Development of a high throughput yeast-based screening assay for human carbonic anhydrase isozyme II inhibitors

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Abstract
Carbonic anhydrase (CA; EC 4.2.1.1) catalyzes the reversible hydration of carbon dioxide (CO₂) to bicarbonate and proton. There are 16 known isozymes of α-CA in humans, which differ widely in their kinetics, subcellular localization and tissue-specific distribution. Several disorders are associated with abnormal levels of CA, and so the inhibition of CA has pharmacological application in the treatment of many diseases. Currently, searching for novel CA inhibitors (CAI) has been performed using in vitro methods, and so their toxicity remains unknown at the time of screening. To obtain potentially safer CAIs, a screening procedure using an in vivo assay seems to have more advantages. Here, we developed a yeast-based in vivo assay for the detection of inhibitors of the human CA isozyme II (hCAII). The yeast Saccharomyces cerevisiae mutant deprived of its own CA (Δnce103 strain) can grow under a high CO₂ condition (5% (v/v) CO₂) but not at an ambient level. We constructed Δnce103 strains expressing various levels of hCAII from a plasmid harboring the hCAII gene arranged under the control of variously modified GAL1 promoter and relying on the expression of hCAII for growth under low CO₂ condition. Using a multidrug-sensitive derivative of the Δnce103 strain expressing a low level of hCAII, we finally established a high throughput in vivo assay for hCAII inhibitors under a low CO₂ condition. Cytotoxicity of the candidates obtained could be simultaneously determined under a high CO₂ condition. However, their inhibitory activities against other CA isozymes remains to be established by further investigation.

Keywords: Human carbonic anhydrase isozyme II, Saccharomyces cerevisiae, Yeast-based assay, Resazurin, NCE103, Carbonic anhydrase inhibitor

Introduction
Carbonic anhydrases (CAs, EC 4.2.1.1) belong to the metalloenzymes family of proteins. They are a class of enzymes catalyzing the simple but physiologically essential process of carbon dioxide (CO₂) hydration to bicarbonate and proton (CO₂ + H₂O ↔ HCO₃⁻ + H⁺). Overall, CAs play important roles in pH regulation, fluid balance, carbonic acid reactions, calcification, bone resorption, tumorigenicity and in other pathological and physiological processes, such as gluconeogenesis, ureogenesis and lipogenesis (Supuran and Scozzafava 2000). Six different genetically distinct CA families have been described to date; namely the α- (present in vertebrates, bacteria, algae and cytoplasm of green plants), β- (predominantly in bacteria and algae), γ- (mainly in archaea and some bacteria), δ- and ζ- (present in marine diatoms) and η-CA (present in parasite) (Del Prete et al. 2014; Lindskog 1997; Supuran 2008). Sixteen α-CA isozymes have been found in humans, which differ widely in their kinetics, subcellular localization, tissue-specific distribution and susceptibility to different inhibitors. However, they all play important physiological roles, as briefly outlined above (Supuran and Scozzafava 2000). Many human CA isozymes are established therapeutic targets for the treatment of a wide range of disorders (Sly and Hu 1995; Supuran 2008; Supuran and Scozzafava 2000). Indeed, antiobesity, antiepileptic, anticancer and antiglaucoma drugs based on CA inhibitors (CAIs) are presently used,
and they target various human CA isoforms (Carta and Supuran 2013; Masini et al. 2013; Monti et al. 2013; Scozzafava et al. 2013; Supuran 2008).

Sulfonamide compounds, which are classical CAI, have been used as commercial drugs for the treatment of glaucoma, epilepsy, edema and altitude sickness (Supuran 2008). However, they can inhibit all CA isoforms non-specifically, diluting the drug effectiveness and causing undesired side effects from the off-target inhibition. Furthermore, a small but significant percentage of patients cannot be treated with sulfonamide-based compounds owing to their sulfa allergy (Lomelino et al. 2016). Thus, safer CAIs are required. In previous studies, CAIs were screened for by in vitro methods using a biochemical strategy, but this approach has several disadvantages. For example, it provides no information about drug uptake into cells, drug stability and, in particular, the cytotoxicity of the compounds (Bilsland et al. 2013). Such disadvantages could be improved by using an in vivo assay.

The yeast Saccharomyces cerevisiae has emerged in the last few decades as a powerful organism for the study of many human enzymes. The deep genetic information available on this organism has allowed it to become an increasingly popular model for pharmacological and/or drug discovery studies (Daniel et al. 2005). The Δnce103 null mutation leads to the loss of endogenous CA activity and inhibits the growth of the yeast cells under an ambient CO2 concentration (Clark et al. 2004) due to the low level of available bicarbonate ions in the absence of CA activity.

