Yeast resistance and its reversion

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RESUMO

Actualmente, a resistência a drogas é um dos grandes problemas na terapia de diferentes patologias e também na biotecnologia e agricultura.

O objectivo do presente estudo foi elucidar a resistência a drogas em leveduras patogénicas ou alimentares, que mais frequentemente são encontradas. Assim, diferentes antifungicos clínicos e novos compostos recentemente sintetizados foram testados por um método para avaliar a resistência e sua reversão que foi previamente desenvolvido.

Foi testado um painel de diferentes drogas para reverter a resistência. Mostramos que alguns destes moduladores foram capazes de reverter eficazmente a resistência.

Palavras-Chave: Resistência em leveduras; compostos antifúngicos; reversão de resistência.

ABSTRACT

Nowadays, drug resistance is one of the major problems in the therapy of different pathologies and also in agriculture and biotechnology.

The aim of the present study is to elucidate drug resistance and its reversion in some of the more common pathogenic and foodborn yeasts. Thus, different clinical antifungal agents of more common use as well as new synthesized compounds were tested by a previously developed method to detect resistance and its reversion.

A panel of different drugs was tested to lower the resistance to the antifungal agents. We have shown that some of these were able to lower resistance.

Key-Words: Yeast resistance; antifungal agents; reversion of resistance patterns.

1. INTRODUCTION

An increasing number of resistant cells are emerging in different biological fields. Among them it is well established that *Candida* species displays increasing resistance to antifungal therapy by mechanisms not yet well established (White *et al*., 1998). On the other hand, the opportunistic infections by "non pathogenic" yeast are also increasing, namely in AIDS, other immunodepressed patients, transplanted patients and those in critical conditions admitted to the intensive care units (Schwenke, 1992; *Sajben et*

al., 1993; Krcmery, 1998; Lamb *et al*., 1997). Hence, nowadays any yeast species can be considered, in exceptional cases, as a potentially pathogenic one.

Multidrug resistance (MDR) is exhibited by a wide variety of prokaryotic and eukaryotic cells and among drug resistance mechanisms has been identified as one of the more generalized (Prudêncio, 2000). This mechanism is associated with the active efflux of toxic compounds from the cells and is mediated by active efflux proteins (AEP) and is present in fluconazole and other azole resistant *Candida* species (Clark, *et al.*,1996; Carlson *et al*., 1996; Sanglard *et al*., 1995; Parkinson, *et al*., 1995; Marichal and Vanden Bossche, 1995; Walsh, 1997; Hernaez, 1998).

In order to further elucidate the mechanism(s) underlying drug resistance in yeasts, the clinical antifungal agents fluconazole, 5-fluorocytosine and amphotericin B were selected in previous studies (Prudêncio *et al.,* 2000) among those of more common use.

For this purpose the studies were conducted in isolates of *Candida spp.* namely, *Candida albicans*, *Candida tropicalis* and *Candida krusei*. The yeast species *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* were selected, since they were already reported as resistant to antimicrobial agents, being the resistance mechanism related to the induction of AEP (Prudêncio, 2000) and additionally *Issatchenka Orientalis*.

In a second phase new drugs were tested. Primaquine is a drug widely used against malaria disease in the last forty years, due to its action against different forms of malaria parasites. However some problems are still raised: rapid metabolic inactivation to form carboxyprimaquine and serious blood toxicity, with particular ability to induce oxidation of oxyhemoglobin to methemoglobin (Constantino *et al*., 1999; Brueckner *et al* 2001).

One possible way to reduce the primaquine toxicity is its transformation into an imidazolidin-4-one (Gomes *et al* 2004; Araújo *et al* 2005). Compounds of this kind are being synthesized to develop new approaches for the implementation of more effective treatments against malaria (Gomes *et al* 2004). Recently it has been observed that primaquine derivatives could also have antifungal activity (Thomas *et al,* 2000). The compound used in the present work was the 5-isopropyl-3-[4-(6-methoxy-quinolin-8-ylamino)-pentyl]-2,2-dimethyl-imidazolidin-4-one (ValPQacet) that was synthesized through acylation of the anti-malarial primaquine with L-valine and subsequent reaction of the resulting a-aminoamide with propanone (Gomes *et al* 2004).

