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Effects of Acridine Derivatives on Ca^{2+} Uptake by *Candida albicans*

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Abstract

The effects of several acridine derivatives, and chloroquine, which has a similar lateral chain to quinacrine, but with a quinoline nucleus, were studied on a strain of *Candida albicans*. Parameters estimated were: a) dichloromethane/water partition coefficients; b) uptake by cells; c) effects on respiration, d) effects on the acidification of the medium; e) efflux of K^+; f) uptake of $86Rb^+$ and $45Ca^{2+}$, and d) effects on growth of cells. Results obtained in general: a) most of them showed a low hydrophobicity; b) most of them were significantly taken up by cells; c) acridine orange, acridine yellow, quinacrine and nonyl acridine orange inhibited respiration; d) acridine orange, quinacrine and nonyl acridine orange inhibited acidification of the medium. The most significant finding was that acridine orange, quinacrine and nonyl acridine orange at 60 µM or 120 µM, and acriflavine at 120 µM produced an efflux of K^+, an inhibition of $86Rb^+$ uptake, and a remarkable many fold increase of $45Ca^{2+}$ uptake. Acridine orange and acridine yellow produced only a decrease of duplication time; with the concentrations used, only nonyl acridine orange inhibited growth. It is suggested that quinacrine may be used as an adjuvant or topical agent against candidiasis. Chemical derivatives of some of the dyes might also be used against pathogenic fungi.

Keywords Acridine derivatives; Ca^{2+} uptake; *Candida albicans*

Introduction

The diversity of organic molecules synthesized for more than one century is enormous; many of them are dyes with most varied industrial uses to stain all kinds of materials. Due to large-scale production and extensive application, synthetic dyes can cause considerable environmental pollution and are serious health-risk factors [1]. They may potentially generate ROS (reactive oxygen species), leading to oxidative stress (OS) and toxicity [2]. Methylene blue, for instance, at concentrations above 5 µM increases intracellular ROS and OS as evidenced by oxidation of glutathione (GSH), vitamin C and dihydrofluorescein [3], but in earlier studies almost a century ago [4], or more [5], treated several patients with malaria, using methylene blue.

Cationic molecules include synthetic dyes with considerable structural basic diversity, such as the derivatives of acridine, triphenylmethane, phenazines, phenotiazines, and quinoline derivatives. Some belong to the imine-iminium class rhodamines, malachite green, fuchsine, crystal violet auramine and cyanins. Many of them are characterized by a basic structure with amino groups with a variable degree of substitutions of -H, by methyl or ethyl groups.

The majority of them are toxic to animals, but many have been synthesized and tested regarding their capacity to inhibit growth or even kill bacteria, fungi or protozoa, responsible for different human or animal infections

Malachite green has been extensively used as a topical fungicide and parasiticide in the fish industry throughout the world [6-8]. It is well known, however, that leucomalachite green, in spite of having two dimethylated amino groups does not appear to affect living cells. Crystal violet was commonly used for treatment of oral and vaginal candidiasis or for sterilization during operations until the 1960s. However, triphenylmethane derivatives have been banned in almost all the world, mainly because of studies that revealed chronic toxicity and carcinogenicity of gentian violet in mice [9].

Auramine is a diphenylmethane imine with two dimethylated amino groups with structural resemblance to acridine derivatives. In yeast, it causes DNA damage, aneuploidy and mitotic recombination. It is used in some countries as a food colorant and mostly to colour smoke in firework displays and in military applications. This dye induces forward mutations in Salmonella typhimurium in the presence of metabolic activation [10], and generates DNA strand breaks in primary cultures of rat hepatocytes [11] and human cell line HuF22 in the absence of metabolic activation [12].

Acridine orange is a slightly cationic fluorochrome stain capable of permeating cell and organelle membrane structures. It is commonly used in fluorescence microscopy and flow cytometry analysis of cellular physiology and cell cycle status. Acridine yellow has been used as a topical antiseptic, as a fluorescent stain in histology and more recently to estimate and measure the plasma membrane potential in Saccharomyces cerevisiae [13].

Early studies on the effects of cationic agents on microorganisms were limited to whether they inhibited growth, mainly of bacteria and fungi, but then it was found that ethidium bromide at rather low concentrations inhibits the transport of K+ in yeast, but at higher ones produces its efflux [14]. Later on, it was found that this compound, as well as other cationic dyes significantly stimulate Ca^{2+} uptake [15]; similar effects of other cationic dyes were found [16,17].

More recent work on the effects of cationic agents on ion homeostasis in yeast was started by Courchesne [18] who found a fungistatic action of amiodarone apparently due to an alteration of Ca^{2+} homeostasis in *S. cerevisiae* [19]. These studies were continued by Muend and Rao [20], and later by Maresova et al. [21], who found a plasma membrane hyperpolarization that drives Ca^{2+} influx into *S.*

cerevisiae. This was also found with other antifungals, as ketoconazole, miconazole [22], and fluconazole [23,24]. This hyperpolarization was confirmed and explained [25] by the primary efflux of K+ produced by the drugs (see Discussion).