Human carbonic anhydrase isozyme II (hCAII) is the most efficient isozyme in CO2 hydration and is a highly abundant CA isozyme in cells (Supuran and Scozzafava 2000). Furthermore, hCAII is the only isozyme so far reported in which its overexpression can complement the growth defect of the yeast Δnce103 null mutant strain (Clark et al. 2004). Thus, in this study, we aimed to develop a novel yeast-based assay in a 96-well format for the high throughput screening of CAIs against hCAII.

Here, a highly sensitive Δnce103 null yeast strain expressing hCAII was constructed and used for screens in a resazurin microtiter plate assay (REMA). The developed yeast-based assay enables high-throughput, live-cell, target-based screening to identify compounds that could inhibit hCAII activity. Hence, it could be a potential tool for accelerating the discovery of non-sulfonamide-based CAIs to be used for the treatment CA-related diseases, such as glaucoma.

Materials and methods

Microbial strains and cultivation media

Escherichia coli DH5α [F-endA1 hsdR17 (r− K/m − K) supE44 thi-1 λ-recA1 gyrA96 ΔlacU169 (φ80lacZΔM15)]

Table 1 Yeast strains used in this study

| Yeast strain | Genotype | Reference |
|--------------|----------|-----------|
| W303-1A      | MATa ade2-1 his3-11 leu2-3,112 trp1-1 can1-100 | Yeast Genetic Resources Center, Japan |
| AS01         | MATa ade2-1 his3-11 leu2-3,112 trp1-1 can1-100 nce103::loxP | This study |
| AS02         | MATa ade2-1 his3-11 leu2-3,112 trp1-1 can1-100 nce103::loxP erg3::loxP | This study |
| BY25929      | MATa ade2-1 his3-11 leu2-3,112 trp1-1 can1-100 yrr1::TRP1 pdr1::hisG pdr3::hisG | Yeast Genetic Resources Center, Japan |
| BY25929.1    | MATa ade2-1 his3-11 leu2-3,112 trp1-1 can1-100 yrr1::TRP1 pdr1::hisG pdr3::hisG erg3::loxP-URA3-loxP | This study |
| BY25929.2    | MATa ade2-1 his3-11 leu2-3,112 trp1-1 can1-100 yrr1::TRP1 pdr1::hisG pdr3::hisG erg3::loxP | This study |
| BY25929.3    | MATa ade2-1 his3-11 leu2-3,112 trp1-1 can1-100 yrr1::TRP1 pdr1::hisG pdr3::hisG erg3::loxP | This study |
| BY25929.4    | MATa ade2-1 his3-11 leu2-3,112 trp1-1 can1-100 yrr1::TRP1 pdr1::hisG pdr3::hisG erg3::loxP | This study |
| AS03         | MATa ade2-1 his3-11 leu2-3,112 trp1-1 can1-100 yrr1::TRP1 pdr1::hisG pdr3::hisG erg3::loxP nce103::loxP-URA3-loxP | This study |

(Thermo Fisher Scientific, USA) was used in the construction and transformation of recombinant plasmids. All clonings were performed using E. coli DH5α grown at 37 °C in Luria–Bertani medium (Titan Biotech LTD., India) containing 0.1 g L−1 ampicillin (T.P. Drug Laboratories, Thailand) (Sambrook et al. 1989). The S. cerevisiae strains used in this study are listed in Table 1. Yeast transformants were grown at 30 °C in synthetic dextrose (SD) medium or synthetic raffinose (SR) medium [6.7 g L−1 yeast nitrogen base (YNB) without amino acids (Difco Laboratories, USA) and 20 g L−2 of either glucose or raffinose (Difco Laboratories, USA), respectively, containing only essential amino acids (Sigma Aldrich, USA)]. Uracl auxotrophic yeast strains were selected by 5-fluoroorotic acid (5-FOA) medium containing 6.7 g L−1 YNB with ammonium sulphate and without amino acids, 20 g L−1 glucose, 1 g L−1 5-FOA (Zymo Research, USA) and essential amino acids as well as uracil. For the functional complementation, Western blot analysis and two step quantitative reverse transcription-polymerase chain
reaction (qRT-PCR) experiments, the yeast transformants were induced for hCAII expression by cultivation in synthetic galactose (SG) medium (6.7 g L⁻¹ YNB without amino acids, 10 g L⁻¹ raffinose, 20 g L⁻¹ galactose; Difco, USA) containing 0.2 g L⁻¹ adenine, 1 g L⁻¹ leucine and 0.1 g L⁻¹ histidine (SG + Ade + Leu + His). The Δnce103 strain was grown under the high-CO₂ condition (5% (v/v) CO₂) using an AnaeroPack (Mitsubishi Gas Chemical, Japan).

