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Research Article

Synergistic Effect of Dichloroacetate and Fluconazole on the Growth of *Saccharomyces cerevisiae*

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Abstract

The development of resistance to antifungals is a relevant issue during the treatment of infections. Using *S. cerevisiae* cells, we studied the effect of dichloroacetate (DCA), on the growth of the microorganism in the presence of fluconazole (FCZ). The results obtained show that DCA (2.0 - 3.0 mg/ml), sensitizes yeast cells to growth inhibition by fluconazole.

Results indicate that the external pH reduction produced by DCA decreases the electrochemical proton gradient necessary for the extrusion of toxic compounds to yeast metabolism. This effect favors a synergism strategy leading to lower drug doses and reducing drugs side effects.

ABBREVIATIONS

DCA: Dichloroacetate; FCZ: Fluconazole; CFU: Colony Forming Units

INTRODUCTION

Studies using yeasts as a model system have provided valuable information on a variety of cellular processes. Metabolic similarities between mammalian and yeast cells allow the yeast to be used to elucidate the effect of drugs in similar situations [1-3].

The compound dichloroacetate (DCA), has been used for the treatment of congenital lactic acidosis [4-7].

Results obtained in *Saccharomyces cerevisiae* indicate that it affects the activity of the pyruvate dehydrogenase complex (PDH) [8-11].

A problem that arises when using drugs in different therapies is the development of cell resistance to the recurrent use of these drugs. This fact forces the use of higher doses of drugs with the consequent presence of undesirable side effects [12].

One way of dealing with this situation is to use combinations of drugs that, through synergy, allow the necessary doses to be diminished or limit the emergence of resistant phenotypes [10].

Considering the acidification capacity of yeasts under fermentation conditions, this paper proposes to analyze: 1) What effect does DCA have on the acidification capacity of *S.cerevisiae*? and 2) What effect does the phenotype obtained on resistance to the antifungal fluconazole when yeasts grow in the presence of DCA?

Based on these objectives, the effect of DCA on the external acidification of the yeast was determined in a rich medium (1% yeast extract, 1% peptone, and 2% glucose) and then antifungal resistance was tested.

The results obtained show that DCA, in doses between 2.0 - 3.0 mg/ml, decreases the external acidification produced by the yeast and sensitizes the cell to the inhibition of growth by fluconazole.

MATERIALS AND METHODS

Strains, media, and growth condition

Saccharomyces cerevisiae strain MMY2 (*MAT* a *ura3*), was used in all the experiments. Assays were carried out in YPD medium with the following compositions: 1% yeast extract, 1% peptone and 2% dextrose. In a medium with maltose as a carbon source, the carbohydrate was added at a 2% final concentration. The solid medium contained 2% agar and 1 % in top agar. Cells grew at 30°C with constant agitation. When the dichloroacetate (DCA), effect was studied medium was supplemented from a 30.0 mg/ml solution of the drug.

Fluconazole was added from a 15 mg/ml water solution. The Optical Density (OD), of each yeast cell suspension was determined at 570 nm and used to calculate the necessary

dilutions needed to reach 350-500 colony forming units (cfu), on solid YPD plates.

Acidification assays

Cells from MMY2 strain grew in YPD medium for 19 hours. Thereafter cells were fractionated and two aliquots of 3.0 ml were added with a DCA stock solution (30.0 mg/ml, MR Pharma SA), to obtain a final concentration of 2.0mg/ml and 3.0mg/ml. Also, two aliquots of 3.0 ml were separated as controls. Incubation was continued for 30 minutes. After the incubation, the cells were suspended with sterile H_2O and then centrifuged at 5000 rpm in a clinical centrifuge. Then cells were suspended with sterile H_2O to a final volume of 3.0 ml. An aliquot of 1.0 ml was removed to determine viability and the rest were used for acidification assay. Acidification was initiated by the addition of glucose to a 15 mM concentration. The pH value was determined with pH-meter Hanna (HI98103), after 5minutes of glucose addition when the reading stabilizes. In a test where Bromocresol Purple (BP), was used it was included in top agar at 85 mg/ml final concentration.

