Role of the ABC transporter TruMDR2 in terbinafine, 4-nitroquinoline *N*-oxide and ethidium bromide susceptibility in *Trichophyton rubrum*

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A single-copy gene, designated *TruMDR2*, encoding an ATP-binding cassette (ABC) transporter was cloned and sequenced from the dermatophyte *Trichophyton rubrum*. The ORF of *TruMDR2* was 4048 nt and the deduced amino acid sequence showed high homology with ABC transporters involved in drug efflux in other fungi. The encoded ABC protein predicted 12 transmembrane segments (TMSs) and two almost identical nucleotide-binding domains (NBDs) arranged in two halves in a (TMS₆–NBD)₂ configuration and could be classified as a member of the multidrug-resistance (MDR) class of ABC transporters. Northern blot analyses revealed an increased level of transcription of the *TruMDR2* gene when mycelium was exposed to acriflavine, benomyl, ethidium bromide, ketoconazole, chloramphenicol, griseofulvin, fluconazole, imazalil, itraconazole, methotrexate, 4-nitroquinoline *N*-oxide (4NQO) or tioconazole. Disruption of the *TruMDR2* gene rendered the mutant more sensitive to terbinafine, 4NQO and ethidium bromide than the control strain, suggesting that this transporter plays a role in modulating drug susceptibility in *T. rubrum*.

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INTRODUCTION

Trichophyton rubrum is a cosmopolitan filamentous fungus that can infect human keratinized tissue (skin, nails and, rarely, hair) and is the causal agent of 80–90% of all chronic and recurrent dermatophytoses (Fernández-Torres *et al.*, 2000). This pathogen, which normally causes well-characterized superficial infections, can also cause deep dermal invasion in immunocompromised patients (Smith *et al.*, 2001; Squeo *et al.*, 1998). Griseofulvin, terbinafine and itraconazole are widely used to treat dermatophytosis. Although in routine clinical practice the response to these antifungals is usually satisfactory, the occurrence of strains that are insensitive to antifungal agents (Mukherjee *et al.*, 2003; Osborne *et al.*, 2005) could play a relevant role in the failure of antifungal treatments. Thus, studies of the mechanisms of antifungal resistance are crucial for a more rational use of drugs, to minimize or overcome resistance.

A clinical isolate of *T. rubrum* submitted to a mutagenic treatment presented simultaneous resistance to griseofulvin and tioconazole *in vitro*, suggesting the existence of a

multidrug-resistance (MDR) mechanism based on cellular efflux involved in this event (Fachin *et al.*, 1996). The *T. rubrum TruMDR1* gene, which encodes an ATP-binding cassette (ABC) transporter, is also differentially expressed in the presence of unrelated toxic compounds, including the antifungals griseofulvin and itraconazole (Cervellati *et al.*, 2006). ABC transporters are highly conserved ATPases, ubiquitous from bacteria to humans, and this mechanism protects cells against the cytotoxic effects of compounds by reducing the accumulation of toxic compounds in the cell. In eukaryotes, most ABC transporters are composed of two similar halves, each consisting of a cytoplasmic nucleotide-binding domain (NBD) and six transmembrane segments (TMSs). The NBDs of ABC transporters contain conserved amino acid sequences, called the Walker A and Walker B motifs (Walker *et al.*, 1982), and the ABC signature (Ames *et al.*, 1990). Based on their topology, the ABC transporters can be subdivided into subfamilies or clusters. The best-characterized among these are those involved in MDR [(TMS₆–NBD)₂] or in pleiotropic drug resistance [(NBD–TMS₆)₂] (Wolfger *et al.*, 2001). ABC transporters have been described in several fungi and yeasts (De Hertogh *et al.*, 2002; Del Sorbo *et al.*, 2000) and may also be required for phytoalexin tolerance and virulence in phytopathogenic fungi such as *Botrytis cinerea* (Schoonbeek *et al.*, 2001; Vermeulen *et al.*, 2001), *Gibberella pulicaris* (Fleissner *et al.*, 2002), *Magnaporthe grisea* (Urban *et al.*, 1999) and

Abbreviations: ABC, ATP-binding cassette; MDR, multidrug resistance; NBD, nucleotide-binding domains; 4NQO, 4-nitroquinoline *N*-oxide; TMS, transmembrane segment.