Chemicals
Acetazolamide (AZA) and FK506 were purchased from Sigma Aldrich, USA. Avicennin was a gift from Dr. Warinthorn Chavasiri, Department of Chemistry, Faculty of Science, Chulalongkorn University, Thailand.

Plasmid construction
All plasmids used in this study are shown in Table 2. The hCAII cDNA was obtained from Krungkrai et al. (2005). A full-length GAL1 promoter (nucleotide positions 1–451) fused with the Flag epitope tag at the C-terminal of hCAII (c-Flag hCAII) was obtained by PCR amplification from the pAG414GAL (c-Flag hCAII) vector (Panthan 2011) using the oligonucleotide primers pGAL1.4_hCAII Fw and NotI_hCAII Rv (Table 3) with the reverse primer (one of pGAL1.3_hCAII Fw, pGAL1.2_hCAII Fw or pGAL1.1_hCAII Fw) with the reverse primer NotI_hCAII Rv (Table 3) to amplify a GAL1 promoter with either three, two or one Gal4 binding site domains, respectively. All PCR reactions were performed as described above. The resulting plasmids with three, two and one Gal4 binding sites were designated as pGAL1.3_hCAII, pGAL1.2_hCAII and pGAL1.1_hCAII, respectively.

Table 2 Plasmids used in this study

| Plasmid Description | Reference |
|---------------------|-----------|
| pAG414GAL (c-Flag hCAII) | Panthan (2011) |
| pRS414 (AmpR, URA3, CEN) | Addgene, USA |
| pGAL1.1_hCAII (AmpR, URA3, CEN, P_GAL1.1_hCAII) | This study |
| pGAL1.2_hCAII (AmpR, URA3, CEN, P_GAL1.2_hCAII) | This study |
| pGAL1.3_hCAII (AmpR, URA3, CEN, P_GAL1.3_hCAII) | This study |
| pGAL1.4_hCAII (AmpR, URA3, CEN, P_GAL1.4_hCAII) | This study |
| pUG72 (AmpR, loxP-URA3-loxP) | Euroscarf, Germany |

To modulate the transcription level of hCAII under the control of the GAL1 promoter, modification of the number of Gal4 binding sites (Cottier et al. 2006; Giniger et al. 1985; Hong et al. 2008; Liang et al. 1996; Marmorstein et al. 1992) was performed by PCR amplification from the pAG414GAL (c-Flag hCAII) plasmid using the desired forward primer (one of pGAL1.3_hCAII Fw, pGAL1.2_hCAII Fw or pGAL1.1_hCAII Fw) with the reverse primer NotI_hCAII Rv (Table 3) to amplify a GAL1 promoter with either three, two or one Gal4 binding site domains, respectively. All PCR reactions were performed as described above. The resulting plasmids with three, two and one Gal4 binding sites were designated as pGAL1.3_hCAII, pGAL1.2_hCAII and pGAL1.1_hCAII, respectively.

Gene disruption
A PCR-based gene disruption method (Gueldener et al. 2002) was employed in the yeast strain W303-1A and BY25929 background (Table 1). A disruption cassette, containing the loxP-URA3-loxP sequence of pUG72 (EUROSCARF, Germany) flanked at both sides with a short homology sequence of 40 bp at the 5’ and 3’ termini of the target gene, was amplified by PCR using oligonucleotide primers (Table 3) as follows. The disruption cassette loxP-URA3-loxP for the ERG3 gene (Accession No. M64989.1) was amplified using oligonucleotide primers D-ERG3_Fw and D-ERG3_Rv, whereas the other disruption cassettes were amplified using the primer pairs D-TRP1_Fw and D-TRP1_Rv for TRP1 (Accession No. NM_001180315.3) and D-NCE103_Fw and D-NCE103_Rv for NCE103 (Accession No. NM_001182875.3).

The respective amplified disruption cassette was transformed into the yeast cells and successful disruptants were confirmed by PCR using the gene-specific oligonucleotide primers (Table 3).

Yeast transformation
Yeast cells were transformed by the lithium acetate method with 1 µg plasmid or 5 µg DNA fragment, respectively (Gietz et al. 1995). In addition, 50 µg of carrier DNA, deoxyribonucleic acid sodium salt from salmon testes (Sigma Aldrich, USA), was added to enhance the transformation efficiency (Gietz et al. 1995).

Yeast complementation experiment
Complementation of the Δnce103 yeast strain was performed as follows. The exponential growth phase of the yeast transformants in 3 mL SR + Ade + Leu + His medium was diluted to a final 10⁶–10⁷ cells mL⁻¹ and 5 µL of each serially diluted culture were spotted onto the
surface of SG + Ade + Leu + His agar medium. The plates were incubated at 30 °C for 3–4 days under either the high- or the low-CO₂ condition.

