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Article

Berberine reverses multidrug resistance in *Candida albicans* by hijacking the drug efflux pump Mdr1p

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ABSTRACT

Clinical use of antimicrobials faces great challenges from the emergence of multidrug-resistant pathogens. The overexpression of drug efflux pumps is one of the major contributors to multidrug resistance (MDR). Reversing the function of drug efflux pumps is a promising approach to overcome MDR. In the life-threatening fungal pathogen *Candida albicans*, the major facilitator superfamily (MFS) transporter Mdr1p can excrete many structurally unrelated antifungals, leading to MDR. Here we report a counter-intuitive case of reversing MDR in *C. albicans* by using a natural product berberine to hijack the overexpressed Mdr1p for its own importation. Moreover, we illustrate that the imported berberine accumulates in mitochondria and compromises the mitochondrial function by impairing mitochondrial membrane potential and mitochondrial Complex I. This results in the selective elimination of Mdr1p overexpressed *C. albicans* cells. Furthermore, we show that berberine treatment can prolong the mean survival time of mice with blood-borne dissemination of Mdr1p overexpressed multidrug-resistant candidiasis. This study provides a potential direction of novel anti-MDR drug discovery by screening for multidrug efflux pump converters.

1. Introduction

Considering the high mortality of fungal infections in immunocompromised patients and the limited number of effective and safe antifungal drugs, the development of new antifungals and/or antifungal therapeutics is critical [1]. Widespread and repeated use of current antifungals, particularly azoles, however, has led to the rapid occurrence of antifungal resistance [2]. Current approaches for novel antifungals discovery are usually targeting essential fungal genes or metabolic pathways, which are very likely to generate drug resistance over time.

One major mechanism underlying fungal drug resistance is the overexpression of drug excretion transporters. There are two types of such transporters in the most commonly seen clinical fungal pathogen *C. albicans*. The *C. albicans* drug-resistance (CDR) transporters, like Cdr1p and Cdr2p, which belong to the ATP-binding cassette (ABC) family, use ATP as their energy source for drug excretion [3]. The other type is the major facilitator superfamily (MFS) transporter, such as Mdr1p (also known as the benomyl/

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methotrexate resistance protein). This superfamily is a drug/H^+ antiporter that uses the proton gradient across the cytoplasmic membrane for drug excretion [4–6]. Enhanced expression of *MDR1* has been correlated with resistance to a variety of structurally unrelated compounds, such as fluconazole and cerulenin [7]. In theory, as a proton gradient drove drug/H^+ antiporter, Mdr1p has the potential to work reversely, importing substrates while exporting H^+. This could provide a novel opportunity for antifungals to fight against multidrug-resistant *C. albicans*.

Berberine is an alkaloid with a long history of medicinal application in traditional Chinese medicine that can be produced by many plant species, such as *Coptis chinensis* (Coptis, goldenthread), *Hydrastis canadensis* (goldenseal), and *Berberis vulgaris* (barberry). Berberine has demonstrated significant activities on antimicrobial [8], anti-tumor [9], anti-inflammatory [10], anti-diabetes [11], lower-cholesterol [12], and compromise-mitochondrial functions [13]. Recent studies indicated that fungal mitochondria might be a potential antifungal target due to the presence of unique DNA/proteins [14].

In this study, we described an unexpected association between the killing effect of berberine and the expression of Mdr1p in *C. albicans*. Instead of being excreted, berberine can hijack the overexpressed Mdr1p to facilitate its own accumulation. Berberine then compromises the function of mitochondria to selectively eliminate the Mdr1p overexpressed multidrug-resistant *C. albicans*.

2. Materials and methods

2.1. Strains and growth conditions

The complete list of strains used in this study is listed in Table S1 (online). Fungal strains are stored in 25% glycerol at −80 °C, while cell lines, ordered from the cell bank, Shanghai Institutes for Biological Sciences, are stored in liquid nitrogen.