Halo formation

From a suspension of $1.0x10^6$ cells/ml, 100 µl portions were dispersed onto plates of YPD medium. Glass-fiber filters (Schleicher & Schuell Inc, catalog N° 3362) 8 mm in diameter, were impregnated with 5-35µl portions of fluconazole (15 mg/ml) with/without 20-40µl of DCA (30.0 mg/ml) and placed in the center of the seeded plates. After incubation at 30°C for 48 hours, the diameters of the inhibition halos were measured and compared.

RESULTS AND DISCUSSION

We have already mentioned that dichloroacetate (DCA), affects cell metabolism, specifically glycolysis. This compound has been used to decrease acidification produced by lactate and to increase the effect of antimicrobial and chemotherapy drugs.

We first decided to establish which dose of DCA slightly affected the growth of the MMY2 strain and whether the presence of DCA in the culture medium of this yeast altered the acidification produced by the microorganism in the extracellular environment.

To evaluate the effect of DCA on the growth of the MMY2 strain, we performed a liquid and a solid medium test in YPD. For the liquid growth medium, we inoculate the medium with a fresh inoculum of MMY2 to obtain an initial concentration of 10^5 cells/ml. On the other hand, we inoculated approximately 300 colonies per plate for the calculation of colony forming units (CFU).

The results obtained for the liquid medium show that growth inhibition is in the range of 10% for 2.0 mg/ml and 25% for a dose of 3.0 mg/ml. For the solid medium, we find that the value of 3.5 mg/ml reduces viability by 20% (data not shown).

Then we use concentrations between 2.0 and 3.0 mg/ml for both liquid and solid media. We decided to use these concentrations with the idea of modifying the phenotype of the MMY2 strain without significantly or dramatically altering cell viability.

Next, we test the effect of DCA on extracellular acidification in cells grown in rich medium YPD. By adding DCA at a concentration of 2.0 mg/ml we see that after 24 hours of growth the control medium reaches a pH value of 5.22 units while the addition of DCA leads to a pH value of 5.31 units. This difference implies that there was a decrease of 22.6 nmol of $H^+/10^6$ cells in the presence of DCA. For 3.0 mg/ml of DCA, the pH reaches a value of 5.54 units which implies a decrease of 63.6 nmol of $H^+/10^6$ cells.

Considering that the YPD medium may contain some pH buffering capacity, we grew the cells in YPD medium supplemented with DCA. After harvesting and washing the cells we measured the acidification capacity on the external medium by adding glucose in a final concentration equal to 15 mM. We find then that control cells produce acidification of 81.37 nmoles of $H^+/10^6$ cells, while with the addition of 2.0 mg/ml of DCA there is a decrease of this value by 65% (Table 1). The presence of DCA in the growth medium produces a phenotype with a decreased ability to acidify the external medium.

To observe this phenotype in a solid medium, we carried out a test with Bromocresol Purple dye [13]. For this purpose, we distributed approximately 200 colonies in the medium containing dye in increasing amounts of DCA.

If the DCA produces a decrease in the acidification capacity of the yeast, we would expect to see a modification in the diameter of the halo produced by the dye around each colony. Bromocresol Purple (BP) has a yellow color at pH less than 5.2 and turns purple-blue at higher pH values (pKa = 6.3).

The results obtained are shown in Figure 1. The higher concentration of DCA leads to a fainter color of yellow around the colony compared to the control conditions. We also observed that the diameter of the colonies is not modified by the addition of dye or top agar. We obtained a similar result by using maltose as a carbon source (data not shown). Considering that maltose uses a different transport system than glucose, the effect we observed response to a lower acidification capacity of the yeast

Table 1: Acidification of the external medium in cells incubated with DCA.