Two step quantitative reverse transcription-polymerase chain reaction (qRT-PCR)
The recombinant strains were cultured in SG + Ade + Leu + His medium and then total RNA was extracted using the Masterpure yeast RNA purification kit (Epicentre, USA), as recommended by the manufacturer. For the first stage RT-PCR, aliquots of total RNA (1 µg) were converted into complementary DNA (cDNA) using RevertAid Reverse Transcriptase (Thermo Fisher Scientific, USA) and the respective gene-specific primers (Table 3). All second stage qRT-PCR were run using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, USA). The plates containing the qRT-PCR mix were transferred to the CFX Connect Real-Time PCR Detection System (Bio-Rad, USA) and thermal cycled at 95 °C for 3 min followed by 35 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s. Relative gene expression levels were calculated using the 2^(-ΔΔCT) method (Livak and Schmittgen 2001).

Western blotting analysis
Yeast cells cultured in the inducible (SG + Ade + Leu + His) medium were lysed by vortexing with acid-washed glass beads of 0.45–0.55 diameter (Sigma Aldrich, USA) in sodium dodecyl sulfate (SDS)-sample buffer and subjected to 12% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis using the rabbit anti-Flag (DYKDDDK tag) primary antibody (Cell Signaling, USA) and horseradish peroxidase conjugated donkey anti-rabbit IgG as the secondary antibody (Cell Signaling, USA). Protein signals were detected by Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, USA).

Resazurin microtiter plate assay
The REMA was performed in 96-well plates as follows. Briefly, 1 µL of test compound was dissolved in 80 µL of SR + Ade + Leu + His medium to the

| Table 3  Oligonucleotide primers used in this study |
|-----------------------------------------------|
| Primer name | Primer sequence (5′–3′) |
| A. Primers used in the PCR amplified disruption cassettes for gene disruption |
| D-ERG3_Fw | ATTTCTACTTTCTATGATTCGTTTCCTTGGCAGTGAAGC |
| D-ERG3_Rv | TCTTGACGACGTAAAGGAAAGAAAAAGATGGAACAAACAAGATAGGCCCAGTA |
| D-NCE103_Fw | TACAAATTTCAATTATACACATCAGACGCTGAAGCCTGCTACGC |
| D-NCE103_Rv | CCCGCTACTTGTGGAAATGTTCTTTATATATGTATAGTAGGGCATAGTG |
| D-TRP1_Fw | GTCGGTTATTTAATTCCACAGGTTAGTCTGTCCATTTGTCAGCAGG |
| D-TRP1_Rv | CTATTTCTAGAGGTTATTCAGGGTTGATAGGGC |
| B. Primers used for the confirmation of successful gene disruption in yeast chromosome |
| C-ERG3_Fw1 | CGAAAGCGCGTTTTCGCTATGTAG |
| C-ERG3_Rv1 | ATTTGTGCTTCCTTGGCAGTGTG |
| C-ERG3_Fw2 | TTTCAACAGTTTATACATCATAGCAGT |
| C-ERG3_Rv2 | GAAATTCGCGGTATTAAAGCTTTCAGC |
| C-NCE103_Fw1 | GTCAACATGACGCTTACAAAGC |
| C-NCE103_Rv1 | ATTCGCCGTATTACGGTACG |
| C-NCE103_Fw2 | CTACACCTGGAATGTGATAGG |
| C-NCE103_Rv2 | GACATTTGCTGAGATGAGG |
| C. Primers used in the PCR amplification of the GAL1 promoter derivative fused with hCAII |
| pGAL1.4 Fw | TACAGTAAAGCTACAAACAGGATAGAGGAGCCGG |
| pGAL1.3 Fw | TACAGTAAAGCTACAAACAGGAGCGGCCGAGCAGG |
| pGAL1.2 Fw | TACAGTAAAGCTACAAACAGGAGCGGCCGAGCAGG |
| pGAL1.1 Fw | TACAGTAAAGCTACAAACAGGAGCGGCCGAGCAGG |
| NotI_hCAII Rv | TTTTCTTCTTGCCGCGTCTTTCCTATATCAGTTATCATCTCTTTG |
desired concentration and aliquoted into each well. The AS03(pGAL1.1_hCAII) yeast cells cultivated in SR + Ade + Leu + His medium were then added into each well of the 96-well plate at the appropriate cell density for 10 µL. The test plate was incubated at room temperature for 30 min and then 10 µL of 20% (w/v) galactose was added into each well and incubated at 30 °C under an ambient atmosphere (low CO2) or 5% (v/v) CO2 condition (high CO2) for the appropriate incubation time. A stock solution of 0.1 mg mL⁻¹ resazurin sodium salt (Sigma Aldrich, USA) prepared in distilled water was added to each well to a final concentration of 0.03 mg mL⁻¹ and further incubated at 30 °C in the dark until the color of the wells without the test compound changed from blue to pink, which indicated the growth of yeast cells. The minimal effective dose was defined as the lowest concentration of the drug that could inhibit the growth of yeast cells and so prevent the color change of resazurin. In addition to the color observation in the test wells, the ratio of the optical densities between resorufin (OD572) and resazurin (OD600) was evaluated to determine the level of reduction of resazurin to resorufin. Measurement of the yeast culture turbidity at 660 nm (OD₆₆₀) (Amberg et al. 2005) was also performed before the addition of resazurin.