The GenBank/EMBL/DBJ accession number for the *TruMDR2* sequence of *Trichophyton rubrum* is AF291822.

Mycosphaerella graminicola (Stergiopoulos *et al.*, 2002) and for secretion of endogenous secondary metabolites and xenobiotics in *Aspergillus nidulans* (Andrade *et al.*, 2000). However, the role of ABC transporters in dermatophytes is still poorly understood.

In this paper, we report on the isolation, molecular cloning and functional analysis of *TruMDR2*, an ABC transporter-encoding gene of *T. rubrum*, which may be involved in modulating susceptibility to terbinafine, 4-nitroquinoline *N*-oxide (4NQO) and ethidium bromide. Furthermore, we describe the standardization of a transformation system in *T. rubrum* that was used for disruption of the *TruMDR2* gene.

METHODS

Strains and growth conditions. The *T. rubrum* clinical isolate H6 (ATCC MYA-3108) used throughout this study was cultured as described previously (Fachin *et al.*, 1996, 2001). *Escherichia coli* strain LE392 was used for amplification of the phage library and strain DH5 α was used for transformations and plasmid amplification. All transformants were selected on Luria broth medium (LB) containing ampicillin (50 $\mu\text{g ml}^{-1}$) and cultured at 37 °C overnight. The plasmid pCSN43, used for gene disruption, contained a gene encoding hygromycin phosphotransferase (*hph*) under the control of the promoter and terminator of the *A. nidulans trpC* gene (Fachin *et al.*, 1996, 2001; Staben *et al.*, 1989).

Construction of genomic libraries. DNA of the H6 strain was extracted from mycelia harvested after 72 h of incubation on liquid Sabouraud glucose medium at 28 °C and partially digested with *Sau3A*I. The fragments were sized by electrophoresis on a 0.7% low-melting-point agarose gel and purified from the gel. For construction of the plasmid library, vector pUC18 *Bam*HI/BAP (Amersham Biosciences) was used to clone fragments of approximately 3–6 kb. For the lambda library, λ GEM11 (Promega) was used to clone fragments of approximately 15–23 kb. The bacterial library had 3×10^4 clones and the phage library had 10^5 p.f.u.

Gene cloning and sequencing. A fragment of the *TruMDR2* gene was cloned by PCR amplification using degenerate primers and *T. rubrum* H6 genomic DNA as template. Primers 5'-GCMTTSG-TGGGYCCMTCNGG-3' and 5'-AGVGCRGADGTRGCYYTCRTC-3' were designed to the conserved amino acid sequences of the Walker motifs in ATP-binding domains of presumptive eukaryotic ABC-type transporters. Approximately 200 ng H6 isolate DNA, extracted as described by Raeder & Broda (1985), was used as template. PCR involved 30 cycles of denaturation at 94 °C, annealing at 60 °C and extension at 72 °C. The amplified products were resolved by electrophoresis on a 1% agarose gel. A sharp band of 380 bp was recovered from the gel, cloned into the pGEM-T vector (Promega), sequenced and used as a probe for screening of libraries. Hybridizing clones from phages were digested and subcloned into the pUC18 plasmid vector. The complete sequence of the *TruMDR2* gene was determined by using the PHRED/PHRAP/CONSED package. The copy number of *TruMDR2* in the genome of the H6 strain was determined by Southern blot analysis. Gene-specific probes were prepared and genomic DNA was digested with restriction enzymes *Apa*I and *Spe*I, which do not cleave the *TruMDR2* gene. The Random Primer DNA Labelling System (Invitrogen) was used to generate radioactively labelled oligonucleotide probes with [α - ^{32}P]dCTP. DNA or clone transfer to membranes, hybridization, plasmid DNA

preparation, phage DNA purification and digestion with restriction enzymes were carried out using standard protocols (Sambrook *et al.*, 1989).

