



Characterization of the kinetics and mechanisms of inhibition of drugs interacting with the *S. cerevisiae* multidrug resistance pumps Pdr5p and Snq2p

Tomáš Hendrych^a, Marie Kodedová^a, Karel Sigler^b, Dana Gášková^{a,*}

^a Charles University, Faculty of Mathematics and Physics, Institute of Physics, Ke Karlovu 5, 121 16 Prague 2, Czech Republic

^b Institute of Microbiology, CR Academy of Sciences, Vídeňská 1083, 142 20 Prague 4, Czech Republic

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ABSTRACT

We have developed a novel screening method that measures the kinetics and potencies of inhibitors of the yeast multidrug resistance pumps Pdr5p and Snq2p. The assay uses the potentiometric fluorescent probe diS-C₃(3) (as a benchmark substrate of both pumps) to distinguish drugs with minimal effects on plasma membrane potential as a marker of side-effects on membrane function and integrity. Using FK506, its structural analog rapamycin and enniatin B, we showed that our assay can also be used to determine the minimum drug concentration causing an immediate inhibitory effect and to compare the inhibitory potencies of the drug on the two pumps. We found that the protonophore CCCP effectively inhibits the transport of diS-C₃(3) by both pumps and confirmed the activation of membrane H⁺-ATPase by CCCP.

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1. Introduction

Major clinical problems in the treatment of cancer are caused by multidrug resistance (MDR) proteins which expel a variety of structurally and functionally unrelated anticancer drugs from the cells [1–3]. Most inhibitors of MDR proteins, especially those targeting P-glycoprotein exhibit cytotoxicity [4–6].

The major multidrug exporters of *S. cerevisiae* are the full-size ABC transporters Pdr5p and Snq2p. They show limited sequence similarity with mammalian P-glycoprotein and have a reverse order of their transmembrane and nucleotide-binding domains [7]. Despite these differences, Pdr5p interacts with many of the same substrates and inhibitors as P-glycoprotein [8–14]. Thus, yeast Pdr5p can be used to screen for compounds that may be effective inhibitors of P-glycoprotein. Various benchmark substrates of Pdr5p, such as rhodamines [11], cycloheximide and cerulenin [15], antifungal azoles [13,16], steroids [14] and others [17,18] have been used in various screening models and methodologies that have evolved for identification and characterization of inhibitors/substrates of the pump [11,13,15–17,19].

New efficient inhibitors of MDR pumps found by screening in yeast may be useful therapeutically in conjunction with current drugs to combat yeast and bacterial infections and/or malignancies. Combination therapies may result in the usage of drugs at lower concentrations.

All current yeast screening systems for identifying new inhibitors of Pdr5p are based on the same principle – different accumulation of a benchmark substrate in cells overexpressing Pdr5p (either due to the presence of the *PDR1-3* allele or the presence of a plasmid) in the presence and absence of

inhibitors. A lesser amount of the benchmark substrate accumulates in the absence of inhibitors, while the presence of inhibitors facilitates increased accumulation within cells on the level of an isogenic Pdr5p-deficient strain.

A number of compounds have been found to efficiently reduce Pdr5p's ability to export its benchmark substrates [8,11,13,16,19–22]. Some of them, such as FK506, which have been assumed to be noncompetitive inhibitors [10,14,15], were later found to be Pdr5p substrates. Their inhibitory effects are caused by a low efflux rate or a higher affinity for the pump than the benchmark substrate [23].

The main goal of these screening systems is the identification of Pdr5p inhibitors. However, they also lead to a better understanding of substrate/pump interactions by providing insight into the structure of the Pdr5p substrate binding site, the sizes and chemical properties of substrates [17,18] and the effects of mutations on Pdr5p function [10,23,24].

Our fluorescence method uses the potentiometric fluorescent probe diS-C₃(3) as a benchmark substrate. DiS-C₃(3) is a substrate for Pdr5p and for Snq2p [25]. Our screening system allows us to monitor with high sensitivity and in real time changes in the activities of both pumps. Furthermore, screening is performed on cells in well-defined physiological states, does not require any pretreatment or preincubation and allows us to monitor effects on cellular membrane potentials. We present here an efficient strategy for identifying pump inhibitors with minimal side effects on membrane potential or membrane integrity.

2. Materials and methods

2.1. Yeast strains

Yeast strains used in this study were *S. cerevisiae* US50-18C (*MATα*, *PDR1-3*, *ura3*, *his1*) and its mutants AD1-3 (*MATα*, *PDR1-3*, *ura3*, *his1*,

* Corresponding author. Tel.: +420 221911348; fax: +420 224922797.

E-mail address: gaskova@karlov.mff.cuni.cz (D. Gášková).

yor1Δ::hisG, *snq2Δ::hisG*, *pdr5Δ::hisG*), AD12 (*MATα*, *PDR1-3*, *ura3*, *his1*, *yor1Δ::hisG*, *snq2Δ::hisG*), AD13 (*MATα*, *PDR1-3*, *ura3*, *his1*, *yor1Δ::hisG*, *pdr5Δ::hisG*) and AD23 (*MATα*, *PDR1-3*, *ura3*, *his1*, *snq2Δ::hisG*, *pdr5Δ::hisG*) [26].