Statistical analysis
Statistical analysis was performed using the GraphPad Prism 5.01 package (GraphPad Software INC., USA) with one-way analysis of variance, followed by the Dunnett post-test. Each determination was performed in triplicate. Statistical significance was accepted at the p<0.05 level.

Results
Construction of a drug-sensitive derivative of the nce103 null mutant of S. cerevisiae expressing various levels of hCAII from modified GAL1-promoter-based expression cassettes
The yeast S. cerevisiae is generally highly tolerant to various drugs, which poses a serious obstacle for their use in a yeast-based in vivo drug assay. The yeast strain BY25929 (obtained from the Yeast Genetic Resources Center, Japan; Table 1), was modified to attenuate the general permeability barriers for drugs by disruption of the ERG3 gene, which is involved in the biosynthesis of ergosterol, a major component of the cell membrane (Hemmi et al. 1995). In addition, the NCE103 gene, which codes for CA, was also disrupted. The drug-sensitive derivative of the nce103 null-mutant strain so obtained was designated as the AS03 strain (Table 1).

The AS03 strain was further engineered to express hCAII at various levels. For this purpose, we used the GAL1 promoter containing four copies of the Gal4-binding site to construct a series of modified promoters containing either four, three, two or one copy(ies) of the Gal4-binding site, as previously described (Cottier et al. 2006; Liang et al. 1996), and these were designated pGAL1.4_hCAII, pGAL1.3_hCAII, pGAL1.2_hCAII or pGAL1.1_hCAII, respectively. The drug-sensitive nce103 null strain (AS03) was then separately transformed with each of these hCAII expression plasmids.

The expression levels of hCAII in the transformants were compared by multiple methods. The functional complementation of the growth defect by the exogenous hCAII gene was examined by growth of the transformants under the control of GAL1 promoter under the low-CO2 condition. All the transformants harboring the hCAII expression constructs could grow similarly well, while those with the empty vector showed a severe growth defect (data not shown).

We next compared the hCAII protein levels of the transformants by Western blot analysis. Protein extracts of the yeast transformants with the plasmid containing an expression construct for the Flag-epitope-tagged hCAII at the C-terminus showed increasing levels of the 29 kDa Flag-hCAII band with the increasing number of the Gal4-binding sites located in the modified GAL1 promoter of the expression constructs (Fig. 1a). Setting the relative protein levels of the transformants with four Gal4-binding sites as 100%, the levels in the other transformants were 82%, 62% and 39% for three, two and one Gal4-binding sites, respectively.

We further compared the mRNA levels of the transformants by two-step qRT-PCR (Fig. 1b). The obtained hCAII mRNA levels were consistent with the hCAII protein levels.

Next, we compared the sensitivity to AZA, a known CAI including against hCAII (Supuran 2008), of three different yeast strains with deletions of various genes responsible for the general drug resistance of the cells that rely on the exogenous hCAII under the low-CO2 condition. Specifically, the strains AS01 (Δnce103), AS02 (Δnce103 Δerg3) and AS03 (Δnce103 Δerg3 Δpdr1Δpdr3 Δyrr1) (Table 1) harboring the pGAL1.1_hCAII expression plasmid were constructed and subjected to the drug sensitivity assay on agar plates containing various concentrations of AZA and measuring their growth under the low-CO2 condition (spot-test assay). The result revealed that the minimal effective dose of AZA for the transformants of the AS03, AS02 and AS01 strains were 12.5, 25 and 50 µM, respectively (Fig. 2).

To evaluate the most appropriate expression level of hCAII to be used in the indicator cells for the
yeast-based assay, the AS03 strain was transformed with the different hCAII expression plasmids and then subjected to the spot test assay on plates containing various concentrations of AZA (0, 3.13, 6.25 and 12.5 μM) under the low-CO2 condition. The result showed that the AS03(pGAL1.1_hCAII) transformant which expressed the lowest level of hCAII was the most sensitive strain. It was, therefore, selected for indicator cells in the yeast-based inhibitor assay.

