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THE DUAL MECHANISM OF THE ANTIFUNGAL EFFECT OF NEW LYSOSOMOTROPIC AGENTS ON THE *SACCHAROMYCES CEREVISIAE* RXII STRAIN

ANNA KRASOWSKA[1]*, LUCYNA CHMIELEWSKA[1], JACEK ŁUCZYŃSKI[2], STANISŁAW WITEK[2] and KAREL SIGLER[3]
[1]Institute of Microbiology, Wrocław University, Przybyszewskiego 63-77, 51-148 Wrocław, Poland, [2]Chemistry Dept., Technical University of Wrocław, Wyb. Wyspiańskiego 27, 50-370 Wrocław, Poland, [3]Institute of Microbiology, Acad. Sci. Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic

Abstract: Quinacrine was used to visualize the intracellular pH changes in the yeast strain *Saccharomyces cerevisiae* RXII occurring after exposure to four recently-synthesized lysosomotropic drugs: DM-11, PY-11, PYG-12s and DMAL-12s. The cells took up quinacrine, mostly accumulating it in their vacuoles. DM-11 and PY-11 gave rise to diffuse quinacrine fluorescence throughout the cells, with the vacuoles staining to a somewhat greater extent than the cytosol. This quinacrine-detected overall acidification of the cell interior is very probably caused by blocking of plasma membrane H^+-ATPase. PYG-12s gave rise to a strong vacuolar accumulation of the dye. Like the vacuolar ATPase inhibitor bafilomycin A_1, DMAL-12s strongly lowered the intensity of quinacrine fluorescence. Owing to its low pK_a, it can penetrate rapidly into the cells and may inhibit vacuolar H^+-ATPase and prevent quinacrine-detectable vacuolar acidification without causing strong cell acidification. Since these drugs were found to penetrate into the cells, their lack of effect may reflect a higher resistance of both plasma membrane H^+-ATPase and vacuolar ATPase to the drugs. Our data indicate that the lysosomotropic drugs under study have a dual action. On entering the cell, they cause intracellular acidification, very probably by inhibiting plasma membrane H^+-ATPase and curtailing active proton pumping from the cells. Furthermore, they interfere with the function of V-type ATPase, causing vacuolar alkalinization and eventually cell death.

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* Corresponding author, E-mail: aniak@microb.uni.wroc.pl
Abbreviations used: DM-n – 2-dimethylamino ethyl esters of saturated fatty acids; PY-n pyrrolidine ethyl esters of saturated fatty acids; DMAL-12s – dodecyl-N,N-dimethylalanine oxalate; PYG-12s – dodecyl-N,N-pyrrolidinoglycine oxalate; DMSO – dimethyl sulfoxide; TMS - tetramethylsilane

Key Words: *Saccharomyces cerevisiae*, Lysosomotropic Drugs, H^+ATPase, V-ATPase

INTRODUCTION

The term "lysosomotropic compounds" was introduced by deDuve ***et al.*** [1]. The unprotonated form of these drugs, which are mostly weak bases, easily penetrates cell membranes, and the protonated form accumulates in acidic cell compartments (vacuoles, lysosomes or endosomes). At pH above the pK_a value of the drugs, their unprotonated forms dominate; thus, at higher external pH values the drugs cross the plasma membrane more easily and their concentration in the vacuoles becomes higher. When they exceed the critical micellar concentration in the vacuoles, lysosomotropic drugs act as detergents and destroy the tonoplast [2]. This results in the vacuolar hydrolytic enzymes causing cell autolysis.

Two well-known and very useful lysosomotropic agents are quinacrine and chloroquine – diprotic weak bases that have been used for over 60 years in the treatment of malaria and rheumatoid disorders [3]. Quinacrine has the properties of a fluorescent dye, and is often used as an intracellular acidification marker [4]; it can conveniently be used in determining H^+-ATPase activity ***in vivo***.

The yeast *Saccharomyces cerevisiae* seems to be an organism highly suited for use in studying the mechanism of the action of lysosomotropic drugs. Yeast vacuoles are acidic, with a vacuolar pH of about 5.5, owing to the action of V-type H^+-ATPase. This enzyme couples the transport of protons into the vacuolar interior with the hydrolysis of ATP, and establishes an electrochemical gradient of protons [5]. Among its other functions, V-type ATPase is involved in the regulation of cytoplasmic pH [6, 7] and acidification of other intracellular compartments [8]. Cytoplasm pH is also regulated by plasma membrane H^+-ATPase, which transports protons out of the cells. Its blocking by vanadate or orthophosphate causes acidification of the cytosol and vacuoles. V-ATPase inhibitors such as macrolide antibiotics (bafilomycin A_1 or concanamycin A) induce vacuolar alkalinisation [5, 9].

