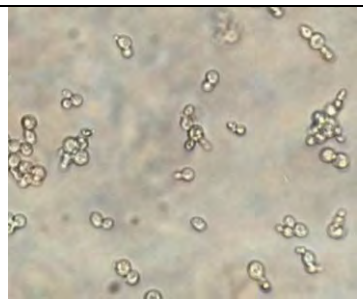
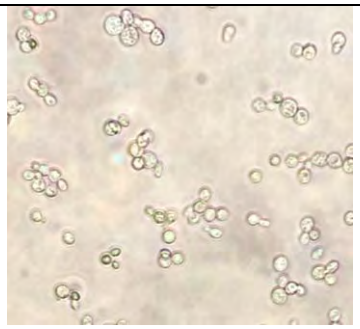
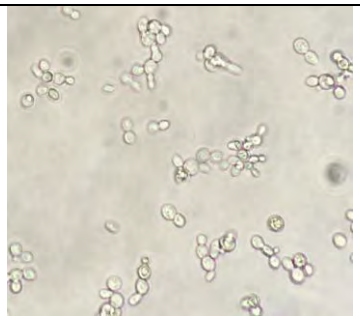


Figure 6 The effect of Cpd1 on the growth of Δ*zds1* overexpressed *MPK1* mutant yeast cells, NSC1 strain. The NSC1 strain (*pYES2::GAL1p-MPK1*) of 5 x10^6 cells/mL was cultivated in uracil drop out SG medium (SG-U) at 30 °C with shaking at 120 rpm. The cell density was measured every 12 h until 48 h of incubation times. The symbols are as follow: ○, 2% DMSO - 1% galactose ; ●, 500 µM Cpd1 + 1% galactose; △, 500 µM Cpd1 - 1% galactose ; ▲, 250 µM Cpd1 + 1% galactose; □, 125 µM Cpd1 + 1% galactose ; ■, 2% DMSO + 1% galactose

Table 4 The effect of Cpd1 on the growth of Δ *zds1* overexpressed *MpK1* mutant yeast cells under induced condition (1% galactose).

Treatment	Phenotype	% elongation buds after cultivated 48 h.
1.5%DMSO (normal control)		0%
500 µM Cpd1 (toxicity control)		0%
500 µM Cpd1		4%
250 µM Cpd1		7%

125 μM Cpd1		18%
1.5%DMSO (negative control)		74%

Effect of Cpd1 on growth of Δzds1 cells overexpressed Mck1

Mizunuma et al. (2001) used genetic analyses of yeast mutants Ca^{2+}-signaling pathways and found that Mck1, a component in the pathways, functions downstream of the Mpk1. To see whether Cpd1 inhibited the Ca^{2+}-signals at the step of Mck1 or not, phenotype of Δ*zds1* cells overexpressed *MCK1* was observed. The calcium hyperactivation caused growth defect (G2/M delay) in Δ*zds1* cells (Mizunuma et al. 1998). The Δ*zds1* overexpressed *MCK1* cells should show the similar phenotypes when cultivated under induced condition. If Cpd1 could inhibit overexpressed Mck1 activity, then the growth defect phenotypes should be alleviated. In the experiment, we popped out chromosomal URA3 of YNS17 strain by cultivating it on medium containing 5-FOA medium (Yeast Genetics Manual, Cold Spring Harbour Lab Press), resulting in the YCY1 strain (the Δ*zds1* Δ*ura3*). Then, YCY1 strain was transformed with plasmid pYES2::*MCK1,* resulting in the NSC2 strain. The yeast transformants were grown in SR-Ura at 30 °C, 120 rpm. To see the effect of Cpd1 on the overexpressed *MCK1* cells, the yeast transformants were cultivated in SG-Ura broth either in the presence or absence of 500, 250 and 125 μM Cpd1 and used 10 μM GSK-3β inhibitor I as positive control. The growth of Δ*zds1* overexpressed *MCK1* cells was monitored. It was found that the growth of the yeast transformant cells overexpressed *MCK1* was severely

defected when cultivated under induced condition (2% galactose). However, in the presence of 500, 250 and 125 μM Cpd1 even cultivated in the induced condition, the growth defect could be restored to the similar extent as that of control (uninduced condition) (Fig. 7 and Table 5). The results suggested Mck1 is a possible molecular target of Cpd1.