RNA extraction and Northern blot analysis. Conidia from *T. rubrum* were incubated in liquid Sabouraud glucose medium for 72 h at 28 °C with shaking at 160 r.p.m. and the resulting mycelia were harvested aseptically and transferred to fresh Sabouraud medium to which different drugs were added. They were incubated for 15 min (based on previous time-course experiments) under the same conditions, harvested by filtration and total mycelial RNA from each treatment was extracted with the Wizard RNA Isolation System (Promega). Approximately 20 μg RNA was electrophoresed on a 1% agarose gel containing formaldehyde, transferred to a Hybond-N⁺ membrane and hybridized with an internal fragment of the *TruMDR2* gene radioactively labelled with [α - ^{32}P]dCTP, as described previously (Sambrook *et al.*, 1989).

Initially, the susceptibility of *T. rubrum* to toxic agents was determined by an agar dilution assay on Sabouraud plates (Cervellati *et al.*, 2006). One hundred microlitres of a conidial suspension of approximately 5×10^5 conidia ml^{-1} was inoculated into Sabouraud glucose agar dishes with various concentrations of the drugs and incubated at 28 °C for 7 days. As a control, the conidial suspension was inoculated into fungicide-free medium with the respective solvent. The subinhibitory concentrations used in Northern blot analysis, which corresponded to the concentrations of the drug at which there was approximately 90% inhibition of macroscopic growth (Cervellati *et al.*, 2006), were as follows: acriflavine, 2.5 $\mu\text{g ml}^{-1}$; benomyl, 2.5 $\mu\text{g ml}^{-1}$; ethidium bromide, 2.5 $\mu\text{g ml}^{-1}$; ketoconazole, 10 $\mu\text{g ml}^{-1}$; cycloheximide, 30 mg ml^{-1} ; chloramphenicol, 50 mg ml^{-1} ; fluconazole, 300 $\mu\text{g ml}^{-1}$; griseofulvin, 2.0 $\mu\text{g ml}^{-1}$; imazalil, 4.0 $\mu\text{g ml}^{-1}$; itraconazole, 30 $\mu\text{g ml}^{-1}$; 4NQO, 10 $\mu\text{g ml}^{-1}$; terbinafine, 0.1 $\mu\text{g ml}^{-1}$; and tioconazole, 0.5 $\mu\text{g ml}^{-1}$. Benomyl was provided by DuPont De Nemours. Imazalil, ketoconazole and itraconazole were provided by Janssen Pharmaceuticals. Cycloheximide was purchased from ICN Biomedicals. Fluconazole, terbinafine and chloramphenicol were provided by the University Hospital of Ribeirão Preto, São Paulo, Brazil. Tioconazole was provided by Pfizer. All other chemicals tested were purchased from Sigma. Acriflavine, ethidium bromide, chloramphenicol and terbinafine were dissolved in sterile water. Benomyl, 4NQO, ketoconazole, itraconazole, tioconazole, imazalil, cycloheximide and griseofulvin were dissolved in DMSO. The final concentration of DMSO never exceeded 1% (v/v).

Disruption of the *TruMDR2* gene. The *Xba*I-*Cl*aI 2.9 kb fragment obtained from the D7P clone, which contains the *TruMDR2* gene, was cloned into the unique *Sma*I site of plasmid pUC18, to generate plasmid pXC (see Fig. 3). This plasmid, digested with *Eco*RV to promote a 135 bp deletion in the *TruMDR2* gene, was used to clone the 2.4 kb *Sal*I fragment hygromycin-resistance gene cassette (*hph*), filling in 5' overhangs with Klenow polymerase (Amersham Biosciences), from pCSN43 to generate p Δ *TruMDR2*, which was used without further modification to transform the H6 strain.