Investigations were done into the antifungal activity and biochemical properties of a series of 2-dimethylamino (DM-n) and pyrrolidine (PY-n) ethyl esters of saturated fatty acids possessing the properties of lysosomotropic compounds [10, 11]. These compounds suppress the growth of *S. cerevisiae* and other yeast-like microorganisms [12], inhibit yeast plasma membrane H^+-ATPase [10] and are substrates of the *S. cerevisiae* PDR transporters [13].

We prepared and investigated the structure, chemical properties and biological activity of new DMAL-12s (N,N-dimethylalanine) and PYG 12s (N,N-dimethylglycine) esters. These newly-synthesized lysosomotropic drugs, which have potent antifungal function, have an interesting dual mechanism of action. Their surfactant-lysosomotropic action strongly affects both plasma membrane H^+-ATPase and V-ATPase and brings about a change in intracellular pH.

MATERIALS AND METHODS

Compounds

DMAL-12s was synthesized in our laboratory by amination of n-dodecyl 2-bromopropionate with dimethylamine in ethereal solution by heating under reflux for 5 hours. The resulting dimethylamine hydrobromide was separated by filtration. The filtrate was then evaporated on a rotary evaporator and the residue was treated with saturated sodium bicarbonate solution and extracted twice with diethyl ether. The extract was dried under $MgSO_4$, and the ether was evaporated to dryness. The oily residue was treated with an equimolar amount of oxalic acid in acetone at room temperature, and the ensuing oxalate was separated by filtration and dried. PYG-12s was analogously obtained as oxalate by amination of n-dodecyl chloroacetate with pyrrolidine. Both compounds and their standards, DM-11 and PY-11, were of analytical grade as checked by GLC analysis of free amino acids (purity > 99.5 %) and ^1H NMR spectra (Bruker Avance DRX_{300} instrument in deutero-chloroform, TMS as internal standard). Quinacrine and bafilomycin A_1 were obtained from Sigma (Poland), methanol and DMSO from P.O.Ch. (Polish Chemical Reagents), and glucose, bacto peptone, technical agar, sodium and potassium phosphates from Difco (Poland). The structures of the test compounds are given in Fig. 1.

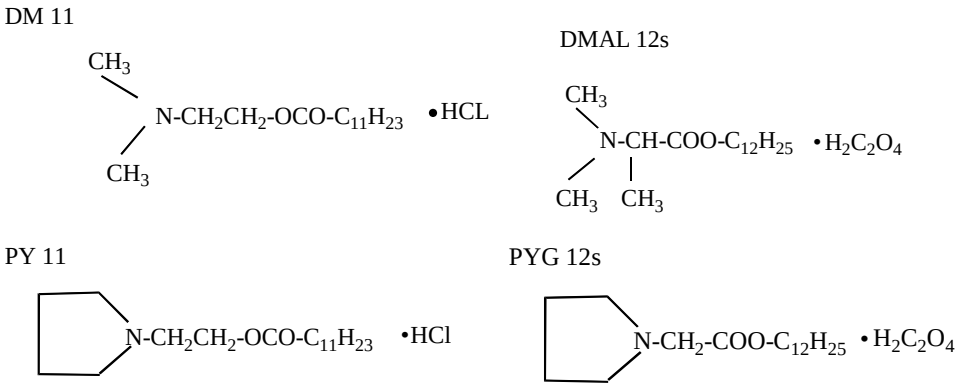


Fig. 1. The chemical structure of the studied lysosomotropic compounds.

pK_a determination

The apparent dissociation constants (pK_a) of the drugs were determined pH-metrically in methanol-water mixtures. An EH-01-4 glass electrode (HYDROMET) and a reference AgCl electrode were used for a calibration procedure based on a four-parameter equation ($pH = \alpha + SpcH + jH\ [H^+] + jOH\ [OH^-]$). This procedure yielded pH readings based on the concentration scale (pcH). The Yasuda-Shedlovsky [14] extrapolation ($p(s)Ka + \log [H_2O] = A/\varepsilon + B$), where ε is the dielectric constant of the aqueous solution of methanol, was

used to derive the dissociation constants (pK_a) by extrapolating from methanol-water solutions.