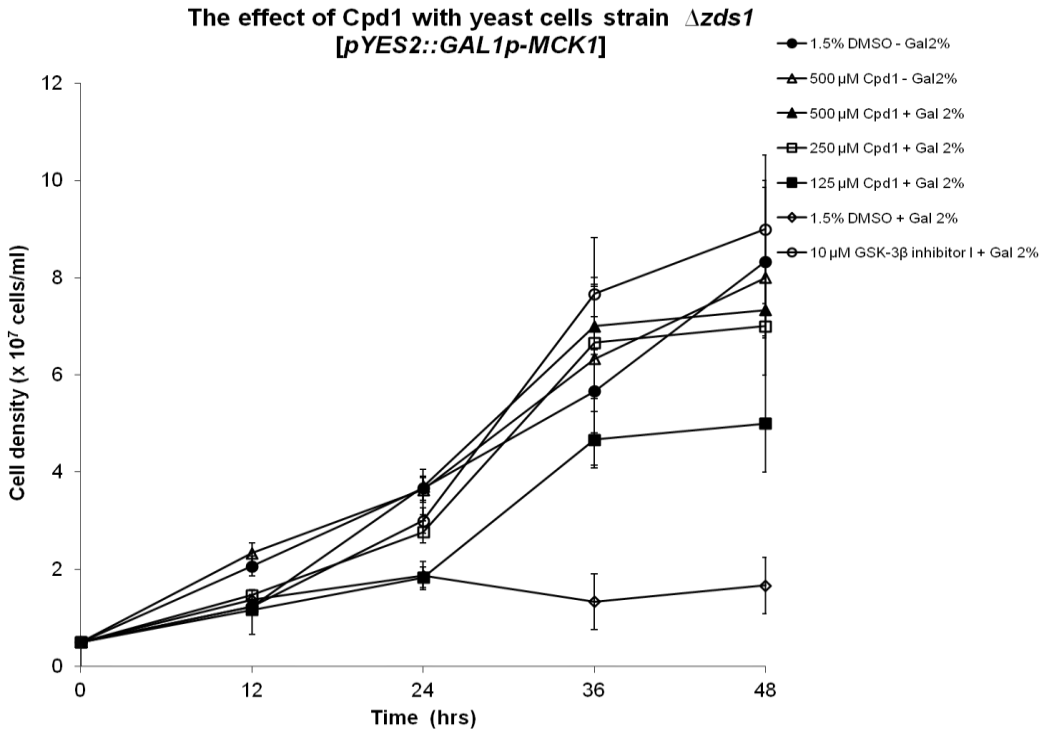
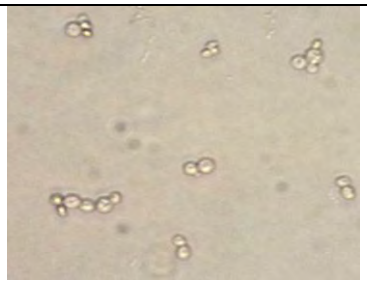
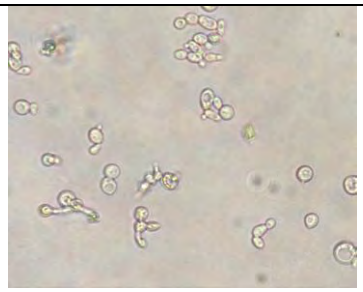
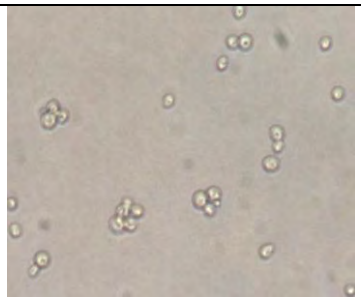
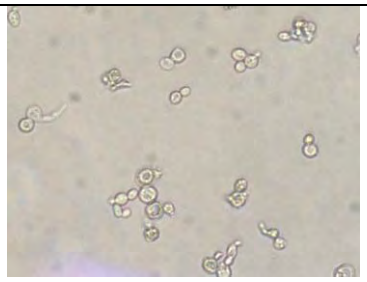
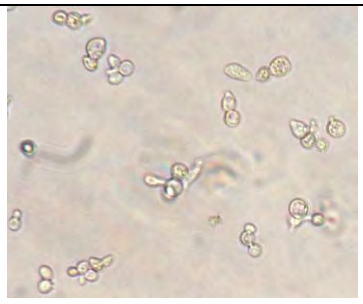
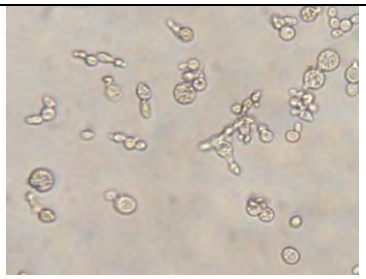
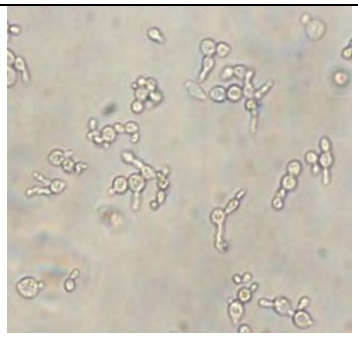


Figure 7 The effect of Cpd1 on the growth of Δ*zds1* overexpressed *MCK1* mutant yeast cells, NSC2 strain. The *cells of YCY1 strain bearing pYES2::MCK1* of 5x10^6 cells/mL were cultivated in uracil drop out SG medium (SG-U) at 30 °C with shaking at 120 rpm. The cell density was measured every 12 h until 48 h of incubation times. The symbols are as follow: ○, 10 μM GSK-3β inhibitor I + 2% galactose ; ●, 1.5% DMSO - 2% galactose ; △, 500 μM Cpd1 - 2% galactose ; ▲, 500 μM Cpd1 + 2% galactose; □, 250 μM Cpd1 + 2% galactose ; ■, 125 μM Cpd1 + 2% galactose ; ◇, 1.5% DMSO + 2% galactose**.**

Table 5 The effect of Cpd1 on the growth of Δ *zds1* overexpressed *MCK1* mutant yeast cells under induced condition (2% galactose).

Treatment	Phenotype	% elongation buds after cultivated 48 h.
1.5%DMSO (normal control)		0%
10 µM GSK 3β inhibitor I (positive control)		7%
500 µM Cpd1 (toxicity control)		0%
500 µM Cpd1		2%
250 µM Cpd1		6%

125 μM Cpd1		23%
1.5%DMSO (negative control)		87%

Effect of Cpd1 on growth of Δzds1 cells overexpressed SWE1

Swe kinase is a component of the Ca^{2+}-signaling pathways in yeast (Fig. 1). To see the effect of Cpd1 on Swe1, the growth of cells overexpressed *SWE1* (the YCR2 strain) was observed in either presence or absence of Cpd1. To induce expression of *SWE1*under control of *GAL1* promoter, the YCR2 strain was cultivated in YPAUR medium containing 2% galactose either in the presence or absence of 500, 250 and 125 μM Cpd1 and used 10 μM GSK-3β inhibitor I, an inhibitor of Swe1 (Chanklan et al. 2008). The growths of the cultures were monitored for 48 h. The results showed that in the presence of 10 μM radicicol, the growth defect could be partially alleviated. However, either in the absence or presence of Cpd1, the mutant cells showed severely growth defect (Fig. 8 and Table 6). This suggested that Swe1 was not the molecular target of Cpd1 in the Ca2+-signaling pathways in yeast.