2.2. Media and cell growth conditions

Yeast was precultured in YPD medium (1% yeast extract, 1% bactopectone, 2% glucose) at 30 °C for 24 h. A small volume (1–10 μl) of inoculum was added to 10 ml fresh YPD medium and the main culture was grown until it had reached the desired phase of exponential growth.

2.3. Fluorescence measurement of diS-C₃(3) accumulation in cells

Cells from the exponential growth phase were harvested, washed twice with double-distilled water and resuspended in citrate-phosphate (CP) buffer of pH 6.0 to OD₅₇₈=0.1. The potentiometric probe diS-C₃(3) (10^{−5} M stock solution in ethanol) was added to 3 ml of yeast cell suspension to a final concentration of 2×10^{−8} M and fluorescence emission spectra of the cell suspensions were measured every 2–5 min on FluoroMax - 3 spectrofluorimeter (JobinYvon - groupe HORIBA) equipped with a xenon lamp. Excitation wavelength was 531 nm, fluorescence range 560–590 nm, duration of one spectral scan 20 s, scattered light was eliminated by orange glass filter with a cutoff wavelength at 540 nm.

The rate and extent of intracellular probe accumulation, a so-called staining curve, records the dependence of fluorescence emission maximum wavelength λ_{max} on the time of staining [27,28]. When appropriate, a tested compound (inhibitor/substrate) was added to a different final concentration during the diS-C₃(3) assay (usually after 20 min of staining). The samples were kept at room temperature and occasionally gently stirred.

2.4. Drug susceptibility assay

Disc diffusion tests were performed as previously described [12] to determine if compounds are substrates of Pdr5p, Snq2 and Yor1p. Briefly, yeast cells grown to post-diauxic phase in liquid YPD medium were washed twice with distilled water and resuspended in CP buffer (pH 6.0). Then they were diluted into top agar (seeded with 2.5×10⁶ cells/ml) and poured onto YPG plates (2% agar, 1% yeast extract, 1% peptone, 2% glycerol). Two types of top agar were used in this study: (1) 1% YPD agar (1% agar, 1% yeast extract, 1% peptone, 2% glucose) and (2) 1% YPG (1% agar, 1% yeast extract, 1% peptone, 2% glycerol, 2% ethanol). The compounds dissolved in

ethanol, DMSO, DMF or as aqueous solutions (2 μl) were spotted onto Whatman paper discs lying on the top of the agar. After 2 days at 30 °C, the plates were photographed and the size of the growth inhibition zones measured.

To determine if a compound is able to inhibit the extrusion of a known substrate (nigericin for Pdr5p and Yor1p, 4-NQO for Snq2p) by the pumps we used a 'double addition' mode of the disc diffusion test. The tested compound (DM-11) was added 15 min after the known substrate.

2.5. Chemicals

The following materials were purchased from the respective companies: diS-C₃(3) (3,3'-dipropylthiacarboxycyanine), DMSO and DMF (Fluka), yeast extract (Serva), bactopectone (Oxoid), glucose (Penta, Czech Republic), citric acid and Na₂HPO₄·12 H₂O (reagent grade) (Lachema, Czech Republic), agar (Dr. Kulich Pharma, Czech Republic) and glycerol (Lach-Ner, Czech Republic). MDR pump substrates or inhibitors, lysosomotropic compound and protonophore used in this study were obtained from the following sources: FK506 and rapamycin (LC Laboratories), enniatin B (Alexis Biochemicals), nigericin from *Streptomyces hygroscopicus* and CCCP (carbonyl cyanide 3-chlorophenylhydrazone) (Sigma), 4-NQO (N-nitroquinoline 1-oxide) (Supelco). The lysosomotropic compound DM-11 (2-dodecanoyloxyethyl-dimethylammonium chloride) was synthesized in the laboratory of Prof. S. Witek (Univ. Wrocław) [29] and kindly provided by Dr. A. Krasowska.

3. Results and discussion

3.1. Validity of the diS-C₃(3) screening assay for identifying inhibitors of Pdr5 and Snq2 pumps

Our fluorescence assay is based on comparing the accumulation of the benchmark pump substrate diS-C₃(3) in Pdr5p- and/or Snq2p-expressing cells (strains AD12, AD13 and US50-18C) versus Pdr5p- and Snq2p-deficient cells (strains AD1-3 and AD23 taken as negative controls) [25,30] in the absence of an inhibitor and after its addition.

