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Proteomic analysis reveals that Adh1p is involved in a synergistic fluconazole and tetrandrine mechanism against *Candida albicans*

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We previously showed that Adh1p participates in fluconazole (FLC) resistance in *Candida albicans* through a mechanism that may involve efflux pumps. We also found that the concomitant use of tetrandrine (TET) and FLC provided a synergistic action against *C. albicans* and that the mechanism of action could be related to inhibition of a drug efflux system. To determine whether Adh1p participates in the synergistic antifungal activity of TET against *C. albicans*, we performed a comparative proteomic study comparing cells treated with FLC and/or TET in FLC-sensitive CA-3 and untreated control cells. Proteins were analyzed using two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), and differentially expressed proteins were identified through matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF/TOF) mass spectrometry. The resulting data were searched against a *C. albicans* protein database. Our analyses identified six differentially expressed proteins; four (Eno1p, Adh1p, Slb1p, and Tdh1p) were down-regulated, and two (Xyl2p, and Cdc19p) were up-regulated. The Adh1p mRNA levels were consistent with the Adh1p protein levels in all of the groups. The results suggest that Adh1p participates in the synergistic antifungal activity of TET against *C. albicans*.

1. Introduction

We previously showed that Adh1p participates in FLC resistance in *Candida albicans* through a mechanism that may involve efflux pumps (Wang et al. 2012). We also found that tetrandrine (TET) can strongly reverse the resistance of *C. albicans* to FLC in vitro (Zhang et al. 2010), in experimental animals and in volunteers with vaginal candidiasis or dermatophytosis (Shi et al. 2011); the mechanism of the action may be related to the inhibition of the drug efflux system (Zhang et al. 2009). Based on our preliminary research, we aimed to determine whether Adh1p participates in the synergistic antifungal activity of TET/FLC against *C. albicans*. Therefore, we used proteomics to identify differentially expressed proteins in untreated control cells and cells treated with FLC and/or TET in fluconazole-sensitive CA-3. We focused on the expression of Adh1p, which was further confirmed at the transcriptional level.

2. Investigations and results

2.1. Protein expression profiles

To obtain stable and comparable 2-D gel map images, total protein samples from *C. albicans* cell strains, either untreated or treated with FLC and/or TET, were prepared in parallel for three independent experiments. Each protein extract was then separated in triplicate by 2-D PAGE. Typical high-resolution silver-stained 2-D maps of four groups with differential protein expression areas are shown in Fig. 1. The results were reproducible within the same protein sample and in samples from independent cell extracts. A total of six spots were differentially expressed.

2.2. Identification of differentially expressed proteins

Differentially expressed proteins were identified based on MALDI TOF MS/MS analysis. A protein score greater than 65 represented confidence limits > 95% in the present study and was considered statistically significant. The proteins identified in this study were named according to the CandidaDB database (http://genolist.pasteur.fr/CandidaDB/), and the biological functions were defined by the *Candida* Genome Database (Table). Our analyses identified six differentially expressed proteins; four (Eno1p, Adh1p, Slb1p, and Tdh1p) were down-regulated, and two (Xyl2p, and Cdc19p) were up-regulated. Interestingly, the expression levels of alcohol dehydrogenase (Adh1p) increased after FLC/TET treatment but decreased after FLC-TET combination treatment.

2.3. Validation of proteomic results by real-time RT-PCR analysis

To validate the differential expression of Adh1p, real-time RT-PCR analysis was performed. The total RNA from the untreated