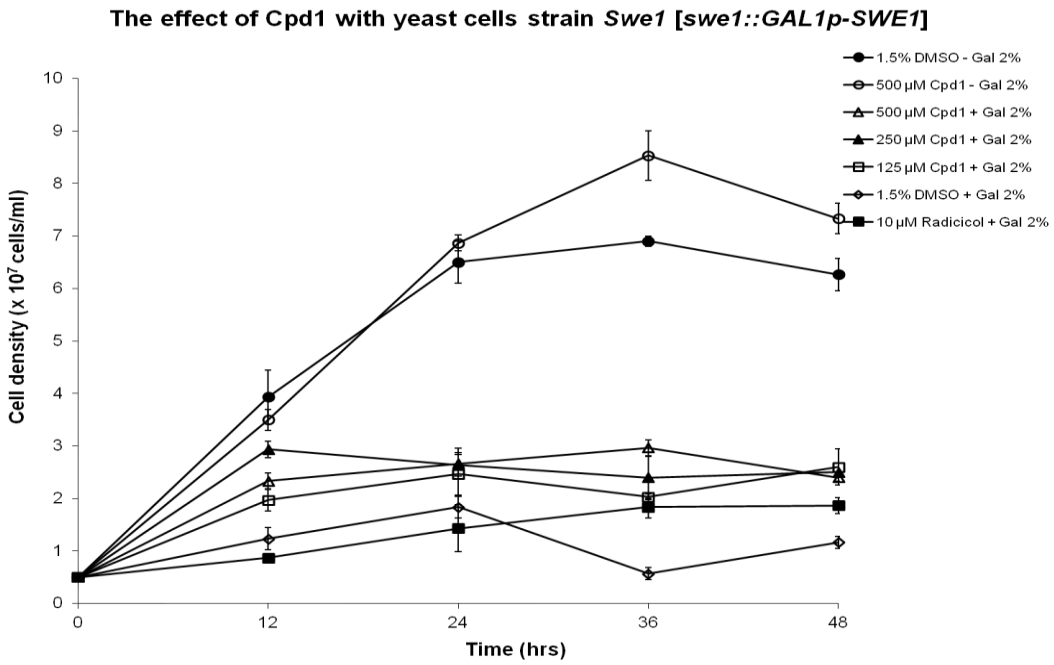
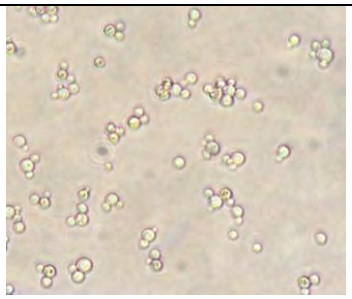
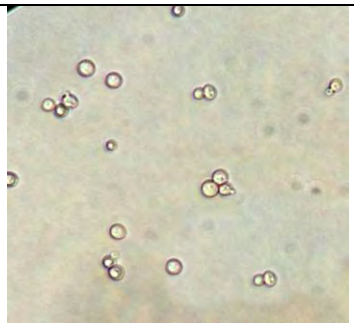
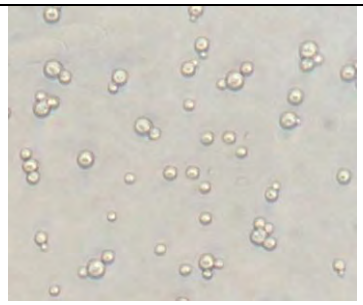
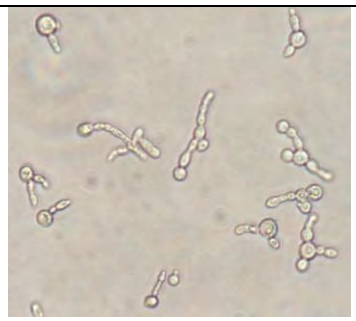
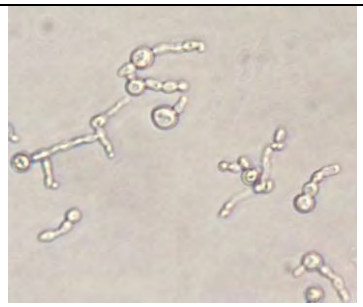
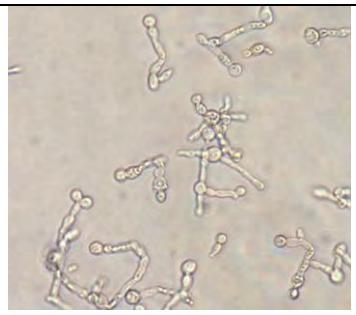
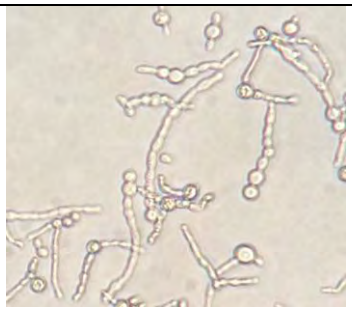


Figure 8 The effect of Cpd1 on growth of cells overexpressed Swe1. The YRC2 strain (Δ*swe1*::*GAL1p-SWE1*) of 5 x10^6 cells/mL was cultivated in YPAUR medium (YP medium with adinine, uracil and 2% raffinose at 30 °C with shaking at 200 rpm. The cells density was measured every 12 h until 48 h of incubation times. The symbols are as follow: ○, 500 µM Cpd1 - 2% galactose; ●, 1.5% DMSO - 2% galactose ; △, 500 µM Cpd1 + 2% galactose ; ▲, 250 µM Cpd1 + 2% galactose ; □, 125 µM Cpd1 + 2% galactose ; ■, 10 µM Radicicol + 2% galactose ; ◇, 1.5% DMSO + 2% galactose**.**

Table 6 The effect of Cpd1 on the growth of Δ *zds1* overexpressed *SWE1* mutant yeast cells under induced condition (2% galactose).

Treatment	Phenotype	% elongation buds after cultivated 48 h.
1.5%DMSO (normal control)		0%

10 µM Radicicol (positive control)		7%
500 µM Cpd1 (toxicity control)		0%
500 µM Cpd1		98%
250 µM Cpd1		97%
125 µM Cpd1		99%

1.5%DMSO (negative control)		99%

Taken together, the results from Fig. 2-8 suggested that *Mck1* was a potential molecular target of Cpd1 in the Ca^{2+}-signaling pathways in yeast. In addition, Cpd 1 showed no cytotoxicity to the yeast tested strains.

Kinase-Glo® Luminescent Kinase Assay Platform

From the result of The Δ*zds1* yeast - based assay showed the molecule target of active compound 5-hydroxy-3,7-dimethoxyflavone called compound1 (Cpd1) from *Kaempferia parviflora* Wall. Ex. Baker was Mck1, homolog human GSK-3β. Then to identify the inhibition activity of Cpd1 on GSK-3β was directly examined in the luminescent kinase assay method using recombinant human GSK-3β as an enzyme and phosphopeptide as a substrate *in vitro*. GSK-3β assays were performed using the Kinase Glo® Plus luminescent kinase assay platform. Regarding the mechanism between protein or enzyme and inhibitor or Cpd1 inhibition, can find ATP-competitive inhibition, non-ATP-competitive inhibition, and substrate-competitive inhibition.

GSK-3β assays were performed using the Kinase Glo® Plus luminescent kinase assay platform in a white 96-well plate with peptide kinase substrate (100 μM) was mixed with recombinant human GSK-3β (0.01098 unit) in a total volume of 15 μl of assay buffer. The efficiency of Cpd1 on enzyme GSK-3β activity of showed an ATP-noncompetitive binding mode to GSK-3β by Dixon plot (Ki =13.04 μM, Figure 16a) and showed a substrate competitive binding mode to GSK-3β with by Dixon plot (Ki = 53.92 μM, Figure 16b).