Setting up a resazurin-yeast-based high throughput screening assay

We next attempted to establish the assay conditions by which hCAII inhibitors could be identified using the AS03(pGAL1.1_hCAII) transformant as the assay cells under the low-CO2 condition.

Resazurin is an oxidation–reduction dye that changes color from blue (resazurin) to pink (resorufin) and further to colorless (hydroresorufin) after reduction by living cells (Rampersad 2012). We chose to examine the viability of the indicator cells in liquid culture having resazurin as an indicator dye in a 96-well plate format.

The AS03(pGAL1.1_hCAII) indicator cells were cultivated in the SG + Ade + Leu + His medium for the induced expression of hCAII under the low-CO2 condition in the absence or presence of AZA as a CAI (Figs. 4 and 5).

To optimize the initial density of the assay cells, we compared the effect of the cell density on the color reaction by varying the initial seeding density from 1 to 100 × 104 cells mL−1. As shown in Fig. 4, the optimal initial cell densities were found to be between 0.5 and 1 × 105 cells mL−1. The OD572/OD600 ratio in the wells containing AZA was approximately half of that in the negative control well. However, when higher cell densities (between 0.5 and 1 × 106 cells mL−1) were used, the OD572/OD600 ratio in the wells containing AZA was higher than that in the negative control well. In contrast, when a lower cell density (1 × 104 cells mL−1) than the optimal one was used, the ratios in the wells with or without AZA did not show any significant difference (Fig. 4a). The OD572/OD600 ratios (Fig. 4a) were consistent with the visually observed color changes (Fig. 4b).

We further investigated various other parameters of the assay, such as the incubation time between the addition of the indicator cells and the test sample, and the incubation time with resazurin before determination of the OD572/OD600 ratio (data not shown). The standard assay conditions were finally established as follows. The indicator cells were cultivated in the presence of the test samples for 24 h at 30 °C, and then incubated with resazurin for 4 h before assaying the OD572/OD600 ratio.

Evaluation of the yeast-based REMA system

To evaluate the yeast-based assay conditions, the dependency of the color reaction on the dose of AZA was examined using serial dilutions of AZA ranging from 0.02 to 25 μM in the SG + Ade + Leu + His medium containing the assay cells at 1 × 105 cells mL−1. The plates were incubated under the low- and high-CO2 conditions to determine the inhibitory effect on the hCAII and the cytotoxicity of AZA, respectively. We found that AZA significantly inhibited hCAII at a dose of 0.31 μM but no cytotoxicity was detected even at the highest concentration of AZA tested (25 μM) under the high CO2-condition (Fig. 5a, b). The results determined by the OD572/OD600 ratio (Fig. 5b) were consistent with the visual color-changes observed (Fig. 5c).
Comparison of the cell metabolism by the REMA method with that determined by the OD660 values revealed that the OD660 values showed consistent results with the OD572/OD600 ratio (Fig. 5a, b), and so could be used as a surrogate indicator of cell growth.

To further evaluate the specificity of the assay system, we compared the effect of AZA with those of avicennin, a CA isozyme IX inhibitor (Davis et al. 2013) and a FK506, a calcineurin inhibitor (Breuder et al. 1994; Liu et al. 1991; Rusnak and Mertz 2000; Shitamukai et al. 2000; Sugiura et al. 2001). The results showed that avicennin at 500 µM (its maximum solubility) showed only a weak inhibitory effect and FK506 up to 10 µM (its maximum solubility) showed no detectable inhibitory
activity (Fig. 5c), indicating the high specificity of our assay system.

Discussion

In this study, a novel yeast-based screening system for the detection of compounds that could inhibit the function of hCAII in vivo was successfully established using a high drug sensitive nce103 null yeast mutant expressing hCAII as indicator cells. *S. cerevisiae* is highly tolerant of various drugs, due to the presence of ATP-binding cassette (ABC) transporter genes resulting in the poor permeability of these drugs through the yeast cell surface (Pieuch and Oblak 2014). Therefore, in this study the yeast strain BY25929 that has been disrupted for the genes encoding transcription activation factors of the ABC transporter genes (*PDR1, PDR3* and *YRR1*) and the ABC gene *YOR1* (Miyamoto et al. 2002), was further modified to attenuate the general permeability barriers for drugs by disruption of the *ERG3* gene, which encodes the C-6 desaturase of the ergosterol biosynthesis pathway (Hemmi et al. 1995). Then, the *NCE103* gene was further deleted along with the drug resistance genes to obtain a higher drug sensitive nce103 null yeast strain designated as the AS03 strain. The Δnce103 strain deprived of its own CA can grow under a high (5%)—but not a low (ambient)-CO₂ condition due to the difference in the availability of bicarbonate ions that are essential for cellular anaplerotic reactions (Aguilera et al. 2005). However, its growth defect phenotype when cultivated under a low-CO₂ condition could be restored by overexpression of hCAII (Clark et al. 2004), or by only a low expression level of hCAII (Fig. 4).