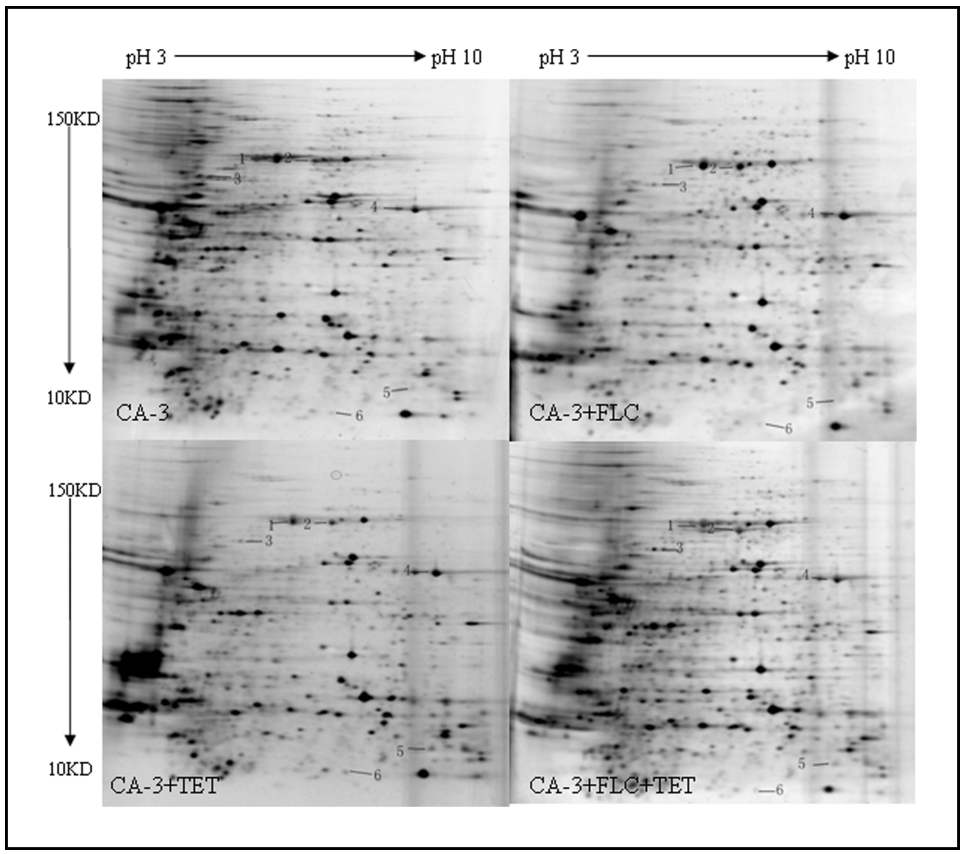

Fig. 1: 2D electrophoresis of whole cell extracts of C. albicans strains. The molecular weight (MW, kDa) and isoelectric point (pI) are indicated along the y-axis and the x-axis, respectively. Spots marked with arrows show significant differences in protein expression profiles among untreated cells or cells treated with FLC and/or TET.

Table: Summary of the identified proteins with different expressions in cells treated with FLC and/or TET in fluconazole-sensitive CA-3 and untreated control cells

Spot	*Accession No.* *	*Proteins* †	*Function description* *	*Protein score*‡	*MW (kDa)*	*pI*	*FLC*	*TET*	FLC + TET
1	232054	Eno1p	Enolase 1	813	47	5.54		↓	↓
2	1168348	Adh1p	Alcohol dehydrogenase	683	37	6.02	↑	↑	↓
3	68485044	Slb1p	Possible sphingolipid long chain base sensory protein	166	35	4.86	↓	↓	↓
4	68472227	Tdh1p	Glyceraldehyde-3-phosphate dehydrogenase	570	36	6.61	↑		↓
5	68482226	Cdc19p	Pyruvate kinase	761	55	6.54		↑	↑
6	238880445	Xyl2p	D-xylulose reductase	76	38	5.63		↑	↑

* Protein accession numbers and description according to NCBI protein database; †: Protein name according to the *Candida albicans* genomic database(CandidaDB); ‡: It is based on NCBInr database using the MASCOT searching program as MALDI-TOF data; "↑", vs control cells; "↓", vs control cells.

cells and the cells treated with FLC and/or TET was isolated in parallel for three separate experiments. Real-time RT-PCR reactions were performed in triplicate with independent RNA isolations. The changes of *ADH1* expression correlated to those observed in Adh1p expression (Fig. 2).

3. Discussion

C. albicans is one of the leading causes of fungal infections that affect immuno-compromised individuals. Combination therapies of antifungal drugs with synergistic antifungal agents, such as tacrolimus (Sun et al. 2008), chemosensitizers (Bulatova and Darwish 2008), cyclosporine (Marchetti et al. 2003), baicalein (Fu et al. 2011) and TET, could be of use for the treatment of candidiasis caused by drug-resistant *C. albicans*. However, the mechanism of the synergistic agent requires further investigation.