Protoplasts were obtained by incubating the mycelium harvested from two Petri dishes in 30 ml lytic solution consisting of 600 mg Glucanex (Novo Nordisk), 0.7 M KCl and 50 mM phosphate buffer (pH 5.8) for approximately 3–4 h at 28 °C. The protoplasts were filtered through glass wool, collected by centrifugation (5 min, 4 °C, 1556 g), washed with 10 ml CT buffer [0.4 M ammonium sulfate, 1% sucrose (w/v), 0.05 M citric acid (pH 6.0)] and collected by centrifugation. They were then washed with 10 ml DT buffer (0.6 M KCl, 0.1 M CaCl₂, 0.01 M Tris/HCl, pH 7.5), collected by centrifugation and the pellet was resuspended in 1 ml DT buffer and transformed with polyethylene glycol, as described by May (1992), using 10 μg plasmid p Δ *TruMDR2*.

Transformants were selected in solid minimal medium (Cove, 1966) supplemented with 1 M sucrose containing 600 µg hygromycin ml⁻¹ at 28 °C. After 15 days, hygromycin-resistant colonies were clearly visible. These colonies appeared at a rate of 1.7 (µg plasmid)⁻¹ and (10⁷ protoplasts)⁻¹. No such colonies were detected when controls lacking DNA were plated on to selective medium.

Toxicant sensitivity assays. Susceptibility of the H6 and Δ *TruMDR2* mutant strains to cytotoxic agents was tested by assessing the MICs of the drugs according to the M38-A microdilution technique proposed by the National Committee for Clinical Laboratory Standards (NCCLS, 2002). Briefly, colonies obtained by growing the strains on Sabouraud plates at 28 °C for 15 days were covered with sterile saline containing 1% Tween 80, conidia were harvested by sterile scraping and the solution was filtered through glass wool. The resulting mixture of mostly non-germinated conidia was transferred to a sterile tube and adjusted spectrophotometrically at a wavelength of 530 nm ranging from 70 to 75% transmittance. These conidial suspensions were diluted 1:50 in RPMI 1640 (Sigma) buffered with MOPS, which corresponded to twice the density needed for the test of approximately 3×10^5 – 5×10^5 c.f.u. ml⁻¹. Each microdilution well containing 100 µl of the diluted (twice) drug concentrations was inoculated with 100 µl of the diluted (twice) conidial inoculum suspensions (final volume in each well was 200 µl). Growth and sterility controls were included for each isolate tested. Microtitre trays were incubated at 28 °C and MICs were recorded after 7 days of incubation. The susceptibility end point was recorded for each strain and for each drug. MIC₁₀₀ was defined as the lowest drug concentration resulting in total inhibition of visible growth.

RESULTS AND DISCUSSION

Isolation and organization of the *TruMDR2* gene

We used degenerate primers based on the sequence of known MDR genes from a variety of organisms to amplify a 380 bp DNA fragment from *T. rubrum*. Cloning and DNA sequence analysis revealed that the amplified fragment encoded a highly conserved amino acid sequence characteristic of proteins containing an ABC. This PCR product was used to screen a genomic DNA plasmid library and a genomic phage library and led to the identification of the complete sequence of a gene named *TruMDR2* that contained an ORF of 4048 nt interrupted from nt 384 to

435 by a putative intron. Analysis of the deduced 1331 aa sequence of the encoded protein, *TruMDR2*, suggested the presence of 12 TMSs and two almost identical NBDs. These domains were arranged in two homologous halves in a (TMS₆-NBD)₂ configuration, as predicted by the program PREDICT PROTEIN (Rost *et al.*, 1995). An ABC was present in the N- and C-terminal halves of *TruMDR2*. The cassette in the hydrophilic moiety of both halves contained almost identical degenerate Walker A motifs (GxSGxGK) with the lysine residue (K), which is highly conserved among members of the MDR transporters (Andrade *et al.*, 2000; Angermayr *et al.*, 1999; Tobin *et al.*, 1997). In addition, the conserved glutamic acid residue (E) (Cutting *et al.*, 1990) was present next to the Walker B motif in both halves of *TruMDR2* (Fig. 1). The *T. rubrum* protein *TruMDR2*, which belongs to the subfamily 3.A.1 (transporter classification system), was 68% identical to AFUMDR1 of *Aspergillus fumigatus* (Tobin *et al.*, 1997), 66% identical to AtrD of *A. nidulans* (Andrade *et al.*, 2000), 63% identical to ABC4 of *Venturia inaequalis* (AAL57243) and 57% identical to AFLMDR1 of *Aspergillus flavus* (Tobin *et al.*, 1997). These results suggest a close evolutionary relationship among these proteins. The putative protein *TruMDR2* had an estimated molecular mass of 145 kDa and an isoelectric point of 6.7. Southern blot analysis of restriction enzyme-digested genomic DNA indicated that the *TruMDR2* gene is present as a single-copy gene (data not shown).

