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# A high-throughput screen to identify novel calcineurin inhibitors

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ABSTRACT

Calcineurin is a eukaryotic protein phosphatase important for many signalling and developmental processes in cells. Inhibitors of this enzyme are used clinically and there is interest in identifying novel inhibitors for therapeutic applications. This report describes a high-throughput assay that can be used to screen natural or chemical libraries of compounds to identify new calcineurin inhibitors. The microtitre plate assay is based on a yeast reporter strain and was validated with known inhibitors and tested in a pilot screen of bacterial extracts.



## 1. Introduction

Calcineurin is a highly conserved, eukaryotic calmodulin-dependent serine/threonine phosphatase type 3CA (formerly PP2B). In mammalian cells, it participates in diverse cellular processes such as signal transduction, cell-cycle regulation, stress response and apoptosis (Rusnak and Mertz, 2000), and has recently been implicated in several important neurodegenerative disorders, including schizophrenia (Kvajo et al., 2010); (Tabarés-Seisdedos and Rubenstein, 2009). In the immune system, calcineurin is centrally involved in maturation of T cells and, for this reason, most immunosuppressive therapies involve the use of calcineurin inhibitors (CNI) (Castroagudín et al., 2011). The most widely used CNIs are cyclosporin A and tacrolimus (FK506), both of which are natural microbial metabolites that bind to intracellular receptors known as immunophilins, which then block calcineurin function. The mode of action of and history of these CNIs has been extensively studied and very well-documented (Liu et al., 1991); (Pritchard, 2005). Interestingly, the main details of the mechanism of action of both cylcosporin A and FK506 were first determined in the budding yeast, *S. cerevisiae*, something made possible by the strong conservation of calcium signalling pathways and calcineurin across the Eukarya (Fox and Heitman, 2002). Although CNI are extensively used for immunosuppressive therapy, most notably in organ transplantation, there are cardiovascular, renal and other side-effects that can occur with long-term use (Rezzani, 2006); (Fung et al., 1991) and thus there is interest in identifying novel calcineurin inhibitors. Furthermore, such molecules may also have potential as novel anti-fungal agents (Steinbach et al., 2007) and applications even as cell biology reagents to study calcium signalling and homeostasis are possible.

The most prolific reservoir of bioactive molecules has always been the natural world and the majority of molecules used in medicine are derived from microbes or plants. Many antibiotics, as well as important drugs such as CsA, tacrolimus (FK506) and sirolimus (rapamycin), are of microbial origin and in the current era of genome biology there is renewed interest in bioprospecting to identify new microbial metabolites with therapeutic applications. The challenge of developing screens to identify specific inhibitory activities remains, however, and modern requirements stipulate that any methods are specific, rapid, high-throughput and automatable in so far as is possible. Current methods to screen for calcineurin inhibitors are limited and do not satisfy these requirements (Fruman et al., 1992); (Enz et al., 1994); (Sellar et al., 2006). The aim of this study was to develop a new screen for calcineurin inhibitors that would be compatible with robotic handling systems for screening large numbers of microbial extracts, metagenomic libraries or combinatorial chemistry libraries.

The screen was developed using yeast reporter strains. In yeast, as in mammalian cells, various extracellular signals lead to a transient increase in calcium ions in the cytoplasm. The cytoplasmic protein calmodulin binds calcium and then activates calcineurin. The targets of calcineurin differ among organisms, with the transcription factor Crz1p, the main target in yeast. Following dephosphorylation by calcineurin, Crz1p translocates to the nucleus where it activates transcription of target genes by binding to a promoter sequence known as the CDRE element (Kraus and Heitman, 2003); (Cyert, 2003). The conservation of calcineurin in Eukarya facilitated the development of this yeast screen.

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## 2. Material and methods

### 2.1. Strains and growth conditions

*Saccharomyces cerevisiae* BY4741 was used as the standard strain for all assays. Typically, yeast strains were grown in YNB medium at 30 °C with appropriate auxotrophic selection to maintain plasmids (Sherman, 1991). Routine lithium acetate transformation (Gietz et al., 1992) was used to introduce the reporter plasmids pMRK212, which carries a *CDRE::lacA* fusion (Serrano et al., 2002), and pRSP97, which carries a *GFP::CRZ1* fusion (Polizotto and Cyert, 2001).

### 2.2. Development of the assay

To prepare yeast cells for the assay, the strain carrying the *CDRE::lacA* fusion was grown overnight and inoculated into fresh medium at an A600 of 0.1. The fresh culture was allowed to undergo 2–3 doublings (approximately 5 h) before cells were harvested by centrifugation and resuspended at an A600 of 0.4. 25 µl of this cell suspension followed by 25 µl of the test reagent (or control solution) was added to each well of a 96-well polystyrene microtitre flat bottomed plate and the plate incubated at 30 °C for 30 min. Next, alkaline stress is provided by adding 25 µl of TAPS (N-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid) pH 8.1 (100 mM stock, final concentration, 33.3 mM) and incubating at 30 °C for 1 h. A β-galactosidase activity assay was then performed as follows. 75 µl of Z-buffer of pH 7.0 (60 mM disodium hydrogen phosphate, 40 mM Sodium dihydrogen phosphate, 10 mM KCl, 1 mM Magnesium sulphate heptahydrate) was added. Cell lysis was by addition 75 µl of lysis buffer (1% N-lauroylsarcosine and 0.5% ß-mercaptoethanol in Z-buffer) and incubation for 30 min at 30 °C. The use of N-Lauroyl sarcosine for cell lysis was to avoid the effect of chloroform on the plastic microtitre plates (Kippert, 1995). Lysis was followed by the addition of 25 µl ONPG solution to each well and incubation at 30 °C until a yellow colour developed in the positive control (typically 20 min). The time was recorded and the reaction stopped by addition of 60 µl of 1 M $Na_2CO_3$. The A405 was then measured in a plate reader (Thermo Multiskan FC) and units calculated. Arbitrary units were defined as follows: Activity = $[A_{405} \times 1000]/[\text{Time (min)} \times \text{Volume of the cells (ml)}]$. The A600 of the cells is not included in the formula as experience showed that although the assay is highly reproducible with respect to the relative values of positive and negative controls, absolute values can vary and all data must be compared to these controls within an experiment. For comparison, all values were then normalised with the negative control (typically ~150 units) given a value of 1.