Whether the increased Ca^{2+} uptake can induce apoptosis and death of cells is not clear. Gamarra et al. [26] have proposed use of amiodarone, which stimulates Ca^{2+} uptake, combined with fluconazole as a possible antifungal treatment against *Candida albicans*, even for a strain resistant to the antifungal. A similar therapeutic approach was also suggested for methylene blue by Schirmer et al. [27,28]. According to a general mechanism proposal, it was decided to evaluate a group of acridine derivatives, to verify whether it can or cannot be applied. Expecting them to inhibit yeast growth, we used the pathogenic yeast *C. albicans* as an experimental subject. Besides amiodarone, among the acridine derivatives tested, three of them had the most interesting effects, producing a remarkable increase of Ca^{2+} uptake by cells, and three of them which either retarded or inhibited growth at the concentrations used.

Materials and Methods

Yeast

Yeast used was *C. albicans*, strain ATCC 10231, reported by the American Type Culture Collection as isolated from a patient with bronchomycosis.

Growth and handling of the cells

The cells were maintained in YPD plates (2% glucose, 2% peptone, 1% yeast extract, 2% agar) and cultures were renewed once a month. To grow cells for the experiments, a loopful from the plates was placed in 0.5 L of the same medium without agar, incubating them for 24 h in 1 L Erlenmeyer flasks in an orbital shaker at 30°C and 250 RPM. So as to have a better appreciation of the effects of the substrates used in the experiments, cells were then starved. To that purpose, they were collected and washed once by centrifugation, and suspended in 250 ml of water. They were placed in a 1 L Erlenmeyer flask and subjected to aeration in the shaker at 30°C for 48 h, required to decrease its endogenous substrates. Finally, cells were collected and washed with water by centrifugation. The final pellet obtained was weighed and suspended in water at a 1:1 ratio (w/v). Cells were maintained on ice and used during the same day.

Compounds tested

The following acridine derivatives were tested: 9-aminoacridine, acridine orange, acridine yellow, acriflavine, auramine, quinacrine and nonyl acridine orange (Table 1). In order to get information about the requirement of the acridine nucleus of quinacrine, chloroquine was also tested because it has the same lateral chain as quinacrine, but with a quinoline nucleus. Likewise, the nonyl derivative of acridine orange was used to test the effect of an additional alkyl chain. In some experiments, the effects were compared to those of amiodarone.

Acridines	Quinolines	With tail
9-aminoacridine. 9-Azaanthracene. Sigma.	Chloroquine (Aralen). 4-N-(7-chloroquinolin-4-yl)-1-N,1-N-diethylpentane-1,4-diamine diphosphate salt. Extracted from Aralen (Merck) pills; with dichloromethane, recrystallized in ethanol and dried.	Acridine orange, nonyl bromide. 3-N,3-N,6-N,6-N-tetramethyl-10-nonylacridin-10-ium-3,6-diamine; bromide. Sigma.
Acridine orange-HCl. 3-N,3-N,6-N,6-N-tetramethylacridine-3,6-diamine. Sigma.		
Acridine yellow. 2,7-dimethylacridine-3,6-diamine; hydrochloride.		
Acriflavine. 3,6-Diamino-10-methylacridinium. Polysciences.		
Auramine. 4-[4-(dimethylamino)benzenecarboximidoyl]-N, N-dimethylaniline		
dimethylazanium; chloride. Sigma.		
Quinacrine (atabrine). 4-N-(6-chloro-2-methoxyacridin-9-yl)-1-N, 1-N-diethylpentane-1, 4-diamine; dihydrochloride. Sigma.		

Table 1: Compounds used in this work, IUPAC name and origin.

Distribution coefficients

They were determined as the concentration ratio of the different dyes between 10 mM MES (morpholinoethane sulfonic acid)-TEA (triethanolamine) buffer, pH 6.0 and an equal volume (3.0 ml) of dichloromethane. The experiments were performed by adding the indicated concentrations of each cationic agent to the two component mixture. The mixture was vigorously stirred in a vortex mixer, and then centrifuged. Afterwards, the upper layer was adequately diluted and its absorbance was measured at its previously determined maximal wavelength. The values obtained were compared to the absorbance of a known concentration of each dye in the same medium. Values are given as the dichloromethane/water concentration ratio.