Transcription of the *TruMDR2* gene is enhanced by cytotoxic agents

To verify the possible involvement of the *TruMDR2* gene in drug transport, the level of transcription of this gene was investigated following treatment of H6 mycelia with antifungal agents and some drugs known as substrates for ABC transporters from other organisms. Northern blot analysis showed that the *TruMDR2* gene was transcribed constitutively at low levels, was enhanced after 15 min of exposure to acriflavine, benomyl, ethidium bromide, ketoconazole, griseofulvin, fluconazole, imazalil, methotrexate and tioconazole, and was highly induced by chloramphenicol, itraconazole and 4NQO, compared with the untreated

N-terminal	Walker A	ABC signature	Walker B	
AN	ALVGPSGSGKSTVV	LLSGGQKQRIAIARAVVSDPKILLDEATSALD		622
AF	ALVGPSGSGKSTVV	LLSGGQKQRIAIARAVVSDPKILLDEATSALD		616
VI	ALVGAFSGSGKSPIV	LLSGGQKQRIAIARAVVSDPKILLDEATSALD		623
AFI	AFVGPSGSGKSTII	SLSGGQKQRIAIARAIKDPKILLDEATSALD		567
TR	ALVGPSGSGKSTVV	LLSGGQKQRIAIARAVVSDPKILLDEATSALD		601
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C-terminal	Walker A	ABC signature	Walker B	
AN	ALVGPSGCGKSTTI	MLSGGQKQRVAIARALLRDPKILLDEATSALD		1275
AF	ALVGPSGCGKSTTI	MLSGGQKQRVAIARALLRDPKILLDEATSALD		1276
VI	ALVGASGCGKSTTI	LLSGGQKQRIAIARALLRNPKILLDEATSALD		1280
AFI	ALVGASGSGKSTTI	MLSGGQKQRIAIARALIRNPKILLDEATSALD		1232
TR	ALVGPSGCGKSTTI	MLSGGQKQRIAIARALIRDPKILLDEATSALD		1258
	:*.	:**:*****:*.		

Fig. 1. Multiple alignment of the *T. rubrum* ABC *TruMDR2* (TR) with representative members of the ABC transporter family. AN, *A. nidulans* (GenBank no. AAD43626); AF, *A. fumigatus* (GenBank no. AAB88658); VI, *Venturia inaequalis* (GenBank no. AAL57243); AFI, *Aspergillus flavus* (GenBank no. AAB88656). Identical residues are marked with asterisks. Dots and colons indicate conservative substitutions.

Fig. 2. Northern blot analysis of the *TruMDR2* gene using total RNA from *T. rubrum* strain H6 cultured in liquid Sabouraud medium for 72 h and treated with the following toxicants for 15 min: (1) control; (2) acriflavine; (3) benomyl; (4) ethidium bromide; (5) ketoconazole; (6) cycloheximide; (7) chloramphenicol; (8) griseofulvin; (9) fluconazole; (10) imazalil; (11) itraconazole; (12) methotrexate; (13) 4NQO; (14) terbinafine; and (15) tioconazole. The 380 bp PCR product was used as the probe. Ethidium bromide-stained rRNA bands are shown for comparison of the quantities of loaded RNA.

control (Fig. 2). These results indicated that *TruMDR2* is easily induced by several chemically unrelated compounds. Semighini *et al.* (2002) observed that the ABC transporter encoded by the *atrD* gene of *A. nidulans* was induced in the presence of imazalil, itraconazole and 4NQO, showing that the *TruMDR2* and *atrD* genes have several common substrates. On the other hand, cycloheximide induces the expression of the *A. nidulans atrD* gene (Andrade *et al.*, 2000), but this induction was not observed for the *TruMDR2* gene (Fig. 2), even though both of these micro-organisms display intrinsic resistance to this compound

(Angermayr *et al.*, 1999; Weitzman & Summerbell, 1995). These results show the relevance of functional analysis of each ABC transporter, mainly in pathogenic fungi, as the proteins have an intrinsic property in each organism, as well as the fact that differences in the lipid composition of membranes may also affect substrate specificity (Andrade *et al.*, 2000).