The accumulation of diS-C₃(3) in control and inhibitor-treated cells is recorded as the dependence of the fluorescence emission maximum wavelength (λ_{max}) on time (see Materials and methods). λ_{max}(*t*) reflects the intracellular probe concentration at given time *t* [31]. Increasing intracellular concentrations are accompanied by a gradual shift of λ_{max} towards longer wavelengths (a red shift); probe efflux results in a decreased intracellular concentration and a blue shift [32].

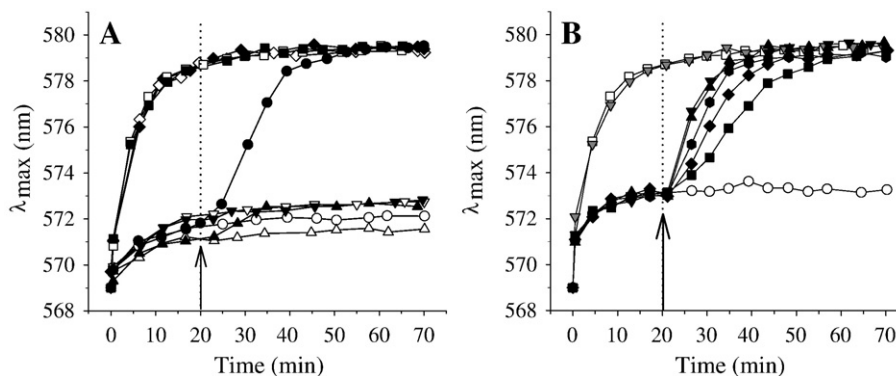


Fig. 1. Inhibitory effect of FK506 on Pdr5p. (A) Staining curves of early exponential cells of AD1–3 (squares), AD23 (diamonds), AD12 (circles), AD13 (inverted triangles) and US50-18C (triangles). Empty symbols – no FK506 added; full symbols – 5 μM FK506 added at 20 min. (B) Staining curves of AD12 cells after the addition of different FK506 concentrations (empty circles – no FK506; full squares – 1 μM; full diamonds – 3 μM; full hexagons – 5 μM; full triangles – 7 μM; full inverted triangles – 10 μM FK506) and AD1–3 cells (empty squares – no FK506; grey inverted triangles – 10 μM FK506). Arrows with dotted lines indicate FK506 addition.

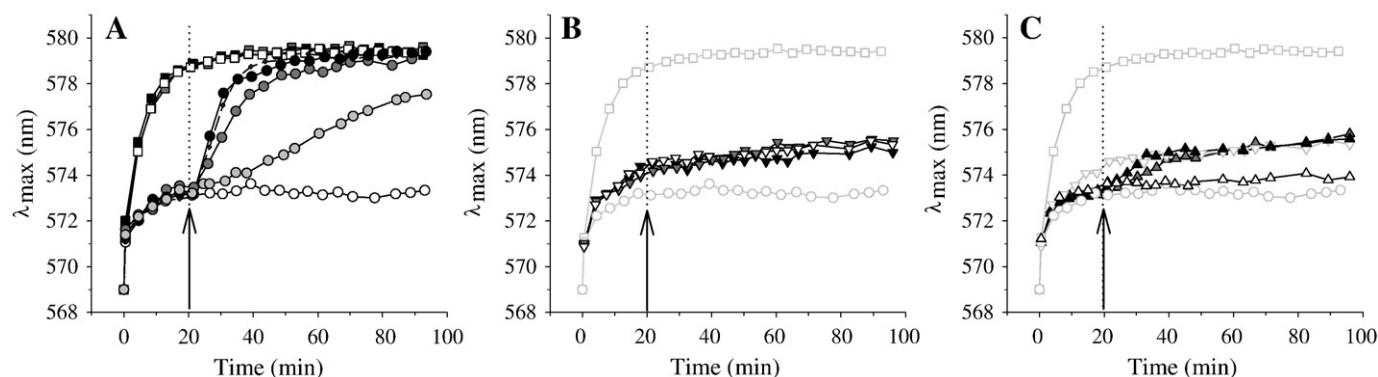


Fig. 2. Comparison of action of enniatin B and rapamycin with the effect of FK506 on MDR pumps. (A) Staining curves of AD1–3 (squares) and AD12 (circles) mid-exponential cells. Empty symbols — no inhibitor added; 5 μ M enniatin B (full symbols), 5 μ M rapamycin (light grey symbols) and 20 μ M rapamycin (dark grey symbols) added at 20 min. Dashed line with crosses — 5 μ M FK506 for comparison. (B) Staining curves of AD13 (inverted triangles) cells. Empty symbols — no inhibitor added; 5 μ M enniatin B (full symbols) and 20 μ M rapamycin (dark grey symbols) added at 20 min. Staining curves of AD1–3 (empty grey squares) and AD12 (empty grey circles) shown for comparison. (C) Staining curves of US50-18C (triangles) cells. Empty symbols — no inhibitor added; 5 μ M enniatin B (full symbols) and 20 μ M rapamycin (dark grey symbols) added at 20 min. Staining curves of AD1–3 (empty grey squares), AD12 (empty grey circles) and AD13 (empty grey inverted triangles) shown for comparison. Arrows with dotted lines indicate drug addition.