Uptake by the cells

To measure the uptake of the dyes, 50 mg of cells were added to the following mixture: 10 mM MES-TEA buffer, 20 µM glucose, and either 60 µM or 120 µM of the dyes, in a final volume of 5.0 ml at 30°C. After 10 min, cells were centrifuged and the absorbance of a convenient dilution of the supernatant was measured at the adequate wavelength. The amount of each dye taken up was calculated by comparing the absorbance values of the supernatant with those from standard concentrations for each dye.

K^+ transport and acidification

The effects of the different agents on potassium transport and acidification of the medium were followed by means of pH and K^+ selective electrodes connected to a pH meter and an adequate acquisition system. Cells, 100 mg wet weight were incubated in 2 µM

MES-TEA, pH 6.0, 20 μM glucose, in a final volume of 10.0 ml. 60 μM and 120 μM concentrations of the dyes were added. Experiments were carried out in a chamber with continuous magnetic stirring, maintained at 30°C.

K^+ efflux

It was found that several of the cationic agents affect the response of a K^+ selective electrode; results are not presented, and the efflux they produced was followed by incubating cells as follows. Cells (62.5 mg wet weight) were incubated for 10 min at 30°C in 10 μM MES-TEA, pH 6.0, 20 μM glucose with two different concentrations of each dye (60 μM and 120 μM) in a final volume of 5 ml. After incubation, cells were centrifuged and K^+ in the supernatant was measured by flame photometry. Total K^+ in the cells was obtained by boiling an equal number of cells with no dye added during 20 min. Results are given as percentage of the total K^+ content of control cells.

Oxygen consumption measurements

Oxygen concentrations were followed with a Clark oxygen electrode connected to a polarization and measurement device (Yellow Springs), collecting the data with a computer in a closed jacketed chamber maintained at 30°C. The medium contained 10 μM MES-TEA buffer, pH 6.0; 20 μM glucose, in a final volume of 5.0 ml. Tracings were started with 50 mg (wet weight) of starved cells to follow the oxygen consumption rate under continuous stirring. When indicated, variable amounts of the dyes were added as shown in the figures.

$86Rb^+$ and $45Ca^{2+}$ uptake

Uptake of 86 Rb^+ or 45 Ca^{2+} was measured by adding 12.5 mg of cells (25 μl of the cell suspension) to 10 μM MES-TEA, pH 6.0, and 20 μM glucose, 0.5 ml final volume, plus the indicated concentrations of the dyes in a water bath at 30°C. After 2 min, 0.6 μM of 86 RbCl, or 50 μM 45 $CaCl_2$ were added, and after two more min, aliquots of 0.1 ml were taken. They were filtered through a 0.45 μm nitrocellulose filter and washed twice with 1 ml of 10 μM $MgCl_2$ and 1 ml of water. The filters were then dried and placed in vials to which scintillation liquid was added, and counted in a Tri-Carb Perkin Elmer scintillation counter. To measure the total counts, 50 μl aliquots of the whole incubation mixture were also dried in scintillation vials and treated as the samples.

Effects on growth

Growth was followed in 100 well plates of a Bioscreen C instrument by measuring the absorbance increase of each sample at 600 nm. To this purpose, 250 μl of cells in YPD medium, adjusted to an optical density of 0.03 were placed in wells of the instrument plates. The indicated concentrations of the different agents were added, and the experiment was run for 24 h at 30°C with high continuous shaking, taking readings each 30 min.

ζ potential

This parameter was obtained from the displacement velocity of cells under an electric field, measured directly under the microscope in a Zeta Meter ZM-75 cell electrophoremeter, which allows calculation of the electrophoretic mobility. From this last parameter, the zeta potential is obtained by the Helmholtz-Smoluchowski equation:

Zeta potential = EM 4π Vt/Dt

EM is the electrophoretic mobility

Vt is the viscosity of the liquid measured in poises, at the temperature of the measurement

Dt is the dielectric constant of the suspending fluid at the temperature of the measurement

4π = 12.57

The medium (13 ml final volume) contained 10 μM MES-TEA buffer pH 6.0, 5 mg of cells (wet weight), and different amounts of each dye. The potential difference applied was 100 V.

Results

Distribution coefficients in dichloromethane/water and uptake by the cells

Dichloromethane/water (MES-TEA buffer, pH 6.0) distribution coefficients and the uptake by cells were studied in the same medium plus 20 μM glucose. Most agents were not particularly hydrophobic (Table 2), except for the nonyl derivative of acridine orange. Regarding their uptake, several were taken by cells in a low proportion, but for acridine orange, acridine yellow, acriflavine at 120 μM, and quinacrine a high uptake was observed. Notably, practically all the nonyl acridine orange present in the medium was taken up, coincidentally with its hydrophobicity.