Finally in an attempt to revert the resistance phenotype of the yeast species to the antifungals tested, a panel of different compounds described as substrates or as inhibitors of MDR proteins in different types of cells (Kolaczkowski *et al*., 1998; Kolaczkowski *et al*., 1996; Krishnamurthy *et al.,* 1998; Paul *et al.,* 1996; Prasad *et al*., 1995; Holló., 1994 and Homoloya 1993; Krishan 1994) were tested.

2. MATERIALS AND METHODS

2.1 Microorganisms and growth conditions:

The yeast species studied were: *Saccharomyces cerevisiae* IGC 4072, *Candida tropicalis* PYCC, *Issatchenka Orientalis* PYCC 3341 and *Kluyveromyces marxianus* IGC 2671 from the Portuguese Yeast Culture Collection (PYCC), Universidade Nova de Lisboa, Portugal. The two yeast species *Candida albicans* H33 and *Candida Krusei* H9 were selected from clinical isolates received in the laboratory of Serviço de Microbiologia da Faculdade de Medicina da Universidade do Porto. These yeasts were

previously identified to the species level by the API 20C system and the means of identification were supplemented when needed by conventional morphological and biochemical methods. Each isolate represented a unique isolate from a patient and was maintained on agar slants (Prudêncio *et al,* 1998) with 2% (w/v) of glucose. Cells were grown in a mineral medium (MGV) with vitamins (van uden, 67) supplemented with 2% (w/v) of glucose and incubated on a mechanical shaker at 25ºC.

2.2 Chemicals

The clinical antifungals 5-fluorocytosine and amphotericin B and verapamil, cycloheximide, vinblastine, taxol, progesterone, corticosterone and sodium fluoride (NaF) were purchased from Sigma Chemical Co. (St. Louis, MO) and □-estradiol, from Merck (Darmstadt, Germany). The agriculture fungicides, benomyl and penconazol were purchased from Riedel-de Haën (Chinosol, Seelze). Fluconazol (Diflucan) was purchased from Pfizer (Laboratórios Pfizer, Lda. Seixal, Portugal) in an intravenous infusion solution of 2 mg/ml.

2.3 Determination of the minimum inhibitory concentrations (MIC) and treatments with the antifungals

The determination of the MIC for the antifungals were performed according to (Prudêncio *et al* 1999). For the treatments with the antifungals, the yeast cells were suspended in PBS with 2% (w/v) of glucose and incubated with fluconazole, 5-fluorocytosine and amphotericin B at room temperature for at least 180 minutes. The concentrations used for the different antimicrobial agents were below the MIC determined for each yeast species and confirmed not to be cytotoxic by counting colony forming units. In the experiments with the modulators these were added in the presence of the antifungals for which the cell was resistant. The modulators were tested independently for cytotoxicity (data not shown), being used at subinhibitory concentrations, according to Table 4. The new compounds obtained by organic synthesis, were included in the medium and the cells were grown in the absence or in the presence of ValPQacet, PQ and NaCl. The specific growth constants (kc) determinations were determined according to (Prudêncio, 1995).

2.4 Statistical analysis

The data were compared by the Student´s *t* test.

3. RESULTS AND DISCUSSION

The minimum inhibitory concentrations (MIC) of ValPQacet, primaquine (PQ) and NaCl, were determined for the yeast species presented in Table 1. Since ValPQacet and PQ are bis-hydrochloride salts, the toxicity of NaCl at the same Cl^- concentration was also tested, as a control for the inhibitory effects of hydrochloride. The results obtained for the MIC determinations are shown in Table 1. For *C. tropicalis* it was not possible to determine the MIC value since the occurrence of a halo at 0.5 mg/L of ValPQacet was also observed for similar concentrations of PQ and NaCl. Further studies are needed to understand this result. In the case of *C. albicans* ValPQacet seems to induce the same effects than PQ.