The *GAL1* promoter was used to control the expression level of hCAII in the yeast transformants. *GAL1* is a strong-inducible promoter that strongly expresses when galactose, but not glucose, is present in the medium. The promoter contains the UAS*GAL* 17-mer sites CGG-N₁₁-CCG (Giniger et al. 1985) in four domains (Gal4 binding sites) that are recognized by the Gal4p homodimer transcription activator (Hong et al. 2008; Marmorstein et al. 1992). Cottier et al. (2006) reported that modifications in the number and type of Gal4 binding sites modulates the level of transcription of the HCMV protease gene, with 100%, 71%, 46% and 16% protein production levels, relative to the original *GAL1* promoter, with four, three, two and one Gal4 binding sites, respectively. In agreement with the above observation, our Western blot analysis and qRT-PCR results showed differential expression of hCAII in direct proportion with the number of Gal4 binding sites in the *GAL1* promoter of the expression

![Fig. 4](See figure on next page.)

**Fig. 4** Optimization of the initial cell concentration using the REMA. A suspension of the yeast strain AS03(pGAL1.1_hCAII) at varying final concentrations of 1 x 10⁶, 5 x 10⁵, 1 x 10⁵, 5 x 10⁴ and 1 x 10⁴ cells mL⁻¹ in 100 µL of SG + Ade + Leu + His medium were added to each well of a 96-well plate with varying concentrations of AZA (0, 6.25, 12.5 and 25 µM). The plates were incubated at 30 °C for 24 h whereupon resazurin was added and 4 h later the OD₅₇₂ and OD₆₀₀ values were measured and expressed as a the OD₅₇₂/OD₆₀₀ ratio and b visual observation. Data are shown as a the mean ± 1 SD derived from three independent repeats, where *, ** and *** represent a significant difference at p < 0.05, p < 0.01 and p < 0.001 levels, respectively, compared to that of the untreated control group, and b representative image of three independent repeats.

**Fig. 5** Determination of the minimal effective dose of the tested compounds. Serial dilutions of either AZA (0–25 µM), avicennin (0–500 µM) or FK506 (0–10 µM) were added in wells of a 96-well plate containing 0.5 x 10⁵ cells mL⁻¹ of AS03(pGAL1.1_hCAII) cells and incubated at 30 °C under a high- or low-CO₂ condition. a The growth of the yeast strain treated with AZA was determined by the OD₆₆₀ value prior to adding resazurin into each well. b Then, after 4 h, the OD₅₇₂ and OD₆₀₀ values were measured and expressed as the OD₅₇₂/OD₆₀₀ ratio. c The colorimetric results of the assay for three tested compounds were also observed visually. Data are shown as (a,b) the mean ± 1 SD derived from three independent repeats, where *, ** and *** represent a significant difference at p < 0.05, p < 0.01 and p < 0.001 levels, respectively, compared to that of the untreated control group, and c a representative image of three independent repeats.
construct as 100%, 82%, 62% and 39% for four, three, two and one Gal4 binding site(s), respectively (Fig. 1).

Taken together, the results of Figs. 1, 2 (at 0 µM AZA) and 3 (at 0 µM AZA) revealed that even the lowest level of hCAII (expressed from the promoter containing a single Gal4-binding site) was sufficient to complement the growth defect of the nce103 null mutant under the low-CO₂ condition.

Thus, using this transformant with the lowest expression level of hCAII (pGAL1.1_hCAII), we compared the sensitivity to AZA with three different yeast strains with deletions of various genes responsible for the general drug resistance of the cells under the low-CO₂ condition. The spot test assay for AZA sensitivity of the three different strains of nce103 null mutant background (AS01, AS02 and AS03; Table 1) revealed the strain showing the highest sensitivity to AZA was AS03(pGAL1.1_hCAII), followed by AS02(pGAL1.1_hCAII) and AS01(pGAL1.1_hCAII), respectively (Fig. 2). These results confirmed the high AZA sensitivity of the chosen indicator cells for our yeast-based assay and showed that CAIs that are effective against hCAII can be assayed for by monitoring the growth of these yeast strains (AS03, AS02 and AS01) relying on hCAII under the low-CO₂ condition.

We chose the AS03 strain, as the most drug-sensitive strain, to examine the effect of the expressed levels of hCAII on the sensitivity to AZA. As expected, the degree of AZA resistance was positively correlated with the hCAII expression level (Fig. 3). Based on these data, the AS03(pGAL1.1_hCAII) transformant, which expressed the lowest level of hCAII (Fig. 1) and was the most sensitive strain, was selected as the indicator cells in the yeast-based inhibitor assay.