Conditions	External pH value	nmol $H^+/10^6$ cells
Control cells	5.32	29.55
Control cells and glucose	4.88	81.37
Incubation with 2.0 mg/ml of DCA and glucose	5.35	27.57
Incubation with 3.0 mg/ml of DCA and glucose	5.98	6.46

Cells from MMY2 strain growth in YPD medium for 19 hours. Thereafter the cultivation was fractionated and two aliquots of 3.0 ml were supplemented with a DCA stock solution (30.0 mg/ml) to obtain a final concentration of 2.0 mg/ml or 3.0 mg/ml.
Also, two aliquots of 3.0 ml were separated as controls. Incubation was continued for 30 minutes. After the incubation, the cells were washed with sterile H_2O and then centrifuged at 5000 rpm in a clinical centrifuge. Then cells were suspended with the sterile H_2O to a final volume of 3.0 ml. An aliquot of 1.0 ml was removed to determine viability and the rest was used for acidification assay. Acidification was initiated by the addition of glucose to a 15 mM final concentration. The pH value was determined with a pHmeter Hanna (HI98103) after 5 minutes when the reading stabilizes.

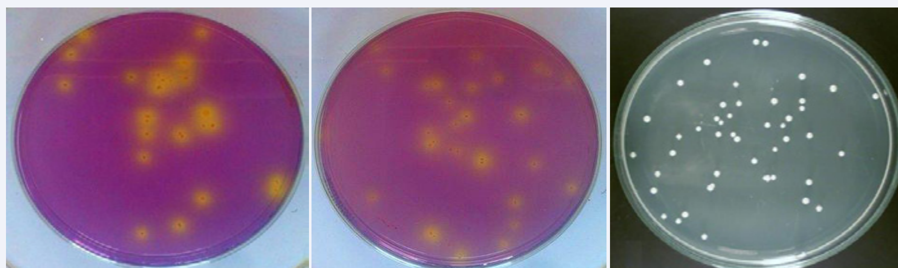

Figure 1 Effect of DCA on plates with top agar with bromocresol purple (BCP). From left to right: a) top agar with BCP, b) top agar with BCP and DCA (2.4 mg/ml) c) control MMY2 without top agar.
The solid medium contained 2% agar and 1% in top agar.
Bromocresol purple (BCP) was included in the top agar at the 85 mg/ml final concentration.

Table 2: Effect of DCA and Fluconazole on yeast growth on solid medium.

	Control	15 µl of Fluconazole (15 mg/ml)	20 µl of DCA (30.0 mg/ml)	15 µl of Fluconazole (15 mg/ml) and 20 µl of DCA (30.0 mg/ml)
Halo surface (mm^2)	2.0 ± 0,3	40.0 ± 0,3	2.0 ± 0,3	65.0 ± 0,3

and is not due to an effect of DCA on the transport of maltose or to the permeability barrier of the yeast [14].

This effect regarding the different acidification capacities has been considered in mammalian cell tests. However, our model system makes it possible to observe the effect directly.

Therefore these results established that low concentrations or in the order of 2.0 mg/ml of DCA do not inhibit the growth of the micro-organism significantly while producing a phenotype with lower acidification capacity of the external environment.

Previous results in our laboratory allowed us to establish that fluconazole-resistant mutants have a higher acidification capacity than wild cells (unpublished data). We, therefore, consider that a lower acidification capacity could alter the sensitivity of the yeast to the antifungal. For this purpose, we performed tests for the formation of halos [15], of inhibition in solid medium YPD (Table 2). We observe that a dose of 20 µl of DCA 30.0 mg/ml solution does not produce any inhibition halo. When adding 15 µl of fluconazole we see that the combination of the two drugs (DCA-fluconazole), increases the inhibition surface by 100 % for the halo formed only by fluconazole. A similar result was obtained in liquid medium YPD where the combination of DCA in final concentration 2 mg/ml and Fluconazole 0.1 mg/ml inhibited growth by 37 % while the antifungal alone reached 9 %.