Strain

The *Saccharomyces cerevisiae* RXII yeast strain was used in the experiments.

Antifungal activity

Antifungal activity was evaluated *in vitro* according to Hussain [15]. Cell survival was determined on a Sabouraud medium (1% bacto peptone, 2% glucose, 2% technical agar) supplemented with ethanol (2% final concentration) solutions of the drugs at appropriate concentrations (1 mM to 1 μM). The pH of the Sabouraud medium was adjusted to 6.0 or 8.0 by adding phosphate buffer (20 mM). The medium (100 ml) was inoculated with 0.1 ml of an overnight pre-culture and incubated for 20 h. The cells were plated on Sabouraud medium plates containing different concentrations of the tested compounds, and the number of colonies was counted after a 72-h incubation at 28ºC. The percentage rate of survival was determined as a mean from three plates.

Quinacrine fluorescence assay

The fluorescent dye quinacrine was used to label the acidic compartments of the cells, essentially as described previously [9]. Cells were grown for 20 h on the Sabouraud medium, pelletted by centrifugation at 3000 rpm for 3 minutes and resuspended in phosphate buffer (50 mM, pH 7.6) with 2% glucose containing equivalent concentrations of the tested compounds. After a 15-minute incubation at 30ºC, the cell suspension was supplied with quinacrine (final concentration 200 μM) and incubated for 15 minutes. The cells were pelletted, and then washed three times with and resuspended in cold phosphate buffer (pH 7.6). They were kept on ice and examined under an Olympus BX-60 fluorescence microscope and in a SFM25 spectrofluorometer (Kontron) within an hour. For fluorescent micrographs, a set of filters was used with an excitation band-pass of 450-490 nm and emission band-pass of 515-545 nm.

RESULTS

Both PYG-12s and DMAL-12s showed a typical pH-dependent lysosomotropic antifungal action that increased with increasing pH from 6.0 to 8.0 (Tab. 1). At pH 6.0, they were less effective against *S. cerevisiae* than DM-11 and PY-11, while at pH 8.0 they strongly inhibited the growth of *S. cerevisiae* (Tab. 1).

The efficiency of the compounds was dependent on their pK_a values (Tab. 2). PYG 12s and DMAL-12s, which had the lowest pK_a, were the most active compounds at pH 8.0.

The penetration of the intracellular pH indicator quinacrine through the plasma membrane strongly depended on the extracellular pH (Fig. 2) quinacrine fluorescence from the cells was only noticeable at external pH higher than 6.5.

Tab. 1. MIC50 [μg/ml] of lysosomotropic drugs tested on *Saccharomyces cerevisiae* RXII.

Compound	MIC50 [μg/ml]	
	pH=6.0	pH=8.0
DMAL 12s	31,2	1,8
DM 11	12,28	3,3
PYG 12s	96,5	0,7
PY 11	55,4	5

Tab. 2. pK_a values of lysosomotropic drugs.

Compound	pK_{a1}	pK_{a2}
DM 11	-	8.06
DMAL 12s	-	6.97
PYG 12s	3.6	7.6
PY 11	-	8.41
quinacrine	7.8	9.72

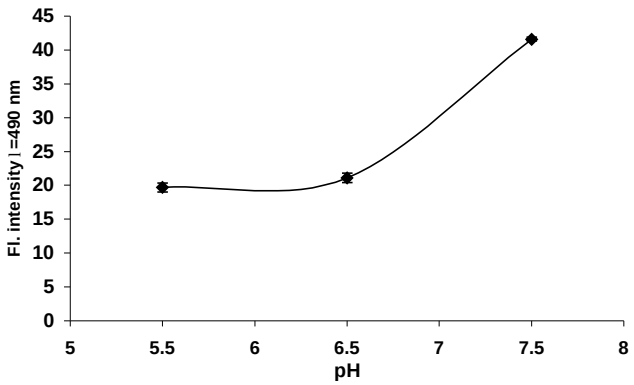


Fig. 2. The dependence of quinacrine (Q) fluorescence intensity in *S. cerevisiae* RXII cells on external pH ± SD (n=3).