Optimizing Kinase Reaction Conditions

ATP standard curve

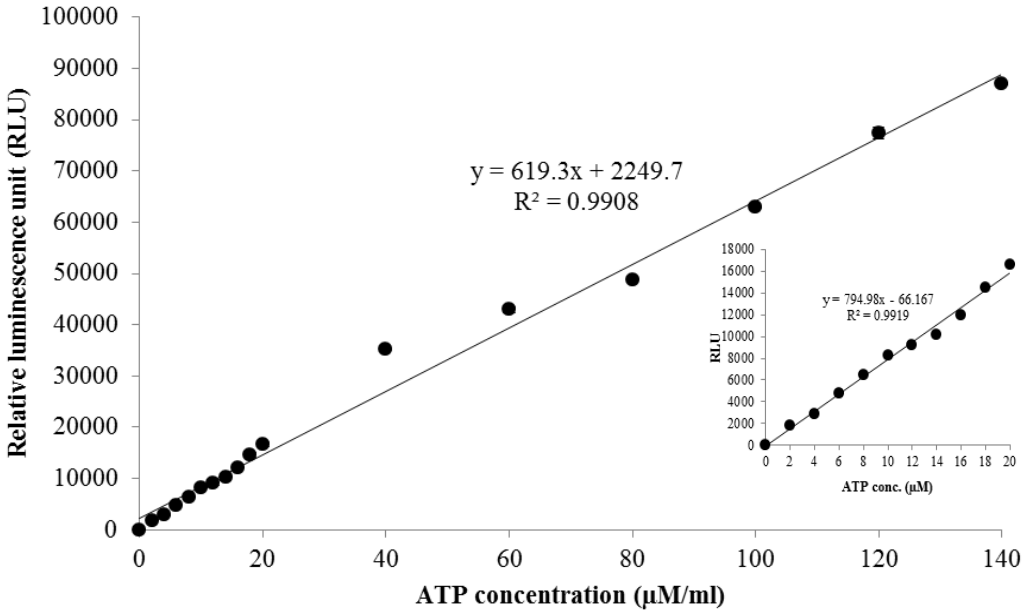


Figure 9 A linear relationship exists between the luminescent signal measured and the ATP concentration in the reaction buffer up to 0-140 µM. The lower ATP concentrations between 0 and 20 µM are also presented in the inset graph and the correlation coefficient (R^2) is 0.9908 for this range. The luminescent signal was recorded 10 min after addition of an equal volume of Kinase-Glo® reagent. The data presented are means standard error of the mean of triplicate wells (n = 3). the slope is the rate of relative luminescence unit (RLU) per 1µM ATP (RLU/1 µM ATP) = 619.3

Determining Optimal Substrate Concentration

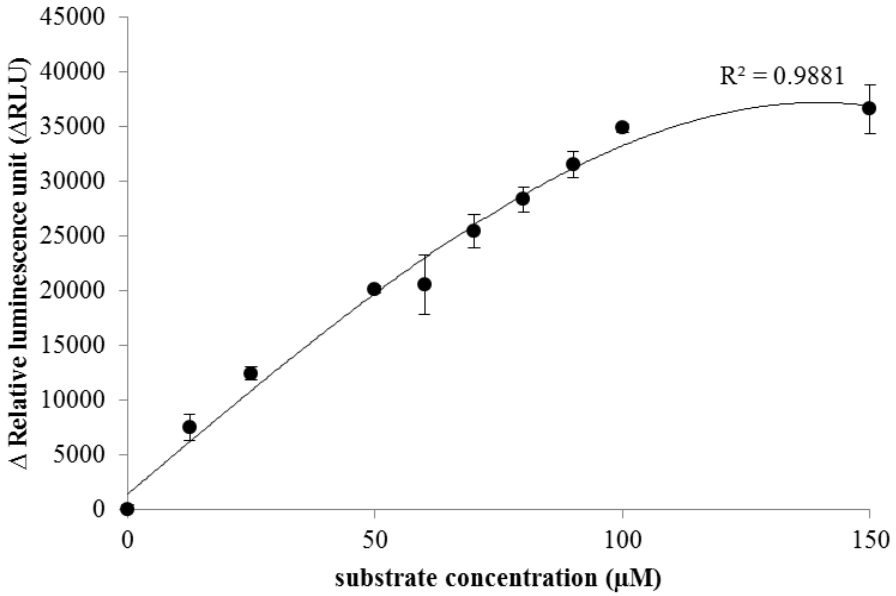


Figure 10 A curve of enzyme activity relationship exists between the relative luminescent signal measured and the peptide kinase substrate concentration in the reaction buffer 0-150 μM and reaction solution were incubate at room temperature for 180 minutes. The early stationary phase is the optimal kinase substrate concentration is 100 μM/ml. The correlation coefficient (R^2) is 0.9881 for this range. The luminescent signal was recorded 10 min after addition of an equal volume of Kinase-Glo® reagent. The data presented are means standard error of the mean of triplicate wells (n = 3).

Determining the Optimum Amount of Kinase

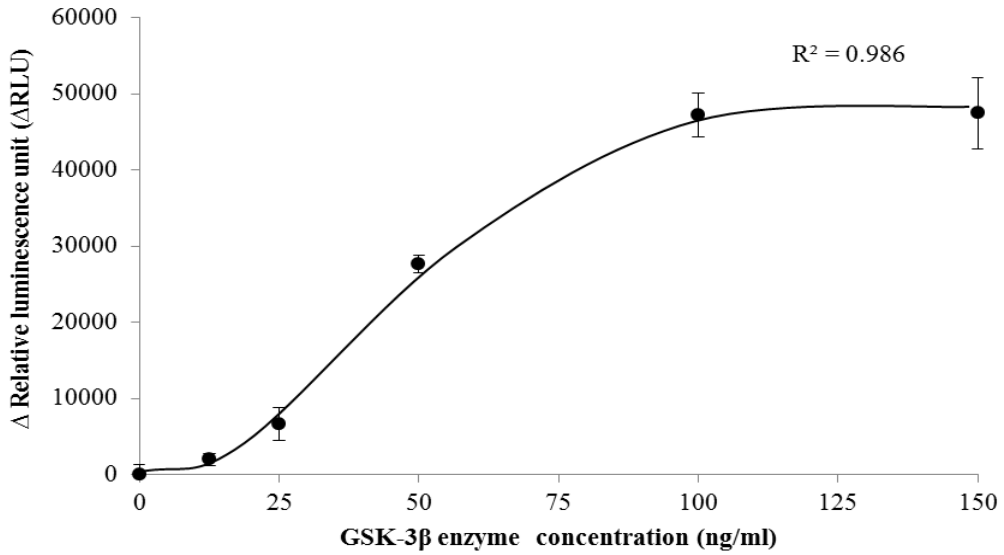


Figure 11 A curve of enzyme activity relationship exists between the relative luminescent signal measured and the human GSK-3β active enzyme concentration in the reaction buffer 0-150 µM and reaction solution were incubate at room temperature for 180 minutes. The early stationary phase is the optimal kinase enzyme kinase concentration is 100 µM/ml. The correlation coefficient (R^2) is 0.9860 for this range. The luminescent signal was recorded 10 min after addition of an equal volume of Kinase-Glo® reagent. The data presented are means standard error of the mean of triplicate wells (n = 3).