3.1.1. Drugs that affect Pdr5p

A comparison of staining curves (Fig. 1A) reveals that pump-expressing cells (strains AD12, AD13 and US50-18C) from an exponentially growing culture have a lower intracellular concentration of diS-C₃(3) than pump-deficient mutants (AD1-3 and AD23) used as negative controls. Because the membrane potentials of pump-deficient and pump-expressing cells in the same phase of growth are identical [25], the differences in intracellular concentrations of diS-C₃(3) reflect probe efflux from the cells by the pumps.

The usefulness of diS-C₃(3) assay for studying the pump inhibition potency of drugs was tested with FK506, a known inhibitor/substrate of the Pdr5 pump [10,14,23]. Addition of 5 μ M FK506 to negative control (AD1-3, AD23) cells does not cause any change in staining (no effect on membrane potential) (Fig. 1A), while its addition to Pdr5p-

expressing AD12 cells leads to the convergence of the staining curve with that of negative control AD1-3 (and AD23) cells. This indicates the inhibition of probe efflux by Pdr5p.

In contrast to AD12, the staining curve for AD13 cells, which only express Snq2p, is unaffected by the addition of FK506 (Fig. 1A), indicating that FK506 has no effect on Snq2p activity.

FK506 addition to parent US50-18C cells having both Pdr5p and Snq2p causes a λ_{\max} increase to the staining level of AD13 cells as a result of Pdr5p inhibition (Fig. 1A). Hence the fluorescence assay clearly shows that FK506 interacts only with Pdr5p.

As seen in Fig. 1B, the speed with which the probe concentrations in the AD12 cells reaches the similar levels as the pump-deficient cells increases with increasing concentration of FK506. At 7 μ M and higher the staining is no longer dependent on drug concentration and the

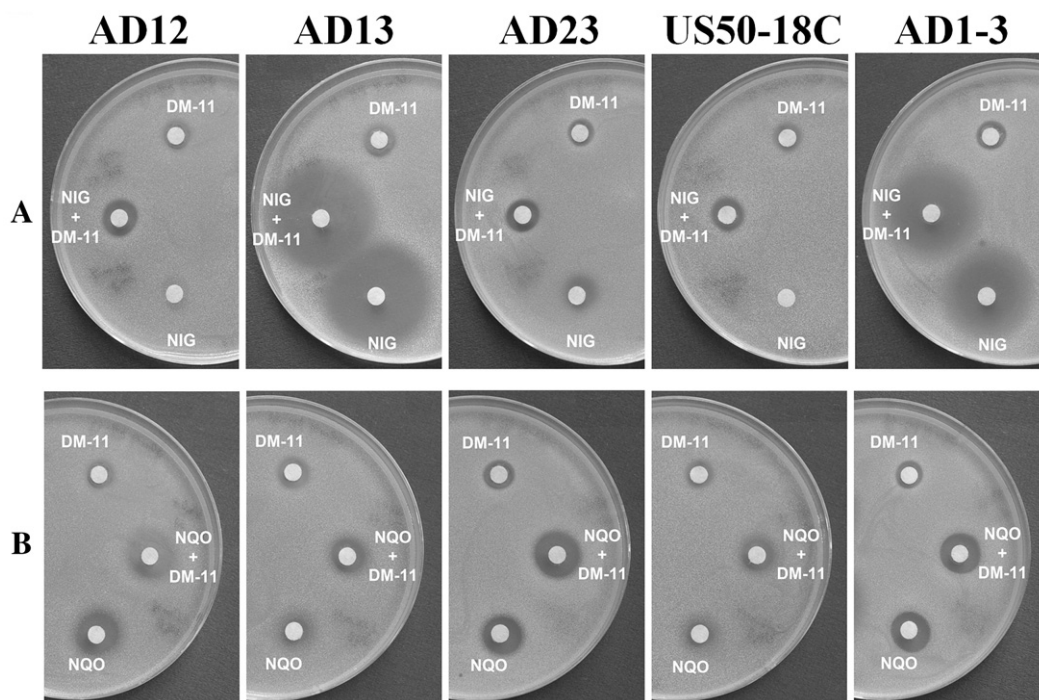


Fig. 3. DM-11 does not interact with Pdr5p, Snq2p and Yor1p either as a substrate or as an inhibitor. (A) Growth inhibition zones measured in a disc diffusion test using YPGE top agar in various pump-expressing strains (AD12, AD13, AD23 and US50-18C) and the negative control AD1-3 exposed to nigericin (NIG, 20 mM; a substrate of Pdr5p and Yor1p), DM-11 (30 mM) and their combinations. (B) Growth inhibition zones measured in a disc diffusion test using YPGE top agar in various pump-overproducing strains (AD12, AD13, AD23 and US50-18C) and the negative control AD1-3 exposed to 4-NQO (NQO, 3.26 mM; a substrate of Snq2p), DM-11 (30 mM) and their combinations.