Table 1 - *Inhibitory effects induced by different concentrations of ValPQacet, primaquine bis-hydrochloride (PQ) and sodium chloride (NaCl). The observation of haloes (growth inhibition) is indicated by (+) and non-observation by (-). The minimum inhibitory concentration (MIC) determined are indicated in bold.*

Yeast species	ValPQacet	PQ	NaCl*	Concentration (mg/L)
S. cerevisiae PYCC 4072	+	+	-	5000
	+	-	-	500
	+	-	-	50
	+	n.d	n.d	**5**
	-	n.d	n.d	0.5
	-	n.d	n.d	0.05
I. orientalis PYCC 3341	+	-	-	5000
	+	-	-	500
	+	-	-	50
	+	n.d	n.d	5
	+	n.d	n.d	0.5
	+	n.d	n.d	0.05
	+	n.d	n.d	0.005
	+	n.d	n.d	**0.0005**
	-	n.d	n.d	0.00005
C. albicans PYCC 3436^{T}	+	+	-	**5000**
	-	-	-	500
	-	-	-	50
C. tropicalis PYCC 3092^{T}	+	+	+	5000
	+	+	+	500
	+	+	+	50
	+	+	+	5
	+	+	+	**0.5?**
	-	+	+	0.05
	-	n.d	n.d	0.005
	-	n.d	n.d	0.0005
	-	n.d	n.d	0.00005

n.d. non determined values; *NaCl concentration always doubled that of ValPQacet and PQ, since these are bis-hydrochloride salts.

The comparison of the MIC values obtained for ValPQacet and PQ, are summarized in Table 2. With the exception of *C. albicans* the potential antifungal application of ValPQacet seems interesting since the inhibitory effects observed were increased 10^5 times for *I. orientalis* and 1000 times for *S. cerevisiae* when compared with the parent drug, PQ. These observations are reinforced when the MIC values of fluconazol (32 mg/L), 5-fluorocitosine (>200mg/L) and amphotericine B (8 mg/L) for *S. cerevisiae* are compared with those obtained for ValPQacet (Prudêncio *et al*., 2000). The values obtained for *I. orientalis* (Table 2) are much lower when compared with the other yeast species tested.

Table 2- *Minimum inhibitory concentration (MIC) of ValPQacet and of primaquine hydrochloride salt (PQ) and the clinical antifungals*, determined for all the yeast species studied.*

Agentes Antimicrobianos (mg/L)	*C. krusei*	*C. albicans*	*S. cerevisiae*	*K. marxianus*	*I. orientalis*
Fluconazol	>128*	10*	32*	0,5*	--
5-fluorocitosine	>200*	>200*	>200*	<10*	--
Amphotericine B	8*	5*	8*	8*	--
ValPQacet	--	5000	5	--	0,005
PQ	--	5000	5000	--	>5000

***** Prudêncio, 2000.

In order to further understand the inhibitory effect induced by ValPQacet, the specific growth constants (kc) for *S. cerevisiae* were determined in the absence and in the presence of 2.5 mg/L and 5 m/L of ValPQacet. Additionally, kc values were compared with those obtained in the presence of 5 mg/L PQ and 10 mg/L NaCl (Table 3). The values obtained further reinforce the idea that NaCl does not seem to inhibit growth in the concentration tested, whereas PQ seems to increase growth slightly at the concentration tested when compared with the control. The inhibitory effects on growth observed in the presence of ValPQacet at the MIC value (5 mg/L) seem to decrease kc by 33%, when compared with the control, whereas at half the concentration (2,5 mg/L) the kc observed is similar to that obtained for PQ, showing a dose/effect relation.