Determining the enzyme activity

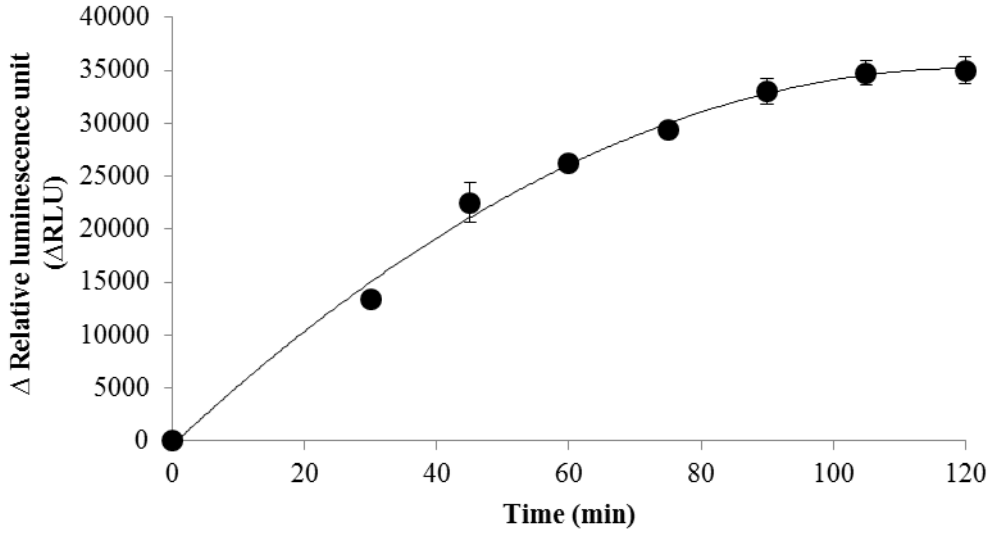


Figure 12 A curve of enzyme activity relationship exists between the relative luminescent signal measured and time in the reaction buffer were incubate at room temperature for 120 minutes. The luminescent signal was recorded 10 min after addition of an equal volume of Kinase-Glo® reagent. The data presented are means standard error of the mean of triplicate wells (n = 3).

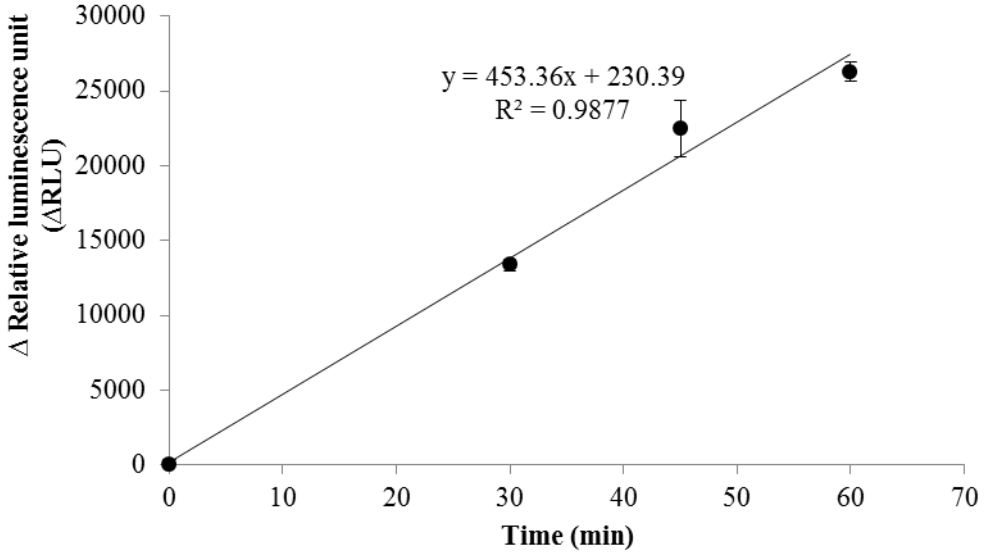


Figure 13 A linear of enzyme activity relationship exists between the relative luminescent signal measured and time in the reaction buffer were incubate at room temperature for 120 minutes. The luminescent signal was recorded 10 min after addition of an equal volume of

Kinase-Glo® reagent. The data presented are means standard error of the mean of triplicate wells (n = 3). The correlation coefficient (R^2) is 0.9860 for this range and the slope of exponential phase is the initial rate of enzyme kinase reaction ΔRLU/min = 453.36 and reversal of initial rate of enzyme kinase reaction to unit of enzyme = 0.01098μM ATP/min and specific activity of enzyme kinase = 7320.539 μM ATP/min/mg protein

Determining the Km and Vmax of enzyme kinase

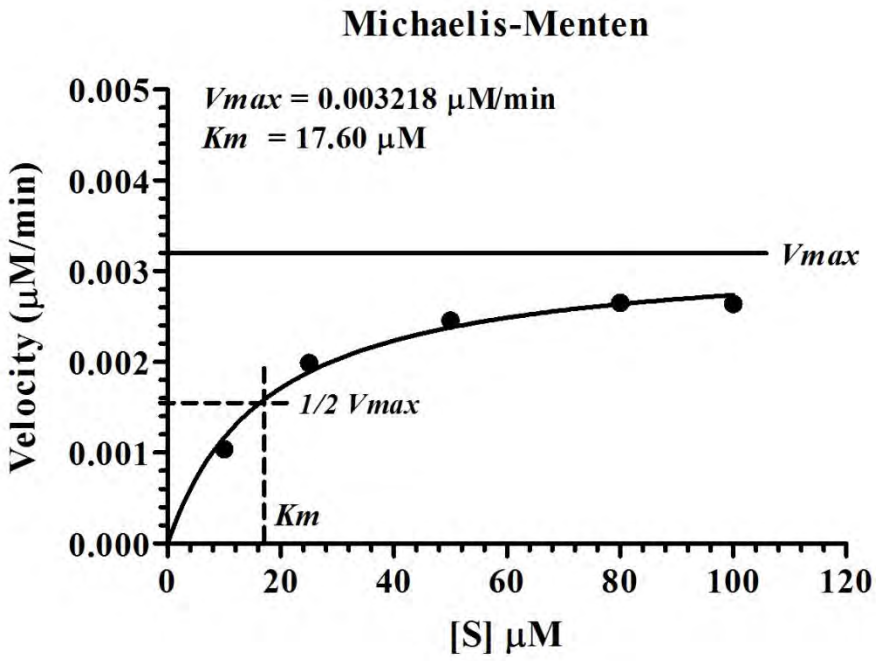


Figure 14 A curve of Michaelis-Menten relationship exists between the velocity of enzyme activity and peptide kinase substrate concentration [S] in the reaction buffer was incubate at room temperature for 120 minutes. The luminescent signal was recorded 10 min after addition of an equal volume of Kinase-Glo® reagent. The maximum reaction velocity (Vmax) = 0.003218 μM/min and the Michaelis constant (Km) = 17.60 μM.