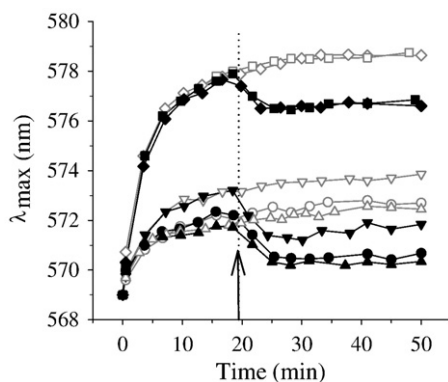


Fig. 4. H^+ -ATPase inhibitor DM-11 lowers the membrane potential of cells but has no influence on the activity of Pdr5p and Snq2p. Staining curves of mid-exponential cells of negative controls AD1–3 (squares) and AD23 (diamonds), and pump-expressing AD12 (circles), AD13 (inverted triangles) and US50-18C (triangles). Empty symbols – no DM-11 added; full symbols – 10 μ M DM-11 added at 20 min. Arrow with dotted line indicates DM-11 addition.

staining curves become parallel with that observed for a negative control. This indicates that the staining is given only by membrane potential [28]. In other words, this concentration is sufficient for an immediate inhibitory effect. The slower change in staining at low FK506 concentrations indicates that inhibition of Pdr5p occurs gradually as the drug passes through the cell wall.

Our assay can determine the minimum drug concentration at which immediate and complete inhibition occurs.

In determining the usefulness of MDR pump inhibitors, it is also important to determine whether the inhibitor has effects on membrane function and integrity. The first indicator of such side-effects is a membrane potential change. Our method permits not only detection of the pump inhibition by a drug but, independently, also reveals the effect of the drug on membrane potential. As shown previously in prestained cells with zero pump activity [33], addition of drugs can result in changes in λ_{\max} . A blue shift indicates plasma membrane depolarization, while a red shift may signify hyperpolarization or membrane permeabilization. The absence of membrane potential changes after the addition of FK506 points to its minimal influence on membrane function and integrity.

No growth inhibition zones and no difference in survival between control and FK506-treated cells were observed in disc diffusion and plating tests (data not shown). Thus FK506, which is known to inhibit

the transport of some amino acids (predominantly tryptophan) [10,23,34] does not affect cell survival of our tryptophan prototrophic strains, even at high concentrations.

We compared the actions of several inhibitors on the pumps to FK506. Enniatin B is a recently discovered inhibitor of Pdr5p [19]. Rapamycin is a structural analog of FK506 that acts on *S. cerevisiae* cells by irreversibly arresting cell in the G1 phase of the cell cycle [35]. Rapamycin has not been tested as a substrate/inhibitor of MDR pumps in yeast but has been shown to interact with the mammalian P-glycoprotein [36].

As seen in Fig. 2A, the inhibitory effect of enniatin B is virtually identical to that of FK506. Inhibition of Pdr5p by 5 μ M rapamycin is much slower than by 5 μ M FK506. Inhibition by rapamycin is still slower at a 4-fold higher concentration. Like FK506, neither of the two drugs affects the Snq2 pump (Fig. 2B, C) and membrane potential (Fig. 2A). In view of the structural similarity of FK506 and rapamycin [36], the rate of passage of both agents through the cell wall barrier should be similar. The slower staining with 5 μ M rapamycin therefore shows that its ability to inhibit Pdr5p is lower than that of FK506.

These data demonstrate that our method can be used to compare the potencies of different inhibitors towards MDR pumps. It should be noted that the assay assesses *in situ* and *in vivo* pump inhibition, which depends on both pump inhibition and permeability through the cell wall. These effects can be quantified by using a suitable parameter. Given the complex phenomena described here, the quantification will be the subject of a forthcoming study.

3.1.2. Compounds that have no effect on pump activity

As an integral part of assessing the usefulness of our method we show that compounds that do not interact with the pumps do not affect the ability of the pumps to transport diS-C₃(3) but can only affect membrane potential. As such compound we used the lysosomotropic compound DM-11, which was shown to inhibit the activity of yeast plasma membrane H^+ -ATPase [29].

Some authors [12, 37] reported that cells deleted in *PDR5*, *SNQ2* and *YOR1* become sensitive to DM-11, which thus seems to be a substrate of Pdr5p, Snq2p and Yor1p. On the other hand, our data show that DM-11 clearly does not interact with Pdr5p, Snq2p and Yor1p either as a substrate or as an inhibitor. As seen in the disc diffusion test on pump-expressing cells (Fig. 3), the zones of inhibition formed in the presence of Pdr5p and Yor1p substrate nigericin (Fig. 3A) or the Snq2p substrate 4-NQO (Fig. 3B) plus DM-11 are similar in size to the individual compounds. DM-11 is therefore