Table 3- *Specific growth constants (kc) determined for S. cerevisiae in the absence and in the presence of ValPQace, PQ and NaCl.*

Concentration (mg/L)	k_c (min^{-1}) in the presence of ValPQacet	k_c (min^{-1}) in the presence of PQ	k_c (min^{-1}) in the presence of NaCl
0	$3.40\times10^{-3}\pm$ 0,34	$3.40\times10^{-3}\pm$ 0,34	$3.40\times10^{-3}\pm$ 0,34
2,5	$4.40\times10^{-3}\pm$ 0,17	n.d	n.d
5	$2.62\times10^{-3}\pm$ 0,16	$4.13\times10^{-3}\pm$ 0,0	$3.56\times10^{-3}\pm$ 0,29

3.1 Reversion of resistance phenotype

Different compounds were tested in an attempt to revert the resistance phenotype obtained previously (Prudêncio, 2000) in *S. cerevisiae* and *C. albicans* (Table 4). These compounds, as referred before, are described as inhibitors or as being transported by MDR proteins in different types of cells or are compounds already used in clinical, but for other purposes. Those of the first group so were tested for competitive or non competitive inhibition of the transporter and in any of this cases if a compound functions as modulator it would increase antifungal retention and hence toxicity, so the resistance would decrease. The second group of compounds was tested for its potential as antifungals in addition to their known clinical functions.

For this purpose the cells were incubated in the presence of the different concentrations of the antifungal agents for witch they exhibit resistance and of a panel of different modulators. The concentrations used for the modulators were subinhibitory and previously confirmed as noncitotoxic by counting colony forming units (CFU).

The results presented in Table 4, show the decrease of the MIC value for *C. albicans* and *S. cerevisiae* in the presence of several the modulators of those tested. These compounds presented the ability to revert the resistance presented to 5-fluorocytosine. For the yeast *C. albicans*, in our experimental conditions, fewer compounds showed an activity to revert the resistance phenotype than for the yeast *S. cerevisiae*.

Table 4 - *Minimum inhibitory concentrations (MICs) of 5-fluorocitosine in the presence of modulators (sub-inhibitory concentrations) in the yeasts C. albicans and S. cerevisiae.*

S. cerevisiae 5-fluorocitosina MIC > *200 mg/L*	*Anfotericina B (5 mg/L)	=100
	*Benomil (50 mg/L)	=100
	*penconazol (10 mg/L)	=200
	*Vinblastina (5 μM)	=50
	*B- estradiol (50 μM)	=50
	*Progesterona (5 μM)	=200
	*Corticosterona (5 μM)	=100
	Acetilsalicilato de Lisina (0,1 mg/L)	=100
	Ibuprofeno (0,1 mg/L)	=200
	Paracetamol (0,1 mg/L)	=100
C. albicans 5-fluorocitosina MIC > *200 mg/L*	Acetilsalicilato de Lisina (0,1 mg/L)	=100
	Ibuprofeno (0,1 mg/L)	>200
	Paracetamol (0,1 mg/L)	=200
	*Penconazol (10 mg/L)	<20

*Prudêncio, 2000

4. CONCLUSIONS

The new primaquine imidazolidin-4-one prepared seem to be interesting as a potential antifungal agent against several yeasts other than *C. albicans*.

The MIC value of new primaquine imidazolidin-4-one prepared for *S. cerevisiae* (5mg/L) indicates its potential application as an antifungal agent, which was further reinforced when compared with the MIC values previously determined (Prudêncio, 2000) for fluconazol (32 mg/L) and penconazol (20 mg/L), two of the most used antifungal agents for clinical and agriculture purposes, respectively.

In *S. cerevisiae* a dose/effect relationship was observed, according to the results obtained for kc values.

Several modulators tested, showed a function in the reversion of the resistance phenotype previously determined (Prudêncio, 2000). These results are promising and may be useful in clinical, biotechnology and agriculture.

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