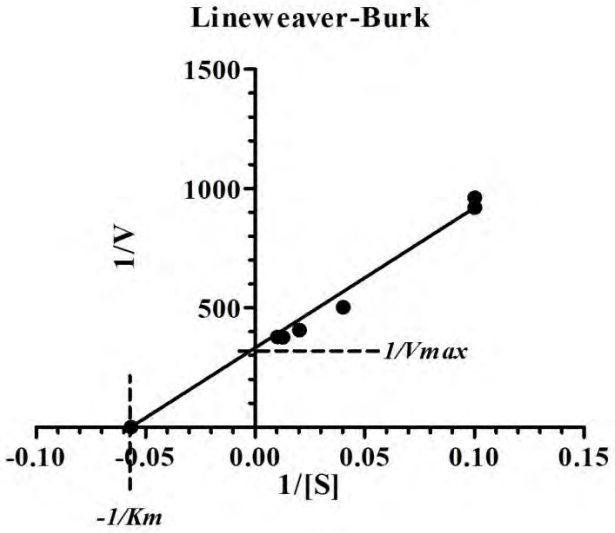


Figure 15 A linear of Lineweaver-Burk plotting relationship exists between the 1/velocity of enzyme activity and 1/peptide kinase substrate concentration [S] in the reaction buffer was incubate at room temperature for 120 minutes. The luminescent signal was recorded 10 min after addition of an equal volume of Kinase-Glo® reagent. The maximum reaction velocity (Vmax) = 0.003218 µM/min and the Michaelis constant (Km) = 17.60 µM.

Determining the percentage of inhibition

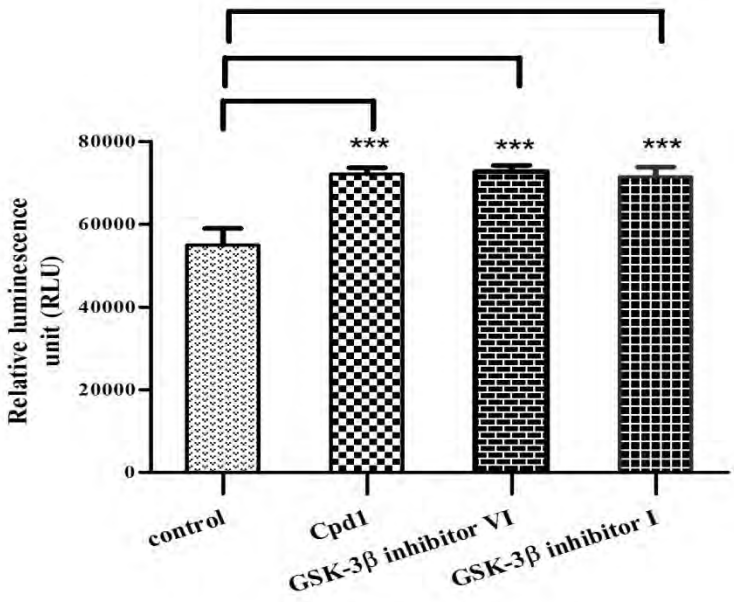


Figure 16 A column relationship exists between the luminescence signal of enzyme activity and GSK-3β inhibitors including 100µM Cpd1, 100µM GSK-3β inhibitor VI, 100µM GSK-3β inhibitor I and control treatment (DMSO) in the reaction buffer was incubate at room temperature for 120 minutes. The luminescent signal was recorded 10 min

after addition of an equal volume of Kinase-Glo® reagent. The %inhibition of all GSK-3β inhibitors were not significant went compare with control and %inhibition of Cpd1= 85.6274%, GSK-3β inhibitor VI = 88.5295% and GSK-3β inhibitor I = 82.6857%.

Determining IC_{50} Values of Kinase Inhibitors

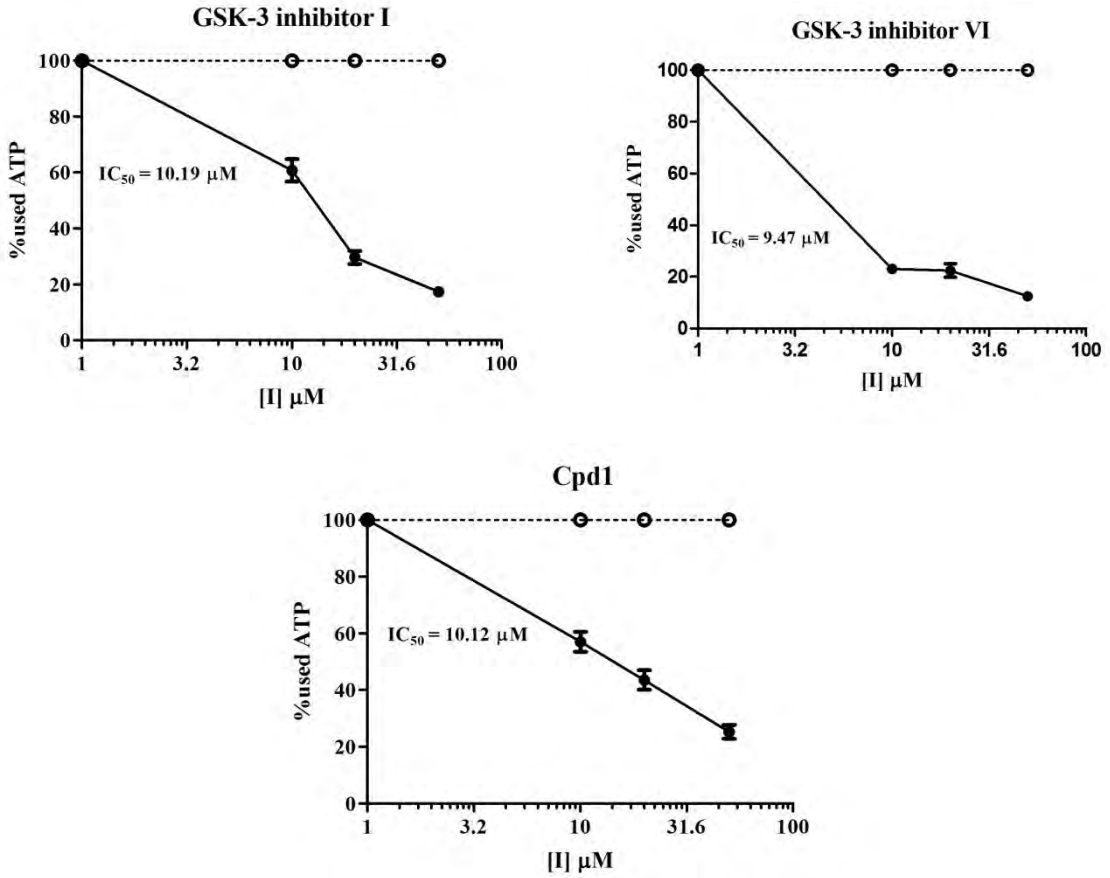


Figure 17 IC_{50} results determined using the Kinase-Glo® Plus Assay for GSK-3 inhibitors including Cpd1, GSK-3β inhibitor I and GSK-3β inhibitor VI varying concentration 0, 10, 20, 50 µM in the reaction buffer contain 100µM ATP. the IC_{50} values reported of Cpd1 = 10.12 µM, GSK-3β inhibitor I = 10.19 µM and GSK-3β inhibitor VI = 9.47 µM. Curve fitting was performed using GraphPad Prism® sigmoidal dose-response (normalized response) software.