High concentrations of quinacrine strongly inhibited the penetration of the dye into the cells (Fig. 3). This is in keeping with the finding that high concentrations of quinacrine inhibit vacuolar ATPase [16]. The highest fluorescence was found at 200 μM external quinacrine, and this concentration was then used in all subsequent experiments.

None of the four lysosomotropic compounds was found to interact with quinacrine or affect its fluorescence (data not shown). The extent to which they affected intracellular quinacrine fluorescence depended on their concentration (Fig. 4).

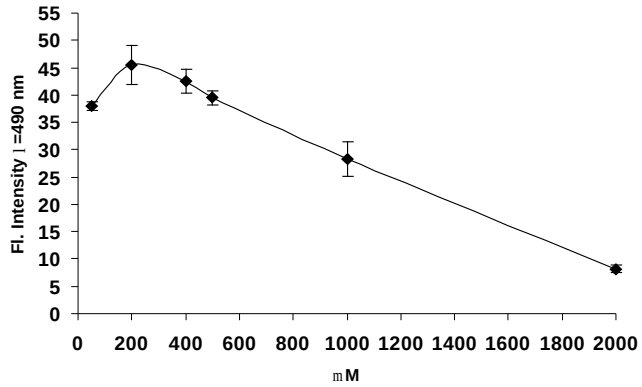


Fig. 3. The dependence of quinacrine (Q) fluorescence intensity in *S. cerevisiae* RXII cells on the external dye concentration. Cells (10^7/ml) were exposed to Q for 15 min. External Q was then washed off and Q fluorescence from the cells measured ± SD (n=3).

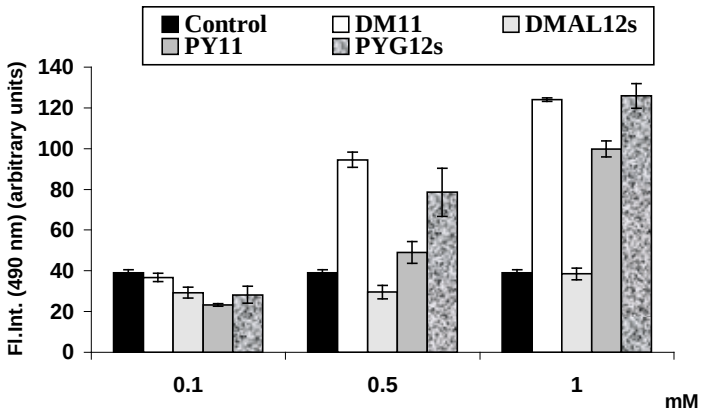


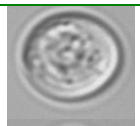
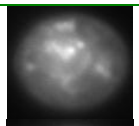
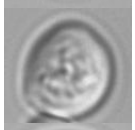
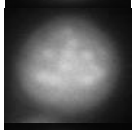
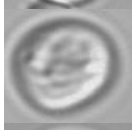
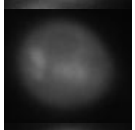
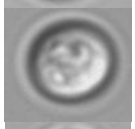
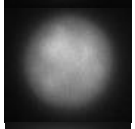
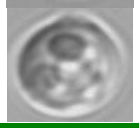
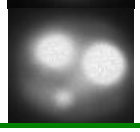
Fig. 4. Changes in Q fluorescence in *S. cerevisiae* cells caused by lysosomotropic drugs ± SD (n=3).

At 0.1 mM, they had only a slight effect on the fluorescence of *S. cerevisiae* whereas at 0.5 and 1 mM their effect was mostly well-detectable, except for that of DMAL 12s. At this concentration, a 15-min application of the compounds as a rule caused an increase in quinacrine fluorescence. This effect was strongest in the cases of DM-11 and PYG-12s (Fig. 4). Again, the exception was DMAL-12s, which failed to enhance quinacrine fluorescence over the control level (Fig. 4).

Quinacrine localization inside the cells was visualized by microscopy (Tab. 3). In *S. cerevisiae* cells, quinacrine was found to accumulate in the vacuoles. PY-11 caused a similar cytosol acidification and PYG-12s caused only vacuolar acidification, while DMAL-12s induced weak quinacrine uptake into the cells

(Tab. 3). Like the vacuolar ATPase inhibitor bafilomycin A_1 (Fig. 5), DMAL-12s strongly lowered the quinacrine fluorescence intensity (Tab. 3).