### 2.3. Testing and validation of the assay

All assays include a well to which no TAPS is added and this serves as the base-line from which an increase in expression following alkaline stress is observed. All other wells have TAPS added as described above and so should see an increase in expression of the *CDRE::lacZ* reporter. One well simply has 25 µl control solution (medium or organic solvent as appropriate to the test) and this is the positive control for the assay, whereas all the test wells have solutions or metabolites pre-added in advance of the TAPS (above). To validate the system, the effects of three known calcineurin inhibitors: FK-506 (Tacrolimus), Cyclosporin A and Ascomycin as well as three other inhibitors/anti-fungal metabolites: Okadaic acid, Rapamycin (Sirolimus) and fluconazole, were tested. Each of these reagents prepared in appropriate stocks and added to a final concentration as follows: FK-506 (1.5 µg/ml) Cyclosporin A (25 µM), Ascomcyin (0.004 µM), Rapamycin (0.78 µM) and Okadaic acid (1.56 µM), Fluconazole (0.125 mM). With the exception of fluconazole (Sigma), all other test reagents were purchased from LC Laboratories, USA.

Following validation, a pilot screen was performed with 81 bacterial extracts. The bacteria used were largely marine-sponge associated bacteria cultured as part of a larger marine biodiscovery project (Kennedy et al., 2009). The bacteria were cultured for 7–14 days in SYP broth (10 g starch/l, 4 g yeast extract/l, 2 g peptone/l, 33.3 g Instant Ocean/l);to obtain supernatant. These extracts were prepared from bacterial culture supernatant by treating cell-free supernatant with amberlite XAD-16 resin for 2–4 h, which was then washed with water and eluted with methanol. This concentrated methanol extract was diluted 1 in 10 with YNB media for the assay. All experiments are carried out in triplicate.

### 2.4. Fluorescence microscopy

Epifluorescent microscopy was used to visualize the GFP-Crz1p fusion protein in yeast cells with or without alkaline stress and with or without inhibition of calcineurin. Strain BY4741 carrying the pRSP97 reporter plasmid was grown to mid-log phase (A600 ~0.8) in YNB media at 30 °C. Cultures were split and exposed to alkaline stress by treating with 30 mM KOH (or control treatment). Individual cultures were also contemporaneously treated with FK506 or putative calcineurin inhibitors to observe the effect on Crz1p localization. Following addition of KOH and the putative drug incubation followed for 15–30 min at 30 °C in a dark room before cells were visualised by epifluorescent microscopy. For visualization, 25 µl of each sample is taken and visualized with DIC and GFP using an I3 filter (emission of 450–490 nm and excitation of 510 nm). The image is then visualized and captured by using the Leica Florescence Microscope.

## 3. Results and discussion

### 3.1. Development of a high-throughput assay of calcineurin activity

The basis of the high-throughput assay to identify calcineurin inhibitors developed in this study is that calcineurin activity can be detected by the activity of a *CDRE::lacZ* gene fusion, which is carried on the pMRK212 plasmid. A cytoplasmic calcium spike is triggered by alkaline stress (addition of TAPS buffer at pH 8.1) and this in turn activates calmodulin, calcineurin and Crz1p, which then binds to the CDRE element on the reporter construct activating expression. Expression of the *CDRE::lacZ* gene fusion can be detected using a modified assay for the β-galactosidase enzyme. A major criterion for the assay was that it could be performed in a 96 well microtitre plate, thereby being amenable to HT robotic methods if desired. Although all individual aspects of the system are routine in yeast, integration into a single assay required various modification and optimizations mentioned in the methods. This culminated in the high-throughput assay illustrated in Fig. 1. The specificity of the assay was validated by assessing the effects of known calcineurin inhibitors as well as inhibitors of other classes of phosphatase and anti-fungal compounds (Fig. 2). The induction of *CDRE::lacZ* expression by alkali stimulation is seen by the increase from lane 1 to lane 2. The inclusion of the calcineurin inhibitors FK506 (lane 3), ascomycin (lane 4) or cyclosporin A (lane 5) prevents the induction, thereby demonstrating how calcineurin inhibitors can be identified. It is important for the assay that the effect is specific to calcineurin inhibition, therefore Rapamycin, an immunosuppressive drug with a distinct mode of action from CNIs (lane 6), and Okadaic acid, which inhibits the class of PP2A phosphatases (lane 7) were also tested. Neither of these potent molecules prevented induction of *CDRE::lazZ* expression. In addition, it was confirmed that the assay is independent of inhibitory effects on yeast growth; for example, inhibitory effects of the anti-fungal drug fluconazole has no effect on the assay (data not shown).

### 3.2. Application of the calcineurin inhibition assay in a pilot screen

A pilot screen was carried out to validate the assay and to demonstrate its applicability for high-throughput approaches. A library of