	Hydrophobicity		Uptake	
Dye	**60 μM**	**120 μM**	**60 μM**	**120 μM**
9AA	1.3 ± 0.1	0.9 ± 0.2	13.2 ± 8.4	62.0 ± 5.0
AO	4.5 ± 0.3	2.7 ± 0	147.0 ± 1.8	295.0 ±1.8
AY	2.6 ± 1.8	2.2 ± 1.5	117.3 ± 8.6	265.2 ± 6.2
Acr	0.2 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	164.7 ± 21.0
AU	1.9 ± 0.9	1.6 ± 0.4	0.0 ± 0.0	6.7 ± 2.3
Q	1.5 ± 0.7	1.1 ± 0.3	5.3 ± 2.7	30.0 ± 2.1
Chl	0.1 ± 0.0	0.1 ± 0.1	90.4 ± 11.8	200.8 ± 34.8
Nonyl AO	111.6 ± 40.3	107.3 ± 45.0	145.7 ± 0.6	295.3 ± 0.7

9AA: 9-Aminoacridine; AO: Acridine orange; AY: Acridine Yellow; Acr.: Acriflavin; AU: Auramine; Q: Quinacrine; Chl: Chloroquine; Nonyl AO: nonyl acridine orange

Table 2: Partition coefficients in dichloromethane (DCM)/water and uptake by the cells of the different agents tested in the experiments.

Partition coefficients were determined by adding the indicated concentrations of the dyes to two equal volumes of dichloromethane and 10 mM MES-TEA buffer, pH 6.0. After vigorous stirring in a vortex, the tubes were centrifuged and the concentration of the dye remaining in the aqueous layer was measured in a spectrophotometer at its respective maximal absorbance wavelength. Final concentration was calculated by comparing the absorbance values to those of a standard curve of each dye. Values are given as the ratio of the final concentrations in dichloromethane over those in water. Uptake was measured by incubating the cells (62.5 mg) in 10 μM MES-TEA, pH 6.0, 20 μM glucose, with the respective concentrations of the dyes,

centrifuging the cells, measuring the absorbance of the supernatant at the maximal wavelength, and comparing it against a standard concentration curve. Values given as the nmoles taken by the cells from a total of 150 nmoles at 60 µM, and 300 nmoles at 120 µM ± standard deviations. n=3.

Effects on respiration

Departing from the idea that cationic agents can be accumulated by the cells due to the electric plasma membrane potential difference (PMP), and that also an electric membrane potential difference exists in the inner mitochondrial membrane, it was considered that the dyes may be also accumulated by this organelle and alter respiration of the cells. Figure 1 shows only the tracings of dyes that produced an effect.

A delayed inhibition was produced by acridine orange. Acridine yellow and quinacrine produced a moderate inhibition while nonyl acridine orange produced a full inhibition of respiration. Moreover, the acceleration by 10 µM CCCP (carbonyl cyanide 3-chlorophenylhydrazone) was absent in the presence of these dyes.

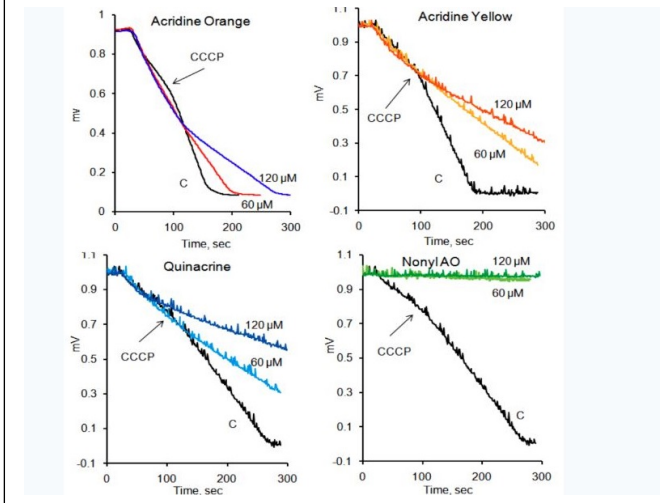

Figure 1: Effects of several organic cationic compounds on respiration by C. albicans. Cells, 50 mg, wet weight were added at the beginning of the tracing to 10 mM MES-TEA buffer, pH 6.0, 20 mM glucose, in the presence of the indicated concentrations of the dyes, final volume 5.0 ml. CCCP (10 µM) was added where indicated by the arrows. 1 mV= 2000 natg O. Only those dyes affecting respiration are presented. Graphs are representative of 4 different experiments.

Inhibition of the acidification of the medium

Transport of cations, mainly K, is affected by the PMP generated by the plasma membrane H^+-ATPase that pumps protons outside of the cells, so, the effect of the different agents on the acidification of the medium was estimated (Figure 2). Only those dyes affecting this parameter are shown. Quinacrine produced a moderate inhibition, acridine orange and its nonyl derivative produced an almost full inhibition of H^+ pumping.