Determining The inhibition pattern of Cpd1 on human GSK-3β

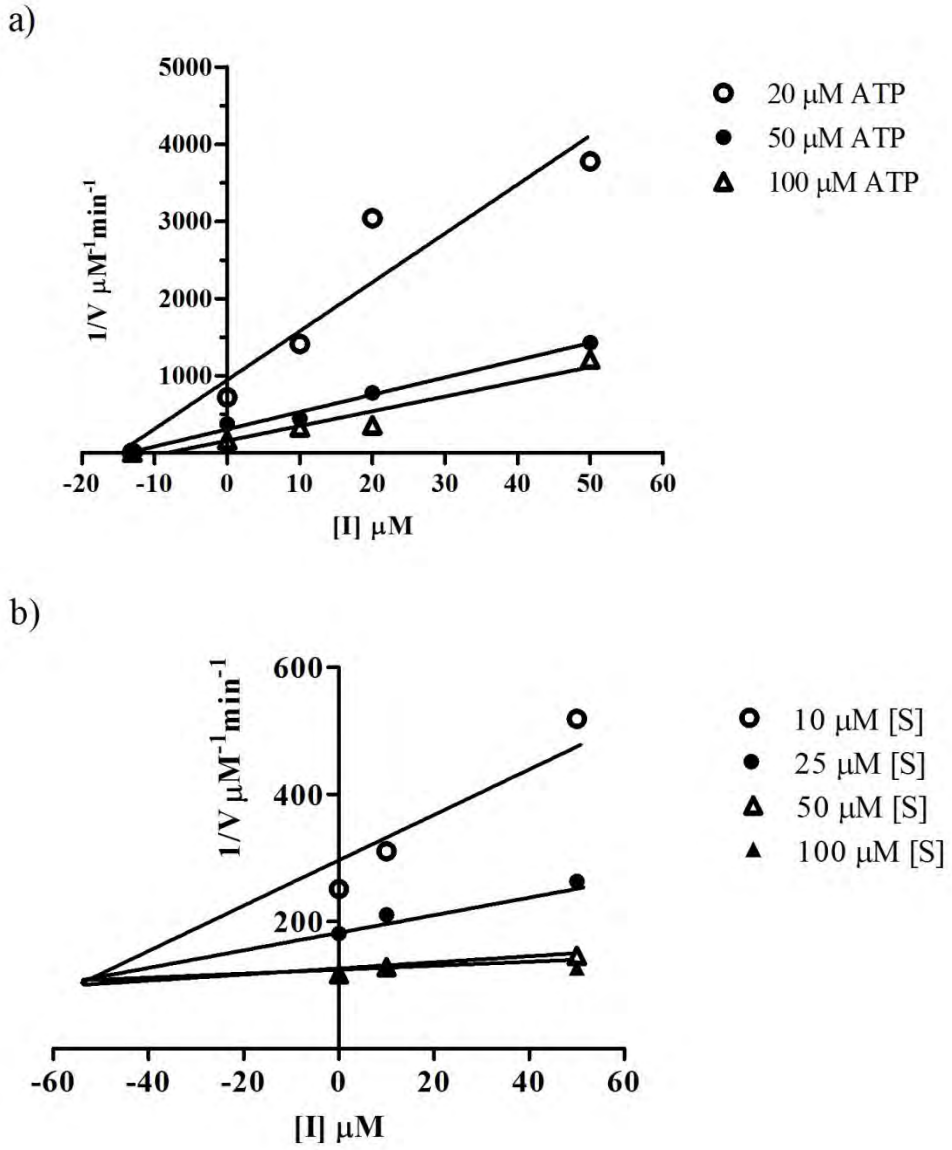


Figure 18 Dixon plot of Cpd1 against GSK-3β. a): Double-reciprocal plot of kinetic analyses was performed using three ATP concentrations (20 (○), 50 (●), 100 (△) μM) and four Cpd1 concentrations (0, 10, 20, 50 μM). The GSK-3β reaction was performed with a constant concentration of peptide kinase substrate (100μM). The result show Ki = 13.04 μM on pattern ATP-noncompetitive inhibition. b): Double-reciprocal plots of kinetic analyses were performed using four peptide kinase substrate concentrations (10 (○), 25 (●), 50 (△), 100(▲) μM) and three Cpd1 concentrations (0, 10, 50 μM) with a constant concentration of ATP (100 μM). The result show Ki = 53.92 μM on pattern substrate-competitive inhibition. The GSK-3β reaction was performed in the reaction buffer for 120 minutes. Curve fitting was performed using GraphPad Prism® (linear regression) software.

Conclusions

To identify the new molecular target of active compound 5-hydroxy-3,7-dimethoxyflavone called compound1 (Cpd1) from *Kaempferia parviflora* Wall. Ex. Baker. The results suggested that *Mck1* was a potential molecular target of Cpd1 in the Ca^{2+}-signaling pathways in yeast. In addition, Cpd 1 showed no cytotoxicity to the yeast tested strains. GSK-3β assays were performed using the Kinase Glo® Plus luminescent kinase assay platform with peptide kinase substrate (100 μM) was mixed with recombinant human GSK-3β (0.01098 unit). The efficiency of Cpd1 on enzyme GSK-3β activity of showed an ATP-noncompetitive binding mode to GSK-3β by Dixon plot (Ki =13.04 μM, Figure 16a) and showed a substrate competitive binding mode to GSK-3β with by Dixon plot (Ki = 53.92 μM, Figure 16b). These data showed that Cdp1 is a new ATP noncompetitive GSK-3β inhibitor and might be expected to be a useful functional compound as anti-type-2 diabetes, anti-Alzheimer's disease, and anti-inflammation agent.

Future perspectives

Effects of Cpd1 will be examined *in vivo* study on human cell line that involve in Type-2 diabetes and Alzheimer's disease.

Acknowledgement

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References

Boonkerd S, Yompakdee C, Miyakawa T, Chavasiri W: A flavonoid, 5-hydroxy-3,7-dimethoxyflavone, from Kaempferia parviflora Wall. Ex. Baker as an inhibitor of Ca2+ signal-mediated cell-cycle regulation in yeast. *Ann Microbiol* 2014, 64(3):1049-1054.