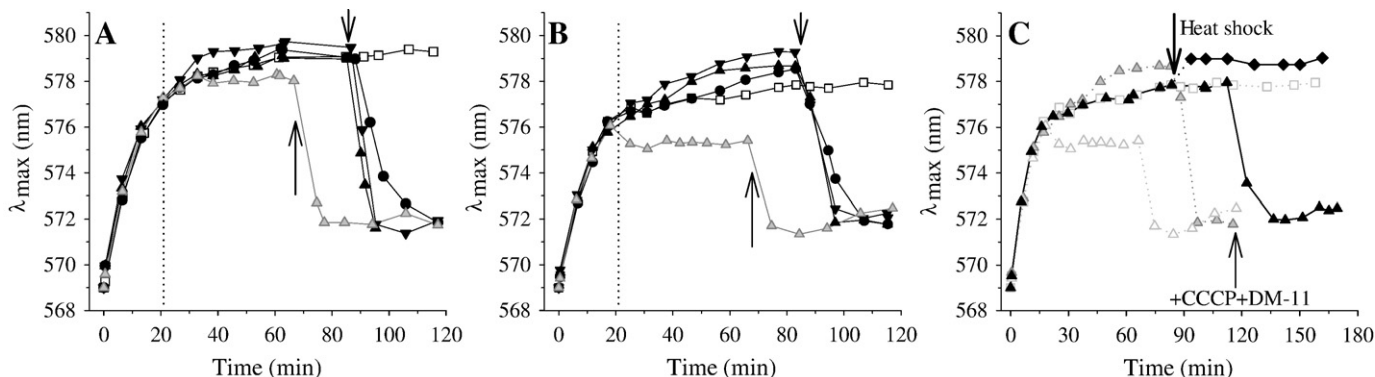


Fig. 5. The protonophore CCCP causes activation of H^+ -ATPase. (A) Effect of CCCP and DM-11 additions (irrespective of the sequence) on staining of early-exponential AD1–3 cells. Empty squares – no compound added; 1 μ M CCCP (full circles), 5 μ M CCCP (full triangles) and 10 μ M CCCP (full inverted triangles) added at 20 min (dotted line) followed by 10 μ M DM-11 (downward arrow). The staining curve after addition of 10 μ M DM-11 at 20 min (light grey triangles) followed by 5 μ M CCCP (upward arrow) is shown for comparison. (B) Effect of CCCP and DM-11 additions (irrespective of the sequence) on staining of late-exponential AD1–3 cells. Symbols as in panel A. (C) Effect of CCCP addition together with DM-11 (cocktail) on staining of late-exponential AD1–3 cells. Full triangles – 5 μ M CCCP plus 10 μ M DM-11 added to intact cells, full diamonds – addition of the cocktail to heat-shock-exposed cells. Upward arrow indicates cocktail addition to both variants. Downward arrow indicates heat-shock pretreatment. Dotted lines with empty or grey symbols are taken from panel B for comparison (empty triangles – 10 μ M DM-11 followed by 5 μ M CCCP; grey triangles – opposite sequence; empty squares – no addition).