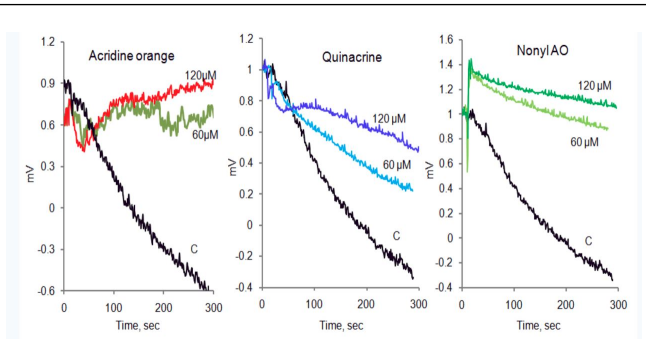

Figure 2: Effects of different dyes on proton pumping by C. albicans. Cells,100 mg wet weight, were added to a chamber maintained at a constant temperature of 30°C containing 2 mM MES-TEA buffer, pH 6.0, 20 mM glucose; final volume 10.0 ml. Only those dyes affecting acidification are presented. Graphs are representative of 4 different experiments.

The efflux of K^+

One possible effect of the different agents is that by modifying the surface charge of the plasma membrane they affect the equilibrium between its electrical potential difference and that of the uptake/efflux system for monovalent cations, as it was described for amiodarone [25]. It is also possible that they interact with the K^+ transporter, blocking the influx and resulting in an efflux of the cation. A significant efflux of K^+ produced by four of the dyes is shown in Table 3, either at 60 µM or 120 µM. The addition of a nonyl hydrophobic tail to acridine orange resulted in an increase of its potency.

Dye	60 µM	120 µM
9AA	00.0 ± 0.00	0.00 ± 0.00
AO	0.65 ± 0.22	0.76 ± 0.19
AY	0.00 ± 0.00	0.02 ± 0.04
Acr	0.12 ± 0.04	0.31± 0.03
AU	0.00 ± 0.00	0.08 ± 0.02
Q	0.42 ± 0.13	0.62 ± 0.12
Chl	0.00 ± 0.00	0.00 ± 0.00
Nonyl AO	0.76 ± 0.19	0.97 ± 0.13

Table 3: Efflux of K+ produced by two concentrations of the different cationic agents on *C. albicans.*

Efflux was measured by incubating cells (62.5 mg wet weight) for 10 min at 30°C in 10 mM MES-TEA, pH 6.0, 20 mM glucose with two different concentrations of each dye (60 and 120µM) in a final volume of 5 ml. After incubation, cells were centrifuged and K^+ in the supernatant was measured by flame photometry. Total K^+ in the cells was obtained by boiling an equal amount of cells with no dye added during 20 min; the cells were then centrifuged and K^+ was measured in the supernatant. Values are given as the fraction of total content of the cation in the cells ± standard deviations. n=at least 3.

86Rb^+ uptake

Their effects on the uptake of 86 Rb^+, reinforce the idea that dyes affect the transport of monovalent cations (Table 3). An almost complete correspondence existed between the effects of the several compounds on K^+ efflux and the inhibition of the uptake of this cation.

Dye	60 µM	120 µM
9AA	1.06 ± 0.30	1.01 ± 0.16
AO	0.38 ± 0.02	0.29 ± 0.07
AY	1.02 ± 0.30	0.72 ± 0.30
Acr	0.85 ± 0.30	0.40 ± 0.07
AU	0.95 ± 0.10	0.88 ± 0.20
Q	0.30 ± 0.10	0.37 ± 0.07
Chl	1.22 ± 0.20	0.93 ± 0.10
Nonyl AO	0.38 ± 0.20	0.15 ± 0.01

Table 4: Effects of different dyes on 86 Rb^+ uptake by *C. albicans*.

Uptake was measured by adding 12.5 mg of cells (25 µl of the cell suspension) to 10 µM MES-TEA, pH 6.0, and 20 M glucose, 0.5 ml final volume, plus the indicated concentrations of each dye in a water bath at 30°C. After 2 min, 0.6 µM 86RbCl, was added, and after two more min, aliquots of 0.1 ml were taken, filtered through a 0.45 µm nitrocellulose filter and washed twice with 1 ml of 10 µM $MgCl_2$ and 1 ml of water. Filters were dried and placed in vials to which scintillation liquid was added, and counted in a Tri-Carb Perkin Elmer scintillation counter. Total counts were measured in aliquots of the whole incubation mixture dried in scintillation vials to be counted. Values are expressed as the fraction of the control without dye ± standard deviations, n=3.

Effects on Ca^{2+} uptake

Again, according to the general scheme considered for the mechanism of action from these cationic agents on yeast, when they produce the efflux of K^+, an increase of the plasma membrane electric potential should be produced, giving rise to the increased uptake of Ca^{2+}.