Tab.3. Nomarski and fluorescent micrographs of Q and lysosomotropic drug-treated cells of *S. cerevisiae* (1000x magnification, computer-adjusted).

Compound	Nomarski micrographs	Fluorescent micrographs
Quinacrine (Q) /control		
DM11 + Q		
DMAL 12s + Q		
PY11 + Q		
PYG12s + Q		

A. B.

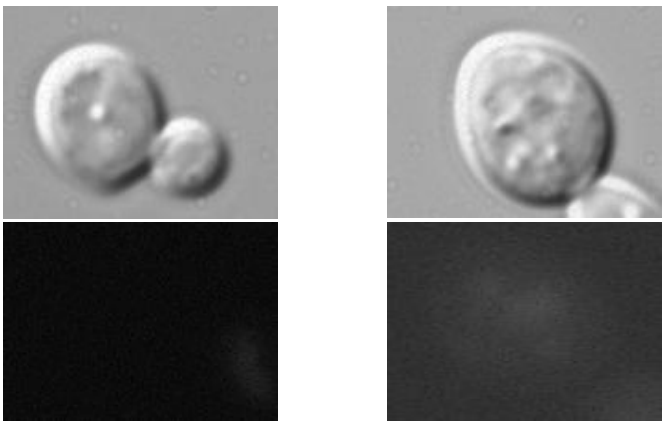


Fig. 5. Q (200 μM) fluorescence from *S. cerevisiae* RXII treated with A. 0.5 μM bafilomycin A_1, B. control

DISCUSSION

The pH-indicator quinacrine has been found to be taken up by *S. cerevisiae* cells within 15 min and then to remain in the cells for 60 min or more without reappearing in the outer medium (data not shown). Increasing the external quinacrine concentration first brings about an enhancement and then a progressive decline of its fluorescence from the cells. This is obviously due to higher intracellular quinacrine concentrations exerting an inhibitory effect on the vacuolar H^+-ATPase, thereby hampering the intravacuolar acidification and the uptake of the dye into the no-longer-acidic vacuoles [16]. This effect of quinacrine is confirmed by the fact that the intensity of quinacrine fluorescence from *S. cerevisiae* RXII cells increases with increasing external pH (quinacrine pK_a – Tab. 2.).

Given this knowledge of quinacrine properties, we can analyze the action of the newly-synthesized lysosomotropic compounds DM-11, DMAL-12s, PY-11 and PYG-12s on the model organism *S. cerevisiae*. As seen from Fig. 4, a 15-minute treatment of cells by increasing concentrations of DM-11, PY-11 and PYG-12s before quinacrine addition brings about an increase in the fluorescence intensity of the dye from the cells. This has been attributed to an overall acidification of the cell interior caused by a blocking of plasma membrane H^+-ATPase. This blocking has previously been documented for DM-11 and PY-11 [17].

DMAL-12s behaves differently. In its presence, the quinacrine fluorescence intensity essentially remains the same as in the control irrespective of DMAL-12s concentration. The reason may be that DMAL-12s, owing to its low pK_a (Tab. 2), penetrates rapidly into the cells and, without affecting the plasma membrane H^+-ATPase and causing overall cell acidification relative to the control, strongly inhibits vacuolar H^+-ATPase before quinacrine is added.

Fluorescence microphotographs (Tab. 3) provide information on the actual localization of quinacrine in cells treated with the lysosomotropic compounds. In the control cells of *S. cerevisiae,* quinacrine appears to be localized mostly in the vacuoles, where it forms strongly stained “islands”. In the presence of DM-11, the dye becomes distributed diffusely throughout the cells, with vacuoles being stained somewhat more than the cytosol. In keeping with the fluorimetric data, DMAL-12s strongly lowers the quinacrine fluorescence intensity. Its action seems to be very similar to that of bafilomycin A_1, a known inhibitor of vacuolar ATPase [16]. PY-11 brings about cytoplasmic acidification and diffuse quinacrine distribution in *S. cerevisiae* cells, while PYG-12s gives rise to a strong unstructured vacuolar localization of the dye.

In conclusion: our data on quinacrine uptake and distribution in *S. cerevisiae* cells indicate that the lysosomotropic compounds under study have a dual action. On entering the cell, they cause intracellular acidification, very probably by inhibiting plasma membrane H^+-ATPase and curtailing active proton pumping from the cells. Secondly, they probably interfere with the function of V-type ATPase, thereby causing vacuolar alkalinization.

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