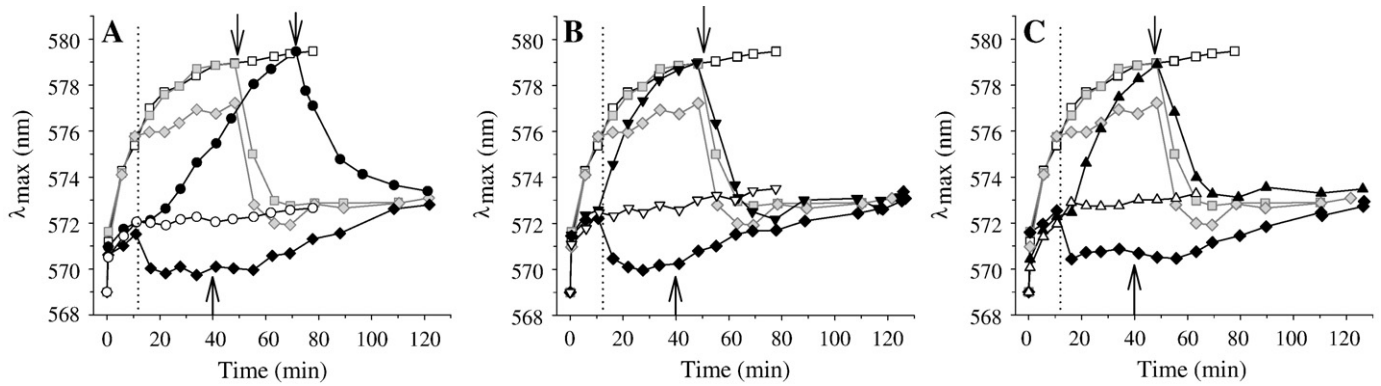


Fig. 6. CCCP inhibits diS-C₃(3) transport by both Pdr5p and Snq2p independent of H⁺-ATPase activity. (A) Effect of CCCP and DM-11 additions (irrespective of the sequence) on staining of mid-exponential Pdr5p-expressing AD12 cells and negative control AD1–3 cells. Empty circles — no compound added to AD12 cells; full circles — 5 μ M CCCP added at 12 min to AD12 cells (dotted line) followed by 10 μ M DM-11 (second downward arrow); full diamonds — 10 μ M DM-11 added at 12 min to AD12 cells followed by 5 μ M CCCP (upward arrow). Empty squares — no compound added to AD1–3 cells; grey squares — 5 μ M CCCP added at 12 min to AD1–3 cells followed by 10 μ M DM-11 (first downward arrow); grey diamonds — 10 μ M DM-11 added at 12 min to AD1–3 cells followed by 5 μ M CCCP (first downward arrow). (B) Effect of CCCP and DM-11 additions (irrespective of the sequence) on staining of mid-exponential Snq2p-expressing AD13 cells compared with negative control AD1–3 cells. Empty inverted triangles — no compound added to AD13 cells; full inverted triangles — 5 μ M CCCP added at 12 min to AD13 cells (dotted line) followed by 10 μ M DM-11 (downward arrow); full diamonds — 10 μ M DM-11 added at 12 min to AD13 cells followed by 5 μ M CCCP (upward arrow). Staining curves and symbols for AD1–3 cells are the same as in panel A. (C) Effect of CCCP and DM-11 additions (irrespective of the sequence) on staining of mid-exponential Pdr5p- and Snq2p-expressing US50-18C cells compared with negative control AD1–3 cells. Empty triangles — no compound added to US50-18C cells; full triangles — 5 μ M CCCP added at 12 min to US50-18C cells (dotted line) followed by 10 μ M DM-11 (downward arrow); full diamonds — 10 μ M DM-11 added at 12 min to US50-18C cells followed by 5 μ M CCCP (upward arrow). Staining curves and symbols for AD1–3 cells are the same as in panel A.

not an inhibitor of these pumps. Moreover, the identical zone size caused by DM-11 in the triple pump deletant AD1–3 and in pump-expressing strains AD12, AD13, AD23 and US50-18C indicates that DM-11 is not a substrate of Pdr5p, Snq2p or Yor1p.

Fluorescence measurements (Fig. 4) reveal that the effect of DM-11 indeed involves lowering of the membrane potential in negative controls (AD1–3, AD23). The decrease in λ_{\max} after the addition of DM-11 reflects the extent of inhibition of the H⁺-ATPase. Cells of Pdr5p- and/or Snq2p-expressing strains in the same growth phase display the same drop in staining relative to the DM-11 untreated controls. Hence DM-11 does not affect the activity of the pumps, i.e. their ability to export the probe from the cells.

3.2. Protonophore CCCP causes activation of H⁺-ATPase and interacts with both Pdr5p and Snq2p

In early exponential AD1–3 cells, in which the probe accumulates solely according to membrane potential, addition of different concentrations of CCCP does not cause any decrease in staining (Fig. 5A). The highest concentration even causes a slight increase in

λ_{\max} . This is surprising since the protonophore should dissipate membrane potential and cause depolarization.

The increase in staining after CCCP addition is still more striking in late exponential cells (Fig. 5B) that have a lower membrane potential [25]. This increase in staining is concentration-dependent, i.e. the same staining level is attained faster at a higher CCCP concentration.

The most likely explanation for this increase in staining is an increase in membrane potential. CCCP is a lipophilic, weak acid [38] that causes intracellular acidification and induces a rapid activation of the H⁺-ATPase. Activation of the H⁺-ATPase has been reported by weak acids [39] and by medium chain fatty acids [40]. A CCCP-induced activation of this enzyme has been described by dos Passos et al. [41]. The participation of the inducible pump Pdr12p, which recognizes CCCP as a substrate (Goffeau, A., personal communication), can be excluded because the export of CCCP anions should not increase the membrane potential [42,43]. In addition, our cultivation and experimental conditions practically exclude a high level of this transporter in the plasma membrane [42]. Induction of Pdr12p, regulated by the War1p transcription factor [44] (but not by Pdr1p and Pdr3p [42]), is observed only under stress caused by some weak organic acids [45].