Dye	60 µM	120 µM
9AA	2.5 ± 1.7	3.6 ± 2.3
AO	27.7 ± 5.0	46.6 ± 4.2
AY	0.5 ± 0.2	1.2 ± 0.6
Acr	2.8 ± 0.5	13.0 ± 2.6
AU	0.8 ± 0.2	0.9 ± 0.3
Q	21.9 ± 5.0	23.3 ± 7.5
Chl	1.2 ± 0.3	1.3 ± 0.3
Nonyl AO	27.3 ± 0.2	21.3 ± 2.6

Table 5: Effects of dyes on 45Ca^{2+} uptake by *C. albicans*.

An almost complete coincidence can be seen in Table 5; those agents not producing an efflux of K^+, also did not produce an increased Ca^{2+} uptake, and those that produced an efflux of K^+ increased Ca^{2+} uptake.

Cells, 12.5 mg of cells (25 µl of the cell suspension) were added to 10 µM MES-TEA, pH 6.0 µM and 20 µM glucose, 0.5 ml final volume, plus the indicated concentrations of the dyes in a water bath at 30°C. After 2 min, 50 µM 45$CaCl_2$ was added, and after two more min, aliquots of 0.1 ml were taken. They were filtered through a 0.45 µm nitrocellulose filter and washed twice with 1 ml of 10 µM, $MgCl_2$ and 1 ml of water. Filters were placed in vials and dried; scintillation liquid was added, to count them in a Tri- Carb Perkin Elmer scintillation counter. To measure the total counts, 50 µl aliquots of the whole incubation mixture were also dried in scintillation vials and treated as the samples. Values are given as times over control ± standard deviations.

Growth inhibition

Significant growth inhibition of *C. albicans* at dye concentrations of 60 µM or 120 µM are shown in Figure 3. Acridine orange and acridine yellow produced a growth delay from 60 µM, and very similar at 120 µM, more pronounce with AY. Most interesting is that nonyl acridine orange at both concentrations produced a complete inhibition of growth. No significant modifications in rates of yeast growth were observed with the other dyes.

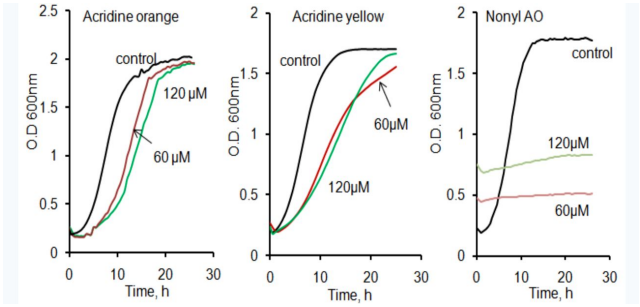

Figure 3: Effects of different dyes on growth of *C. albicans* Curves were followed in 100 well plates of a Bioscreen C instrument by measuring the absorbance increase of each sample at 600 nm. The indicated concentrations of the different agents were added, and the experiment was run for 24 h at 30°C with high continuous shaking, taking readings each 30 min. Only those dyes affecting growth are presented. Graphs are representative of 4 different experiments.

Comparing quinacrine and acridine orange to amiodarone

We decided to compare the effects of the dyes with those of amiodarone, which has been found to produce a notable increase of Ca^{2+} uptake by yeast [18,19], and this agent has been even used to control *C. albicans* infections by combining it with fluconazole [26]. The effects of both quinacrine and acridine orange on Ca^{2+} uptake was compared to those of amiodarone, both by following their effects against time and by testing different concentrations. The time curve (Figure 4a) shows that quinacrine and acridine orange effect was much faster than that of amiodarone. On the other hand, by comparing the respective concentration curves (Figure 4b), also acridine orange effects, but more so of quinacrine required a minimal concentration of

around 25 μM, while amiodarone started increasing Ca^{2+} uptake at a concentration of around 60 μM.

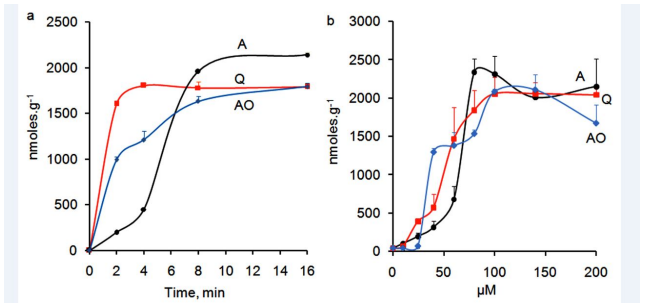

Figure 4: Stimulation of 45Ca2+ uptake by quinacrine (Q), acridine orange (AO), or amiodarone (A) on *C. albicans*; Uptake was measured as described for Table 5, but in a), uptake was followed at different times with 60 μM of each, amiodarone, quinacrine or acridine orange; in b), uptake was measured after an incubation of 4 min with different concentrations of the dyes. Bars indicate the standard deviation from the means. n=3ζ potential.