RPMI 1640 (Invitrogen, Carlsbad, USA) is used according to the manufacturer's protocol. The Yeast Extract–Peptone–Dextrose (YPD) medium consisted of yeast extract 1% (w/v), peptone 2% (w/v), and dextrose 2% (w/v), with 2% (w/v) agar to make a solid medium when needed.

2.2. Antifungal agents and molecular probes

All chemicals were used according to the manufacturers' directions. Fluconazole, itraconazole, sanguinarine hydrochloride, jatrorrhizine hydrochloride, palmatine hydrochloride, chelerythrine, and proflavine were purchased from the National Instisutes for Food and Drug Control, China (purity > 98%). Berberine hydrochloride, cyclophosphamide, rhodamine 123, carbonyl cyanide-m-chlorophenyl hydrazone (CCCP), propidium iodide (PI), and 2,7-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich, USA. Cerulenin was purchased from Alexis, Enzo Life Sciences, USA.

2.3. Antifungal susceptibility assay

Drug susceptibility testing was carried out as described previously [15] in flat-bottom, 96-well microtiter plates (Greiner, Pleidelsheim, Germany), using a broth microdilution protocol modified from the Clinical and Laboratory Standards Institute M-27A methods [16]. The minimum inhibitory concentration (MIC) was determined as the concentration of drugs that inhibit fungal growth by 80% relative to the corresponding drug-free growth control by reading the optical density (OD, A_{600nm}) using a FLUOstar OPTIMA microplate reader (BMG LABTECH, Ortenberg, Germany). Spot assay was performed as described previously [17]. A total of 10 μL samples of ten-fold serial dilutions of cells, suspended in phosphate-buffered saline (PBS), were spotted onto YPD plates in the absence (control) or the presence of tested drugs. Photos were taken after a 48 h incubation at 30 °C. For non-glucose utilization, glucose in yeast extract-peptone agar was replaced with 2% of citrate, glycerol, lactate, or ethanol.

2.4. Berberine accumulation and release assay

All *C. albicans* strains were grown at 30 °C overnight in YPD medium and washed twice with PBS. Cells were resuspended in PBS with approximately 5×10^7 cells/mL (determined by hemocytometer counting). Berberine (32 μg/mL) was added to each sample for incubation at 30 °C. For a 90 min accumulation assay, 1 mL of each sample was taken every 10 min and the supernatant was carefully removed by a pipette after 1 min of centrifugation at top speed, and then the cell pellets were resuspended in 1 mL PBS. For a 60 min release assay, 10 mL of each sample at the time point of 90 min is centrifuged for 5 min at 5000 g, washed once, and then resuspended in 10 mL of PBS. The sampling procedure was similar to the accumulation assay. A total of 150 μL samples were transferred into black 96-well microplates with the clear bottom (Greiner) for fluorescence measurements. Fluorescence measurements of berberine are performed with a BioTek™ Synergy™ Mx Monochromator-Based MultiMode Reader (Thermo Fisher Scientific, Waltham, USA) at 360 nm excitation/520 nm emission wavelengths.

2.5. Accumulation of berberine influenced by CCCP

To investigate the influence of the proton gradient, a final concentration of 20 μg/mL CCCP was added to the samples 30 min before the start of accumulation or release assays. Samples were shaken at 200 r/min (30 °C). After incubation, samples were washed with PBS to remove CCCP, then the berberine accumulation assay was carried out as described above.

2.6. pH-dependent accumulation of berberine

To investigate the influence of pH, cells were incubated in RPMI 1640 media adjusted to pH 4.0, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 and treated with berberine. The berberine accumulation assay is carried out as described above.