TET has been used in the treatment of hypertension, cardiac arrhythmia, and *Angina pectoris* in China since the 1950 s (Chen et al. 2009, Huang et al. 2011), but TET's main mechanism of synergistic antifungal activity against *C. albicans* remains unknown. In this study, we investigated the synergistic mechanism of FLC and TET against *C. albicans*. A comparative

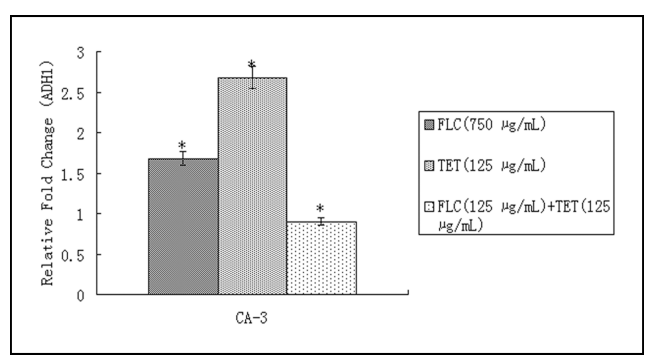

Fig. 2: The expression levels of ADH1 in cells that were either treated with FLC and/or TET relative to untreated cells. The ratios represent the mean ± standard deviations for three independent experiments. *$P < 0.05$ vs control cells.

proteomic study was performed in *C. albicans* strains that were either untreated or treated with FLC and/or TET. We were particularly interested in the striking changes in energy-metabolism-related protein alcohol dehydrogenase (Adh1p) after FLC and/or TET treatment. The expression of Adh1p increased after FLC/TET treatment, but was down-regulated after FLC-TET combination treatment; Adh1p expression was confirmed by real-time RT-PCR. These findings suggest that Adh1p is involved in the synergistic mechanism of TET against *C. albicans*.

Adh1p, located in the cell membrane and cytoplasm of *Candida*, serves as a key enzyme in ethanol fermentation and plays an important role in energy metabolism (Bertram et al. 1996). An increase in Adh1p was also observed in *C. albicans* biofilms (Martinez-Gomariz et al. 2009). FLC has been reported to induce *ADH1* gene expression (Copping et al. 2005). We previously showed that Adh1p is involved in drug resistance in *C. albicans*. In this study, we confirmed that Adh1p is involved in a TET synergistic mechanism. The results may provide more detailed theoretical support for future clinical applications of TET as a synergistic agent.

Two-dimensional gel electrophoresis (2D-PAGE), when combined with the MALDI-TOF/TOF tandem mass spectrometry used in this study, is a powerful tool in proteomics (Monteoliva et al. 2011). The special advantage of this technique is the possibility of high-throughput analysis. In addition to Adh1p, we also observed that other proteins are involved in amino acid biosynthesis (Cdc19p), gluconeogenesis (Eno1p), and other processes. Real-time RT-PCR has the advantages of simplicity, high sensitivity, and high efficacy (Parida et al. 2007). We performed three parallel experiments under identical conditions and implemented corrections using a housekeeping gene (18S rRNA) as the internal reference gene, thereby increasing quantification accuracy.

In summary, differentially expressed proteins were identified in *C. albicans* strains that were either untreated or treated with FLC and/or TET using a proteomic approach. The expression of Adh1p was further verified by real-time RT-PCR. Changes in Adh1p levels after FLC and/or TET treated were monitored, and Adh1p was confirmed to be associated with the synergistic antifungal mechanism of TET against *C. albicans*. However, the pathway through which Adh1p participates in this synergistic action remains unknown; future gene knockout studies are necessary to further understand the mode of action.

4. Experimental

4.1. Candida strains and growth conditions

The experimental strains were FLC -sensitive CA-3(FLC MIC$\leq 8\ \mu g\ mL^{-1}$) from a HIV-infected male who was treated with increasing doses of FLC (generously provided by Theodore C. White, University of Washington, and the Seattle Biomedical Research Institute, Seattle, WA). CA-3 was inoculated into YEPD medium.

4.2. Experimental group

Cultures were grown at 37 °C in a shaker set at 200 rpm until OD of the culture at 600 nm reached a value of 1.0. Cultures were divided into 4 groups: 1. CA-3 group containing culture without any drugs; 2. CA-3 + FLC group containing culture treated with 750 μg mL^{-1} fluconazole; 3. CA-3 + TET group containing culture treated with 125 μg mL^{-1} TET; 4. CA-3 + FLC + TET group containing culture treated with 750 μg mL^{-1} fluconazole and 125 μg mL^{-1} TET.