Most of the compounds tested produced a moderate decrease of the ζ potential (to less negative values), except auramine. Others produced a moderate decrease of the ζ potential of the cells (Figure 5).

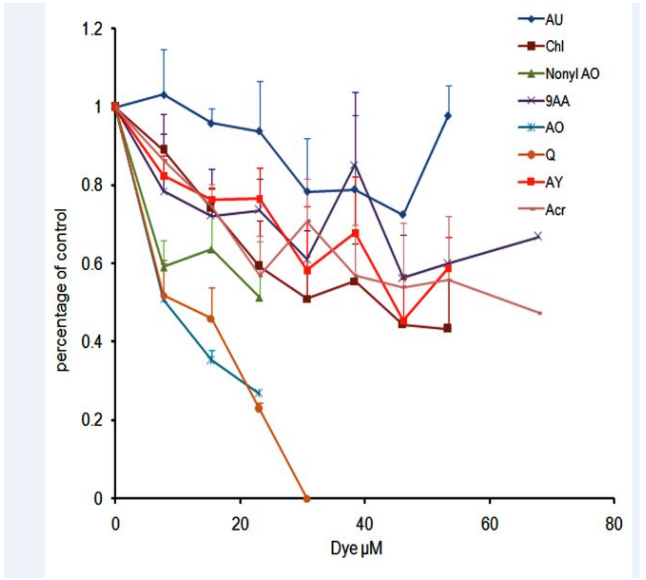

Figure 5: Relative values of the ζ potential of the cells incubated at different concentrations of the dyes in the absence of a substrate. Cells, 5 mg, wet weight, were incubated in 13 ml of 10 mM MES-TEA, pH 6.0 in cold water, and the surface potential was measured by following their movility in an electrophoremeter. From this, the ζ potential was calculated as described under Materials and methods. Values are given as the percentage absolute change compared to the control ± the standard deviation of 5 similar experiments

It is important to mention that quinacrine already at 30 μM produced the total immobilization of the cells under the electric field applied, but also that when higher concentrations were used, the cells not only decreased their migration towards the cathode of the chamber, but then moved in the opposite direction, i.e., they did not only decrease the negative membrane potential of the cells, but reversed it.

Discussion

The results suggest that the uptake of the different agents does not depend, at least as a main factor, on their hydrophobicity; the most relevant property appears to be their positive charge, and all of them were significantly taken by the cells; quinacrine was taken in a smaller proportion than the others, and auramine practically did not enter the cells.

Besides, it appears that net uptake may result in other effects, probably because once taken up, the agents distribute differently inside the cells. In fact, at least considering their effects on respiration, it can be assumed that quinacrine, acridine orange, and nonyl acridine orange, are accumulated in the mitochondria. Quinacrine effect deserves special mention, because although it was not significantly accumulated, it still produced a moderate inhibition of respiration. It is also important that the addition of CCCP did not accelerate respiration, this fact indicates that these dyes affect the maximal oxygen consumption capacity of the cells.

Also, a proton pumping capacity inhibition was observed by the same three dyes, probably related to the respiratory inhibition, since energy supply of *C. albicans* depends mainly on this respiratory capacity [29]. Again, quinacrine effect is relevant, because it was not taken up by the cells to the same degree as the other two dyes, and nonetheless inhibited the proton pumping activity.

The increased Ca^{2+} uptake

The mechanism considered for the many fold increase of Ca^{2+} uptake is as follows: K^+ transport system normally works in such way that the cation is kept within the cell once it is taken up, but uncouplers produce an efflux of K^+ [30], indicating that this system operates in a reversible way through the electric plasma membrane potential difference, in equilibrium with the $[K^+]$out/[Kin] ratio [30]. But the actual [Kout] close to the cell membrane is much higher than that in the surrounding medium, because of the negative charge of the membrane [31]. It is also possible that the dyes bind to the cell membrane due to their cationic and hydrophobic nature, and may directly affect its function and lead to the efflux of the monovalent cation. In fact, during the measurement of the ζ potential, quinacrine at low concentrations reduced the migration velocity of the cells under the electric field, but at higher ones, the migration direction was reversed, indicating that the dye did not only bind to the surface because of its charge but also because of its hydrophobic or other property.

It is so possible to explain the different agent's effects in terms of their binding to cells, which leads to K^+ efflux. This then increases the plasma membrane potential, with the final result of an increased Ca^{2+} uptake. This mechanism appears to operate as described, at least for amiodarone [25], ketoconazole and miconazole [22]. However, other effects are produced, as they also enter the cells.