Boonkerd S, Yompakdee C, Miyakawa T, Chavasiri W: Screening of Thai medicinal plants for inhibitors of Ca2+ signaling using a yeast cell growth-based assay. *RJPBCS* 2011, 2(2):549-557.

Burke D, Dawson D, Strearns T: Method in yeast genetics. A cold spring harbor laboratory course manual. CSHL Press, New York. 2000.

Chomchalow N, Bansiddh IJ, MacBaine C: Amazing Thai Medicinal Plants, in Horticultural Research Institute (HRI), Department of Agriculture and Horticultural Science Society of Thailand (HSST), Bangkok. 2003.

Clapham DE: Calcium signaling. *Cell* 1995, 80:259-268.

Cohen P, Goedert M : GSK3 Inhibitors: Development and therapeutic potential. *Nature reviews* 2004, 3:479-487.

Cunningham KW, Fink GR: Calcineurin Inhibits VCX1-Dependent H1/Ca2+ Exchange andInduces Ca21 ATPases in Saccharomyces cerevisiae. *Mol Cell Biol* 1996, 16(5):2226-2237.

Embi N, Rylatt DB, Cohen P : Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. *European journal of biochemistry / FEBS* 1980, 107(2):519-27.

Espinal MA, Laszlo A, Simonsen L, Boulahbal F, kim SJ, Reniero A, Hoffner S, Rieder HL, Binkin N, Dye C : Global trends in resistance to antituberculosis drugs. World Health Organization-International Union against Tuberculosis and Lung Disease Working Group on Anti-Tuberculosis Drug Resistance Surveillance. *N Engl J Med* 2001, 344(17):1294-1303.

Foskett JK, White C, Cheung K-H, Mak D-OD: Inositol Trisphosphate Receptor Ca2+ Release Channels. *Physiol Rev* 2007, 87(2):593-658.

Gietz RD, Woods RA: Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol* 2002, 350:87–96.

Jope RS, Yuskaitis CJ, Beurel E : Glycogen synthase kinase-3 (GSK3): inflammation, diseases, and therapeutics. *Neurochemical research* 2007, 32(4-5):577-95.

Liberman Z, Eldar-Finkelman H : Serine 332 phosphorylation of insulin receptor substrate-1 by glycogen synthase kinase-3 attenuates insulin signaling. *The Journal of biological chemistry* 2005, 280(6):4422-8.

Lochhead PA, Coghlan M, Rice SQ, Sutherland C : Inhibition of GSK-3 selectively reduces glucose-6-phosphatase and phosphatase and phosphoenolypyruvate carboxykinase gene expression. *Diabetes* 2001, 50(5):937-46.

Mager WH, Winderickx J :Yeast as a model for medical and medicinal research, Trends *Pharmacol Sci* 2005, 26(5): 265-273.

Miller JH: Experiments in molecular genetics. A cold spring harbor laboratory course manual. CSHL Press, New York. 1972.

Mizunuma M, Hirata D, Miyahara K, Tsuchiya E & Miyakawa T: Role of calcineurin and Mpk1 in regulating the onset of mitosis in budding yeast. *Nature* 1998, 392: 303-306.

Mizunuma M, Hirata D, Miyaoka R, Miyakawa T: GSK-3 kinase Mck1 and calcineurin coordinately mediate Hsl1 down-regulation by Ca2+ in budding yeast *EMBO J* 2001, 22(5):1074-1085.

Pisani P, Parkin DM, Bray F, Ferlay J : Estimate of the worldwide mortality from 25 cancers in 1990. *Int. J. Cancer* 1999, 83:870-873.

Putiyanan S, Chansakaow S, Phrutivorapongkul A, Charoensup W: Standard Pharmacognostic Characteristic of Thai Herbal Medicine, Research Book Faculty of Pharmacy, Chiang Mai University, Chiang Mai ISBN: 974-658-299-2. *CMU Journal*, 2004.

Rayasam GV, Tulasi VK, Sodhi R, Davis JA, Ray A : Glycogen synthase kinase 3: more than a namesake. *British journal of pharmacology* 2009, 156(6):885-98.

Rothstein RJ: One-step gene disruption in yeast. *Methods Enzymol* 1983, 101:202-211.

Shitamukai A, Mizunuma M, Hirata D, Takahashi H, Miyakawa T: A positive screening for drugs that specifically inhibit the Ca2+-signaling activity on the basis of the growth promoting effect on a yeast mutant with a peculiar phenotype. *Biosci. Biotechnol. Biochem* 2000, 64: 1942-1946.

Sudwan P, Saenphet K, Saenphet S, Suwansirikul S: Effect of Kaempferia parviflora Wall. ex. Baker on sexual activity of male rats and its toxicity. *Se Asian J Trop Med* 2006,(37):210-215.

Tutulan-Cunita AC, Ohnishi T, Mizunuma M, Hirata D, Miyakawa T: Involvement of Saccharomyces cerevisiae Pdr5p ATP-binding cassette transporter in calcium homeostasis. *Biosci Biotechnol Biochem* 2005, 69(4):857-860.

Wangkangwan W. Positive screening and preliminary characterization of calcium signal inhibitor from plant extracts using *Saccharomyces cerevisiae* mutant. Master degree Thesis. Chulalongkorn University 2007.

Wattanapitayakul SK, Suwatronnakorn M, Chularojmontri L, Herunsalee A, Niumsakul S, Charuchongkolwongse S, Chansuvanich N: *Kaempferia parviflora* ethanolic extract promoted nitric oxide production in human umbilical vein endothelial cells. *J. Ethnopharmacol* 2007:559-562.

Woodgett JR :. Molecular cloning and expression of glycogen synthase kinase-3/factor A. *The EMBO journal* 1990, 9(8):2431-8.

Woodgett JR : Regulation and functions of the glycogen synthase kinase-3 subfamily. *Seminars in cancer biology* 1994, 5(4):269-75.

Output

1. Nuttanich Suanmali, Warinthorn Chavasiri, Tokichi Miyakawa and Chulee Yompakdee. An active compound from *Kempferia parviflora* with inhibitory activity against GSK-3 kinase implicated in Type II Diabetes and Alzheimer's disease. Manuscript in preparation.

2. Nuttanich Suanmali, Warinthorn Chavasiri, Tokichi Miyakawa and Chulee Yompakdee (2016). Yeast GSK-3 kinase like Mck 1, is a likely molecular target of a flavonoid from *Kempferia parviflora* involved in Ca^{2+}-signaling in yeast *Saccharomyces cerevisiae*. To be presented at The 28th Annual Meeting of the Thai Society for Biotechnology and International Conference (TSB 2016) "Natural Resources and Bio-based Innovative Products" November 28-30, 2016. The Empress Hotel, Chiang Mai, Thailand.