In previous works, we have studied the resistance of *S. cerevisiae* to fluconazole in different growing conditions [15,16]. We have observed that the presence of L-proline as a nitrogen source increased the sensitivity to fluconazole and brefeldin [17]. L-proline likely demands an active metabolism of mitochondria that could be detrimental to glycolysis and subsequent extracellular acidification.

Also, the use of glycerol as a carbon source decreases the fermentative metabolism of the cell. This reduction in acidification would lead to a decline in the gradient of protons or driving force necessary to provide or to couple energy for drug expulsion systems. Another contribution in this direction is the increased sensitivity to fluconazole exerted by the potassium ion in the growing medium.

In summary, different growth conditions that alter the acidification capacity of the microorganism may be associated with increased sensitivity to the antifungal fluconazole. In the present work, the lower acidification capacity with DCA increases the sensitivity to fluconazole. The effect of DCA under these conditions would appear to be restricted to fluconazole. Similar assays did not alter the sensitivity of MMY2 strain to Violet Crystal (data not presented). However, it should be noted that this drug has been associated with another transport system [18,19]. The extent of sensitization of yeasts in the presence of DCA against other relevant compounds in medical treatments remains to be assessed in future studies. In addition, it seems unlikely that DCA could alter the permeability of the cell membrane in the presence of glucose [20].

The concentrations obtained in the present work are in the range of those observed in other model systems [21-23].

It is necessary to consider that Fluconazole applications are not limited to intravenous use. There are applications, for example in skin creams that involve higher concentrations than intravenous administration [24,25]. However, the absence of mutagenic effects of DCA in different systems is well established [26]. It should be mentioned that yeast cells, unlike higher eukaryotic cells, have a cell wall. This cell wall acts as an additional barrier protecting the microorganism from, among other factors, sudden changes in the osmolarity of the external environment.

The assays presented were performed on the wild strain MMY2 obtaining similar results on the strain S288c. The results were not altered in deficient respiratory strains (rho°) prepared by treatment with ethidium bromide [27].

For our model system of *S. cerevisiae*, there are sufficient experiments both at the physiological and molecular biological levels on the effect of DCA on the phosphorylation/de-phosphorylation cycles that regulate the activity of the pyruvate dehydrogenase (PDH), complex.

The effect of DCA on the PDH complexes of other yeasts has not yet been established. Future work would show whether DCA has a similar effect on the phenotype of other yeasts. We, therefore, conclude that the decline in the acidification capacity of yeast, induced in this study by DCA, leads to a dramatic change in the susceptibility of the microorganism.

In addition to the important medical use of DCA, the drug generates cellular conditions which prepare a suitable condition for another drug. It is worth remembering or considering that increased lactate is produced not only by genetic diseases but also by stress situations. In a work on the synergistic effect of DCA and salinomycin in cancer cell lines [28], an inhibitory effect of DCA on multidrug transporter activity is demonstrated. The authors rule out an effect on intracellular pH. This situation is similar to that observed in yeast in which the internal pH value does not vary with different culture media. Based on the results presented here, we can interpret that the effect observed with DCA in this cell line may be due to the decrease of the gradient or membrane potential. This characteristic or effect of DCA could extend its consideration at the time of synergy trials with other drugs.

Dichloroacetate could then be considered to be of importance not only in its correct use but concomitantly with the support of other drugs [29,30]. Its low toxicity, low cost, and its already approved use in human beings make it a suitable candidate for further studies.

CONCLUSION

The results obtained show that the DCA (2.0-3.0 mg/ml) affects the growth of MMY2 strain. Concomitantly the presence of DCA in our growing conditions alters the acidification produced by the microorganism on the extracellular medium. As we mentioned, similar results were obtained using maltose as a carbon source (data not shown). The phenotype obtained also shows a greater degree of growth inhibition in the presence of fluconazole.

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