Relating structure to activity

Three of the acridine derivatives increased Ca^{2+} uptake; however, some facts have to be taken in account about the chemical structure of the dyes (Figure 6): a) auramine, not an acridine, did not show any effect; b) acriflavin, with two amino groups, but not methylated, showed some minor effect; c) acridine yellow, also with two not methylated amino groups, was ineffective; d) the nonyl derivative of acridine orange, with a hydrophobic chain, at 120 µM became somewhat less effective, as compared to the original dye; e) acridine orange deserves special mention because it was without any doubt the most effective, mainly in stimulating Ca^{2+} uptake, and has the basic structure of 9-aminoacridine, but the first with a 5 C chain ending in a dimethylated amino group, and the second is a simple dimethyl-diamino acridine; f) the lack of an effect of chloroquine has to be noticed as well, because it has practically the same structure of quinacrine, but a quinoline nucleus instead of the acridine one. Then, in general, it appears that some factors are important: a) the acridine nucleus, b) the existence of methylated amino groups, and c) the addition of an aliphatic chain increases the potency of the dyes. These characteristics may be at least considered as interesting for the possible synthesis of new compounds.

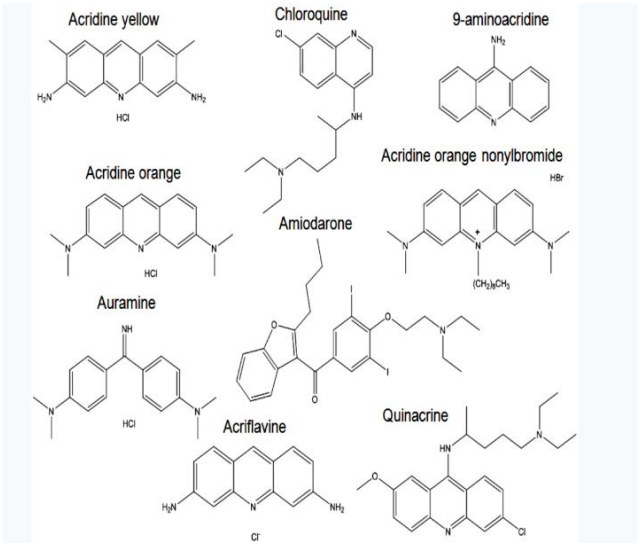

Figure 6: Chemical structure of the dyes. Chemical structure were drawn with ChemDraw Prime version.

Other effects

It was disappointing that only nonyl acridine orange inhibited growth, and acridine orange produced a delay. Acridine yellow deserves a special quote because it did not increase Ca^{2+} uptake, but produced some decrease of the growth rate.

The effects of quinacrine, acridine orange, and nonyl acridine orange on respiration and the proton pumping capacity of the cells are also important, as contributors to the inhibition of growth, definitely clear for nonyl acridine orange, the most hydrophobic of the dyes tested.

Quinacrine and acridine orange dyes

In order of their general effectiveness, nonyl acridine orange was the only one capable of clearly inhibiting growth, followed by acridine orange. Quinacrine is an interesting compound, somewhat more effective than amiodarone regarding Ca^{2+} uptake. These results would point to nonyl acridine orange as an effective drug against candidiasis; however, no studies about its possible collateral toxicity exist; it has been used mainly in cytology and diagnosis [32]. A similar situation exists regarding acridine orange, although some studies have revealed resistance genes to this compound in S. cerevisiae [33].

Quinacrine produced a remarkable increase of Ca^{2+} uptake, but the absence of an effect of this compound on growth, brings some doubt about the proposal that the increased Ca^{2+} is responsible for the death of yeast cells, as suggested by several authors [18-26]. It appears that in order to inhibit growth, other effects are involved. In this same respect, the many fold Ca^{2+} increase uptake, is most probably accumulated in the vacuole, [34] making it innocuous to the cell. In fact, when following its accumulation under the influence of amiodarone by the fluorescence increase of aequorin Courchesne and Ozturk [19] found that the increase of the cation, observed in the cytoplasm, was transient, probably because it was transported into the vacuole.

On the other hand, these findings open the frequent possibility used in therapeutics of combining any of the more effective agents found with another agent, perhaps an antifungal, as already explored by Gamarra et al. [26-28] Out of the three more effective, quinacrine may be a good therapeutic candidate, since it showed a small toxicity and few lateral effects during its use against malaria for many years. On the other hand, nonyl acridine orange, although not knowing its toxicity, might be tried as a local agent against candidiasis. These findings also may be important as a basis for the synthesis of effective compounds.

Finally, aside the effects found, other questions arise, regarding mainly the mechanism of Ca^{2+} transport, particularly because the absence of a correlation between its stimulation and effects on growth indicate that it is not accumulated in the cytoplasm, but probably sequestered in the vacuole. The results obtained also make one think about the possible transporters involved and the mechanism of transport and accumulation of the divalent cation, and in general, the further analysis of this interesting phenomenon.

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