For the operational convenience of a HTS assay for a large number of samples using this yeast-based in vivo assay, we chose to examine the viability of the assay cells in liquid culture with resazurin as an indicator dye using a 96 well-plate format.

The principle of this yeast-based assay was that if tested compounds inhibit hCAII activity, then the nce103 null mutant indicator cells would not be able to grow under the low CO₂-condition but would grow under the high-CO₂ condition if they were otherwise not cytotoxic to the yeast. Although the OD₆₆₀ measurement provided an easy and efficient way to quantify yeast growth over time, metabolic indicators of the cell density, based on the metabolism-dependent reduction of colorimetric dyes, provide an alternative measurement of yeast cell density as well as providing an indicator of yeast vitality (Goughenour et al. 2015). Here, we used the colorimetric indicator resazurin that can also be used as a more objective qualitative indicator. Although the use of resazurin as indicator dye for cell viability has several advantages, care must be taken in setting up the REMA to get a correlation between the OD₅₇₂/OD₆₀₀ ratio and cell turbidity (OD₆₆₀).

During the resazurin incubation, the color of the solution containing the indicator cells in the absence of AZA (negative control) changed from blue to pink and this color change was determined by measuring the OD₅₇₂ value for resorufin and OD₆₀₀ for resazurin and expressed as the OD₅₇₂/OD₆₀₀ ratio. In the absence of AZA, the cells were able to grow under the low-CO₂ condition relying on their expressed hCAII, which led to the color change, while the sample without the indicator cells remained blue. In the sample containing the indicator cells and AZA at a concentration sufficient to inhibit the hCAII, the color remained blue with an OD₅₇₂/OD₆₀₀ ratio that was lower than that without the inhibitor (negative control).

An optimal initial yeast cell density is one of the important parameters of the assay. With too high cell density (0.5–1×10⁶ cells mL⁻¹), the OD₅₇₂/OD₆₀₀ ratio in the test-wells containing AZA was higher than that in the negative control (Fig. 4a), which is due to the extensive reduction of resazurin into resorufin and then hydroresorufin (colorless) in the control-wells (Ramsdell et al. 1935). Whereas, an over twofold difference in the OD₅₇₂/OD₆₀₀ ratio between the control and AZA-treated cells was noted when using a yeast cell density of 0.5–1×10⁵ cells mL⁻¹ (Fig. 4a). This observation is consistent with those determined by visualization of the color change (Fig. 4b). However, at a lower yeast cell density (1×10⁴ cells mL⁻¹) there was no significant difference in the OD₅₇₂/OD₆₀₀ ratio between the control and AZA-treated wells (Fig. 4a). Therefore, under these conditions 0.5–1×10⁵ cells mL⁻¹ was the appropriate initial yeast cell density.

The result showed that the minimal effective dose of AZA determined by the optimized REMA method was 0.31 µM (Fig. 5), which was 20- to 40-fold lower than that determined from the spot test on agar plates (6.25–12.5 µM; Fig. 3). Therefore, the REMA method significantly increased the sensitivity for the detection of this inhibitor compared to the spot test on agar plate. Furthermore, this assay system showed a potentially high specificity (Fig. 5c).

In this study, an in vivo screening method for CAIs that inhibit hCAII was developed utilizing a drug sensitive nce103 null mutant strain expressing and relying on a low level of hCAII as indicator cells in combination with a REMA assay for the HTS of potential novel drugs. The yeast-based in vivo assay system has several advantages over the conventional in vitro biochemical screening assay as follows. Since this screening procedure is based on living yeast cells, in contrast to the
conventional in vitro biochemical screening procedures, only compounds that permeate through the cell surface, and compounds that are metabolically stable in vivo can be screened.

Since the assay is based on the growth inhibition of the assay cells, cytotoxic compounds will be also selected by the screening. These compounds, however, can be easily eliminated by examining the growth of the assay cells under the high-CO$_2$ condition, where the cells do not depend on the hCAII activity for growth.

This screening procedure will potentially facilitate a high throughput screening system for detection of safer hCAII inhibitors that could be further developed as a drug, especially for tropical use in glaucoma treatment. However, the candidates obtained from this screening procedure still require further investigation for their hCAII specificity by screening for inhibitory activity against other CA isozymes.

Abbreviations
CA: carbonic anhydrase; hCAII: human carbonic anhydrase isozyme II; CAI: carbonic anhydrase inhibitor.

Authors’ contributions
AS participated in the experimental design, carried out the experiments and drafted the manuscript. CY designed the experiments and wrote the manuscript. JK provided cDNA containing hCAII. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
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Consent for publication
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