2.7. RNA preparation, sequencing, and transcriptome analysis

Strains were incubated for 16 h at 30 °C in RPMI 1640 medium with shaking with (32 μg/mL) or without berberine. Two volumes of RNAprotect Reagent (Qiagen, Hilden, Germany) were added into the cultures according to the manufacturer's protocol. Cells were harvested, washed twice, and then resuspended into PBS. The cells were then lysed by lysozyme (400 μg/mL, Ready-Lyse™ Lysozyme Solution, Epicenter). Total RNA was purified by RNeasy Mini Kit (Qiagen). Ribosomal RNA was removed by RiboMinus Transcriptome Isolation Kit (Invitrogen) and mRNA is cleaned up using RNeasy Mini Spin columns (Qiagen). The resulting mRNA was used for library construction for RNA-seq using NEBNext mRNA Library Prep Reagent set for Illumina (NEB, Ipswich, USA). Briefly, mRNA was fragmented to the desired length and reverse transcribed into the first-strand cDNA. The single-strand cDNA was used for synthesizing double-strand DNA followed by end repair, dA-tailing, adaptor ligation, and PCR amplification. The library was tested by length determination and quantitative PCR quantification. These constructed libraries were then sequenced by the Illumina platform by paired-end chemistry.

RNA reads were aligned to the *C. albicans* SC5314 assembly using Tophat [18] and quantified with HTseq [19]. The raw read counts were normalized with DESeq2 [20] to estimate gene expression and identify differential gene expression. Differential gene expression was identified using the threshold of the parametric $P < 0.05$ and a fold change of at least 2 and the false discovery rate (FDR) < 0.2. Gene ontology analysis was performed at the *Candida* genome database (CGD, www.candidagenome.org) and FungiFun2 (https://elbe.hki-jena.de/fungifun/). Enrichment maps were constructed with Cytoscape 2.8.3 (http://www.cytoscape.org) and the Enrichment Map v1.2 plug-in using the default settings (http://www.baderlab.org/Software).

2.8. Assay of the reactive oxygen species (ROS) measurement

As previously described [21], intracellular ROS production was detected by staining cells with the ROS-sensitive fluorescent dye DCFH-DA (2,7-dichlorofluorescein diacetate; Sigma, St. Louis, USA). Cells from 25 mL cultures grown at 30 °C overnight in YPD medium were collected and washed twice with PBS. The pellets were suspended to 10^6 cells in 10 mL of PBS plus 2% glucose and treated with or without 10 μmol/L DCFH-DA for 30 min at 37 °C in dark. Cells from each sample were collected and washed twice with PBS after staining. The cells were resuspended with PBS plus 2% glucose and treated with berberine at the indicated concentrations for 60 min 30 °C. The fluorescent intensity was measured using a FACScan flow cytometer (Becton Dickinson, San Jose, USA). PI was added to each sample to detect dead cells. The mean fluorescence for ROS was quantified only in live cells.

2.9. Assay of protoplast and mitochondrial preparations

Cells were grown in 250 mL of YPD broth overnight at 30 °C, then washed and resuspended in RPMI 1640 medium. After incubation with or without 20 μg/mL berberine for 1 h, cells were harvested by centrifugation (5000 r/min for 10 min) and washed with 50 mL of cold water and buffer A (1 mol/L sorbitol, 10 mmol/L $MgCl_2$, and 50 mmol/L Tris-HCl, pH 7.8). Protoplasts were made by digesting the cell wall with Zymolyase 20 T for 1 h at 30 °C. The mitochondrial fraction was obtained by Percoll-density-gradient centrifugation as described previously [21], and suspended in 1 mL of buffer D (0.6 mol/L mannitol, 10 mmol/L Tris-HCl, pH 7.0). The protein content was determined by the Biuret method.

2.10. Assay for the measurement of mitochondrial membrane potential

The mitochondrial membrane potential was determined by staining cells with rhodamine 123 [22]. Cells from 25 mL cultures grown at 30 °C overnight in YPD medium were collected and washed twice with RPMI 1640 medium. The pellets were resuspended in RPMI 1640 medium to 10^6 cells/mL. Berberine was added at the indicated concentrations. After 60 min incubation, the samples were treated with or without 10 μg/mL rhodamine 123 for 15 min at 37 °C in dark. Cell fluorescence in the absence of rhodamine 123 was used to verify that background fluorescence is similar among strains. Cells from each sample were collected and washed twice with PBS after staining. Fluorescence was measured using a FACScan flow cytometer (Becton Dickinson).