Disruption of the *TruMDR2* gene

For functional analysis of the *TruMDR2* gene, a disruptor cassette was constructed and inserted into the H6 strain by protoplast transformation, creating a *TruMDR2* deletion allele ($\Delta TruMDR2$) by one-step gene replacement. We replaced 135 bp of the ORF of *TruMDR2* with a gene encoding hygromycin phosphotransferase (*hph*) to form the gene-replacement vector $p\Delta TruMDR2$ (Fig. 3a). Transformants were selected on hygromycin-containing medium and the *TruMDR2* deletion was confirmed by Southern blot analysis. As expected, genomic DNA of the H6 and $\Delta TruMDR2$ strains digested with *EcoRI* and *SacI* restriction enzymes and probed with the P-380 fragment (Fig. 3a) showed a hybridization signal at 694 bp for the H6 strain, whereas the mutant showed a hybridization signal at 2954 bp, which was the length expected for a correct gene-replacement event (Fig. 3b).

Northern blot analysis was carried out with total RNA isolated from mycelia of the $\Delta TruMDR2$ mutant treated

Fig. 3. Disruption of the *TruMDR2* gene. (a) Physical map of clone D7P containing the *TruMDR2* gene and a schematic diagram of its disruption. The arrow indicates the size of the *TruMDR2* gene and the direction of transcription. The regions of the ATP-binding domains (■) and the putative intron (▲) are shown. The P-135 and P-380 lines represent the DNA fragments used as gene-specific probes for *TruMDR2*. Vector $pCSN43$ contains the hygromycin B-resistance gene cassette (*hph*). Restriction enzyme sites: B, *Bam*HI; Cl, *Clal*; E, *Eco*RI; V, *Eco*RV; Sc, *Sac*I; S, *Sal*I; Sm, *Sma*I; X, *Xba*I. (b) Southern blot analysis of $\Delta TruMDR2$. Genomic DNA extracted from the parental strain H6 and H6 transformed with the disruptor cassette ($p\Delta TruMDR2$) was digested with *Eco*RI and *Sac*I and separated by 0.8% agarose gel electrophoresis. The blot was hybridized with the P-380 probe. Lanes 1 and 2 are the H6 strain and $\Delta TruMDR2$ mutant, respectively.

with itraconazole, a strong inducer of *TruMDR2* transcription, and probed with the fragment excised from the *TruMDR2* gene (P-135). No hybridization was observed, confirming that *TruMDR2* had been functionally deleted (data not shown). The Δ *TruMDR2* mutant was viable and grew in Sabouraud medium as well as the control strain H6.

Thus, the DNA transformation method developed for *Neurospora crassa* (Staben *et al.*, 1989) to disrupt or replace genes by homologous recombination events was successfully used here for *T. rubrum*, permitting, in the future, the functional study of other genes of this fungus. It is also noteworthy that the standardization of DNA transformation and disruption systems for *T. rubrum* amplifies the repertoire of molecular techniques for the study of this dermatophyte.

Sensitivity of the Δ *TruMDR2* mutant to toxicants

The susceptibility of the *T. rubrum* strains to the various inhibitory agents was also determined by estimating their MICs in RPMI 1640. Table 1 shows that the Δ *TruMDR2* mutant displayed increased sensitivity to terbinafine, 4NQO and ethidium bromide when compared with the H6 strain. In contrast, there was no difference in the level of sensitivity between the Δ *TruMDR2* mutant and the control strain when submitted to the other drugs tested. However, in spite of