4.3. Comparative proteomic analysis

4.3.1. Protein sample preparation

Cells were cultured for 6 h, then cells were harvested by centrifugation at 10 000 g at 4 °C, and washed with PBS buffer and ice-cold deionized water. Finally cultures were centrifugated again, then wet cells were weighed and transferred to clean 1.5 mL Eppendorf tubes, stored at −80 °C until analysed. To the *C. albicans* cell pellet (100 mg wet weight) were added 500 μL of lysis buffer (7 M urea, 2 M thiourea, 4 % (w/v) CHARPS, 1 % (v/v) DTT, 2 % (v/v) IPG buffer 20 mg mL^{-1} PMSF and 1 % (v/v) protease inhibitors) for 30 min, then cells were freezed rapidly with liquid nitrogen and thawed for 4 times, then sonicated 100 times for 3 s each (285 W,20 kHz) with 9 s interval cooling on ice. After cell lysis, cellular debris was removed by centrifugation for 30 min at 13 200 g and at 4 °C. The clear supernatants were stored at −80 °C until analyzed.

4.3.2. Two-dimensional gel electrophoresis and in-gel tryptic digest

Proteins containing 150 μg were rehydrated in sample buffer (5 M urea, 2 M thiourea, 2% CHAPS, 2% SB3-10, 65 mM DTT, and 0.5% (v/v) Pharmalyte 3–10) and then applied onto pH 3–10 nonlinear IPG Strips (13 cm, GE Heathcare). Isoelectric focusing was carried out on the Amersham Biosciences IPG-phor IEF System at 20 °C with the following program: 30 V for 2 h, 500 V for 1 h, 1000 V for 1 h, and 8000 V for 6 h, finally 2000 V for 10 h. After focusing, IPG strips were reduced (2% w/w dithioerythritol) for 13 min and then alkylated (2.5% w/w iodoacetamide) for 13 min. SDS-PAGE gel electrophoresis was carried out on homogeneous 12.5% (w/w) T, 1.6% (w/w) C polyacrylamide gels (1.0 mm thick) at 12 mA per gel for15 min and then 30 mA for 4 h using a Hoefer SE600 (GE healthcare, Uppsala, Sweden). Proteins were detected by a silver nitrate staining protocol. Gels were scanned on an Image Scanner II (GE Healthcare, Uppsala, Sweden), and images were analyzed using the Image Master 2D Platinum (GE Healthcare, Uppsala, Sweden). The significant protein spots were excised from the gels for in-gel tryptic digest according to Wang et al (Wang et al. 2006).

4.3.3. Mass spectrometry and database search

Peptide mass spectra were obtained on an ABI 4800 plus MALDI TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA). Both the MS and MS/MS data were interpreted and processed by using the GPS Explorer software (V3.6, Applied Biosystems), then the obtained MS and MS/MS spectra per spot were combined and submitted to MASCOT Search engine (V2.1,MatrixScience,London,U.K.) by GPS Explorer software. MASCOT protein scores (based on combined MS and MS/MS spectra) of greater than 65 were considered statistically significant ($p < 0.05$) (Yan et al. 2007).

4.4. Real-time reverse transcription polymerase chain reaction (real-time RT-PCR)

Total RNA samples from *C. albicans* strains were extracted using the trizol method as previously described (Zhang et al. 2009). cDNA was synthesized as recommended by the manufacturer (Takara, Shiga, Japan). The reaction volume of 20 μL consisted of RNA 2 μL, Oligo(dt)18 2 μL, 5X M-MLV Buffer 4 μL, dNTP (10 mM) 1 μL, RNase Inhibitor (40 U/μL) 0.5 μL, Rtase M-MLV(Rnase H-)(200 U/μL) 0.5 μL, with the remaining volume composed of Rnase-free water. The reaction temperatures were 42 °C for 60 min, 70 °C for 10 min.

cDNA fragments of *ADH1* were amplified with the following primers (Henry et al. 2000) 5'-TGTCTGGTTACACTCACGATGG-3', 5'-GCATCGAAAACTGGAGCAGT-3'.

Real-time RT-PCR reactions were performed with SYBR Green I (TaKaRa, Japan), using LightCycler Real-Time PCR system (Roche Molecular Biochemical, Mannheim, Germany). The thermal cycling conditions comprised an initial step at 95 °C for 10 s, followed by 40 cycles at 95 °C for 10 s, 62 °C for 20 s, and 72 °C for 15 s. The change in fluorescence of SYBR Green I in every cycle was monitored by the system software, and the threshold cycle (CT) was measured. 18S rRNA was used as an internal control, data were analyzed using the LightCycler software version 3.5 (Roche Diagnostics, Mannheim, Germany), and the gene expression level was calculated using the formula $2^{-\Delta\Delta CT}$.

4.5. Statistical analysis

All experiments were done at least three times to ensure reproducibility of the results. Data are presented as mean ± standard deviations. MASCOT protein scores of greater than 65 were considered statistically significant ($P < 0.05$).

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