2.11. Assay for the oxygen consumption rate

Oxygen consumption was measured polarographically using a Clark-type electrode (Hansatech Instruments, Norfolk, UK). Cells were grown overnight at 30 °C in 20 mL of YPD broth and diluted in fresh YPD broth the next day for an additional 4 h until exponential growth was achieved. Cells were then centrifuged, washed with PBS, resuspended in RPMI 1640, and then treated with 10 μg/mL berberine for 2 h. Cells were collected, washed with PBS, and resuspended in 2 mL of YPD broth before loading into a sealed 1.5mL glass chamber. The oxygen concentration in the chamber was monitored over a 5–10 min period. The respiratory rate was calculated as the consumption of oxygen per min per mL of cell suspension normalized by A_{600nm} value.

2.12. Complex I (nicotinamide adenine dinucleotide hydrogen (NADH): ubiquinone oxidoreductase) activity assay

Crude mitochondrial preparations were first treated with two cycles of freeze-thawing in a hypotonic solution (25 mmol/L K_2HPO_4, pH 7.2, 5 mmol/L $MgCl_2$), followed by a hypotonic shock in H_2O. A total of 20 μg of mitochondrial protein from each sample was used to measure complex I enzymatic activity. Mitochondria in 0.8 mL of H_2O were incubated for 2 min at 37 °C and then mixed with 0.2 mL of a solution containing 50 mmol/L Tris, pH 8.0, 5 mg/mL bovine serum albumin (BSA), 0.24 mmol/L KCN, 4 μmol/L antimycin A, and 0.8 mmol/L NADH (substrate of Complex I). The reaction was initiated by introducing an electron acceptor, 50 μmol/L 2,3-dimethoxy-5-methyl-6-n-decyl-1,4-benzo quinone (DB). Enzyme activity was followed as a decrease in absorbance of NADH at 340 nm minus that at 380 nm.

2.13. Relative quantification of differentially expressed genes by quantitative real-time PCR (qRT-PCR)

All primers used in this study for qRT-PCR are listed in Table S2 online. RNA isolation, cDNA synthesis, and PCR amplification were carried out as described previously [23]. Triplicate independent qRT-PCRs were performed using the LightCycler System (Roche, Basel, Switzerland). 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 rDNA was used as an internal control, and the relative gene expression level was calculated using the formula $2^{-\Delta\Delta Ct}$.

2.14. Murine model of systemic infection and drug treatment

Mice related experiments were reviewed and approved by IACUC of Guangdong Laboratory Animal Monitoring Institute following the Guide for the Care and Use of Laboratory Animals under the license of IACUC2012006. Specific-pathogen-free female ICR (Crl: CD-1) mice (female, white, about 20–22 g) were used throughout the experiment, and the experiments were carried out as described previously [15]. Before infection, mice were rendered neutropenic by intraperitoneal (i.p.) injection of cyclophosphamide (CY) (Sigma) daily for 3 consecutive days at a dosage of 100 mg/kg body weight. Mice were monitored at designated days after the first CY injection for WBC counts by using a hemocytometer. Mice were then infected with 0.1 mL of 5×10^4 colony forming unit (CFU)/mL cells of *C. albicans* 11# per mouse in warmed saline (35 °C) by the lateral tail vein on day 3 after pretreatment with CY. Berberine (1 mg/kg) and fluconazole (0.5 mg/kg) independently are administered by i.p. 6 h post-infection and once daily thereafter for 3 d; and 0.1 mL of diluent (Dulbecco's phosphate-buffered saline, DPBS) by the same route as the placebo regimens. Data were averaged from three experiments.