our results suggesting that the *TruMDR2* gene modulates terbinafine susceptibility, Northern blot analysis revealed that the level of transcription of this gene, when *T. rubrum* was cultured in the presence of terbinafine, was at the same basal level as that observed in untreated cultures (Fig. 2). It is likely that the terbinafine concentration used in the Northern blot analysis ($0.1 \mu\text{g ml}^{-1}$) was not sufficient to activate transcription of the *TruMDR2* gene, suggesting that the fungus has another mechanism to deal with terbinafine at low concentrations. In *A. nidulans*, it has been shown that terbinafine resistance can be mediated through the overexpression of *sala*, a gene that encodes a salicylate 1-monooxygenase. This enzyme is involved in naphthalene degradation, suggesting that resistance could follow degradation of the naphthalene ring contained in terbinafine (Graminha *et al.*, 2004).

Ethidium bromide and 4NQO are substrates for some multidrug-resistance pumps. Increased expression of two multidrug transporter-like genes is associated with ethidium bromide and ciprofloxacin resistance in *Mycoplasma hominis* (Raherison *et al.*, 2002, 2005). 4NQO, a potent carcinogen, is activated metabolically to a form that reacts with DNA. In *Saccharomyces cerevisiae*, total deletion of the *SNQ1* gene (ABC transporter) was shown to increase the sensitivity of the mutants to 4NQO (Gömpel-Klein & Brendel, 1990).

We cannot rule out the possibility of the *TruMDR2* gene playing a role by modulating the susceptibility to the other drugs that increase the levels of *TruMDR2* transcripts, as observed by Northern blot analysis. However, the presence of additional forms of ABC transporter proteins in *T. rubrum* may compensate for the deletion of the *TruMDR2* gene by acting as an efflux pump to provide resistance to these toxic compounds. This could be the case for itraconazole, an antimycotic agent used to control dermatophytosis, which strongly induced transcription of *TruMDR2* and to which the Δ *TruMDR2* mutant strain presented no change in sensitivity at the itraconazole concentrations assayed (several concentrations were assayed; data not shown). In fact, the *T. rubrum TruMDR1* gene is also induced by many of these drugs, including itraconazole (Cervellati *et al.*, 2006), in spite of *TruMDR1* showing only 29% identity with *TruMDR2*. In *Candida albicans*, increased sensitivity to some antifungals could be observed only when a double-deletion strain, for genes *CDR1* and *CDR2* (ABC transporters), was constructed, because *Cdr1p* was able to compensate for the lack of *Cdr2p* (Sanglard *et al.*, 1997).

In conclusion, the growth phenotype observed for the Δ *TruMDR2* mutant suggests that the *TruMDR2* gene, although present as a single copy, is not essential for the *in vitro* survival of *T. rubrum*. We presume that the *TruMDR2* transporter-mediated toxicant efflux mechanism plays a role in modulating susceptibility to terbinafine, 4NQO and ethidium bromide in *T. rubrum*. A study of the molecular mechanisms of *T. rubrum* resistance/susceptibility is attractive because this organism is an important

Table 1. Susceptibilities of the *T. rubrum* H6 and Δ *TruMDR2* strains to cytotoxic compounds

MIC₁₀₀ corresponded to the lowest concentration of the toxic compound at which there was no macroscopic growth. The results are representative of two independent experiments performed in triplicate.

Antifungal and cytotoxic agents	MIC ₁₀₀ ($\mu\text{g ml}^{-1}$)	
	H6 (wild-type)	Δ <i>TruMDR2</i>
Aciclovir	1.25	1.25
Benomyl	0.37	0.37
Ethidium bromide	1.25	0.75
Ketoconazole	0.06	0.06
Cycloheximide	37.5	37.5
Chloramphenicol	37.5	37.5
Griseofulvin	0.5	0.5
Fluconazole	75	75
Imazalil	2.0	2.0
Itraconazole	0.09	0.09
4NQO	2.5	1.25
Terbinafine	0.64	0.32
Tioconazole	0.06	0.06
Hygromycin*	200	> 6000

*Hygromycin was used to select the hygromycin-resistant colonies after transformation with the plasmid p Δ *TruMDR2*.

human pathogen and is becoming a model for biomedical research on dermatophytes.

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