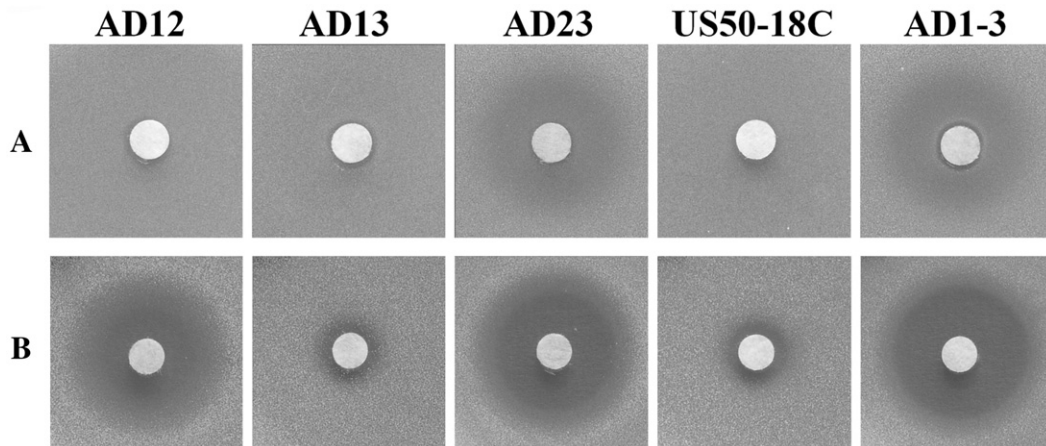


Fig. 7. CCCP is a substrate of both Pdr5p and Snq2p. (A) Growth inhibition zones measured in a disc diffusion test using YPD top agar in variously pump-expressing strains (AD12, AD13, AD23 and US50-18C) and negative control AD1–3 exposed to 10 mM CCCP. (B) Growth inhibition zones measured in a disc diffusion test using YPGE top agar in variously pump-expressing strains (AD12, AD13, AD23 and US50-18C) and the negative control AD1–3 exposed to 5 mM CCCP.

In support of our hypothesis that CCCP activates the H^+ -ATPase, we note that prior inhibition of the H^+ -ATPase with DM-11 prevents the increase in λ_{\max} by a subsequent addition of CCCP. Rather, addition of CCCP leads to a marked depolarization and the staining level is the same as attained with the opposite sequence of additions (Fig. 5A, B).

The CCCP-induced activation of the H^+ -ATPase is an important observation because CCCP is commonly used as a diagnostic tool to indicate cell integrity. CCCP should not be used as the sole marker of cell integrity, but rather it should be used in combination with DM-11 or another H^+ -ATPase blocker. We illustrate the use of DM-11 plus CCCP for determining cell integrity in Fig. 5C. The staining profile of late exponential cells after the simultaneous addition of DM-11 and CCCP (taken from Fig. 5B) is compared to the response of heat shocked cells. In contrast to intact cells, cocktail addition to the heat shocked cells does not cause any drop in the high cell staining. This indicates the membrane-potential-independent interaction of the probe with the components of permeabilized cells [33].

CCCP addition to Pdr5p- and/or Snq2p-expressing cells causes a variably fast increase in λ_{\max} and a convergence of their staining curves with that of the negative control AD1–3 (Fig. 6). We can attribute this effect to a differential ability of CCCP to inhibit probe transport by the two pumps. Although the activity of the two pumps is comparable (the staining levels for strains AD12 and AD13 are practically the same), the rate of λ_{\max} increase in the case of AD13 cells having only Snq2p is much higher than the rate observed in the AD12 strain (Pdr5p). We can attribute this effect to a different ability of CCCP to inhibit the probe transport by the two pumps. The rate of staining observed with strain US50–18C (overproducing both pumps) is a combination of these two effects. Subsequent addition of DM-11 to CCCP-treated cells shows that the cells are intact and behave as a negative control.

This is another important finding in addition to the H^+ -ATPase activation, which of course takes place also in the Pdr5p- and/or Snq2p-overproducing strains. The CCCP-induced inhibition of probe transport by both pumps is documented in Pdr5p- and/or Snq2p-overproducing cells in which an activation of H^+ -ATPase had been prevented by DM-11 (Fig. 6). Even in this case the addition of CCCP is accompanied in all strains by λ_{\max} increase to the level of negative control.

To determine how CCCP inhibits probe export by the pumps, we performed disc diffusion tests (Fig. 7) using both types of top agar (see Materials and methods). In YPD agar, the cells will be growing on a fermentable carbon source and pump activity will be high [25] (Fig. 7A). The absence of zones in Pdr5p- and/or Snq2p-expressing strains AD12, AD13 and US50–18C in contrast to AD1–3 and AD23 clearly shows that CCCP is a substrate of both Pdr5p and Snq2p. In Fig. 7B, where YPGE agar contains a non-fermentable carbon source [11,12], CCCP is a much weaker substrate of Pdr5p than of Snq2p. The growth inhibition zones for strains having Snq2p (AD13, US50–18C) are much smaller than those for AD1–3 and AD23. The zones for AD12 and AD1–3 have almost the same size but a different “clarity”. The negligible difference in the appearance of zones for AD12 and AD1–3 is not given by an extremely low level of Pdr5p in AD12 cells (in comparison with that of AD13) because under the same growth conditions nigericin is still effectively expelled from the cells (see Fig. 3A).

The finding that CCCP interacts with both Pdr5p and Snq2p points to another important diagnostic application of our fluorescence screening system. Namely, the method makes it possible not only to detect if a compound inhibits the probe transport by both pumps but can also document the different ability of the compound to inhibit each of the pumps.

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