2.15. Cytotoxicity evaluation using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The protocol was modified from previous version [24]. Briefly, the testing cells were seeded in a 96-well flat-bottom microtiter

plate with 1×10^4 cells/well and allowed to adhere for 24 h at 37 °C in a CO_2 incubator. Cells were gently washed with fresh medium. Cells were then treated with various concentrations of the target compounds for 24 h in the same cultivation condition. Cells are gently washed with the fresh medium again. Subsequently, 10 μL of MTT working solution (5 mg/mL in phosphate buffer solution) was added to each well and the plate is incubated for 4 h at 37 °C in a CO_2 incubator. The medium was then aspirated, and the formed formazan crystals were solubilized by adding 50 μL of dimethyl sulphoxide (DMSO) per well for 30 min at 37 °C in a CO_2 incubator. Finally, the intensity of the dissolved formazan crystals (purple color) was quantified using the enzyme-linked immuno sorbent assay (ELISA) plate reader at 540 nm.

3. Results

3.1. Berberine susceptibility is inversely correlated with MDR1 expression in C. albicans

To search for natural products that can utilize the drug excretion transporter for importation and accumulation, 29 clinical *C. albicans* isolates were used (Table S1 online) to screen our natural products collection of ~3800 pure compounds and ~100,000 crude extracts. Many of these clinical isolates overexpress drug excretion transporters, which results in fluconazole resistance (Fig. 1a). However, we observed a cluster of one set of the fluconazole-resistant *C. albicans* isolates with *MDR1* overexpression that showed hypersensitivity to berberine (boxed panel of Fig. 1a). Next, we investigated the relationship between Mdr1p overexpression and the hypersensitivity of berberine. Another set of *C. albicans* strains with different expression levels of Mdr1p were used, namely, CaS, CaR, CaDEL, and CaCOM (Table S1 online). CaS, isolated from an AIDS patient G, was proven to be the original fluconazole-susceptible strain with basal Mdr1p expression, while CaR, isolated from the same patient after two years of treatment with fluconazole [25], was highly resistant to fluconazole, and the dominant contributor of fluconazole resistance was Mdr1p overexpression [7,26] (Fig. S1 online). CaDEL was constructed by *MDR1* deletion of CaR [26], and CaCOM was constructed by *MDR1* reconstitution into CaDEL [7]. *MDR1* expression levels of these four strains were confirmed by quantitative real-time PCR (Fig. 1b), and the Mdr1 protein abundance was confirmed by Western blotting [7]. From the spot assay results (Fig. 1c), the growth of those strains with high levels of Mdr1p (CaR and CaCOM) were inhibited by berberine, while they were uniformly resistant to fluconazole (red boxed panel of Fig. 1c). In contrast, CaS and CaDEL, the two strains with basal expression levels of Mdr1p, showed resistance to berberine but were susceptible to fluconazole (Fig. 1c). These results suggested that berberine has a "**s**electively **e**liminate the **M**dr1p **o**verexpressed ***C**. albicans*" (SEMOC) property.

We speculated that berberine would not be the only natural compound with SEMOC. Besides berberine, other compounds, like jatrorrhizine, proflavine, palmatine, and BQM [27] which have higher activity against drug-resistant *C. albicans* with Mdr1p overexpression over wild type *C. albicans* were also identified from our screening (Fig. S2 online).

3.2. Berberine could specifically be accumulated in Mdr1p overexpressed C. albicans cells

Next, we sought to understand the biological mechanism underlying SEMOC property. As berberine has fluorescence emission at 520 nm by 360 nm excitation, intracellular berberine accumulation can be quantitated by fluorescence readout. We measured the rate of accumulation and release of intracellular berberine in all four strains: CaR, CaS, CaDEL, and CaCOM, it showed a progressive and consistent increase in berberine accumulation throughout a 90-min incubation period (left panel of Fig. 1d). The amount of berberine in strain CaR is approximately three times as much as that observed in CaS and CaDEL. We observed that the rate of berberine uptake in Mdr1p overexpressed strains exceeded the rate of release (Fig. 1d), leading to a net accumulation of berberine in CaR and CaCOM strains. This observation suggested that overexpressed Mdr1p might serve as an importer of berberine into *C. albicans* cells.

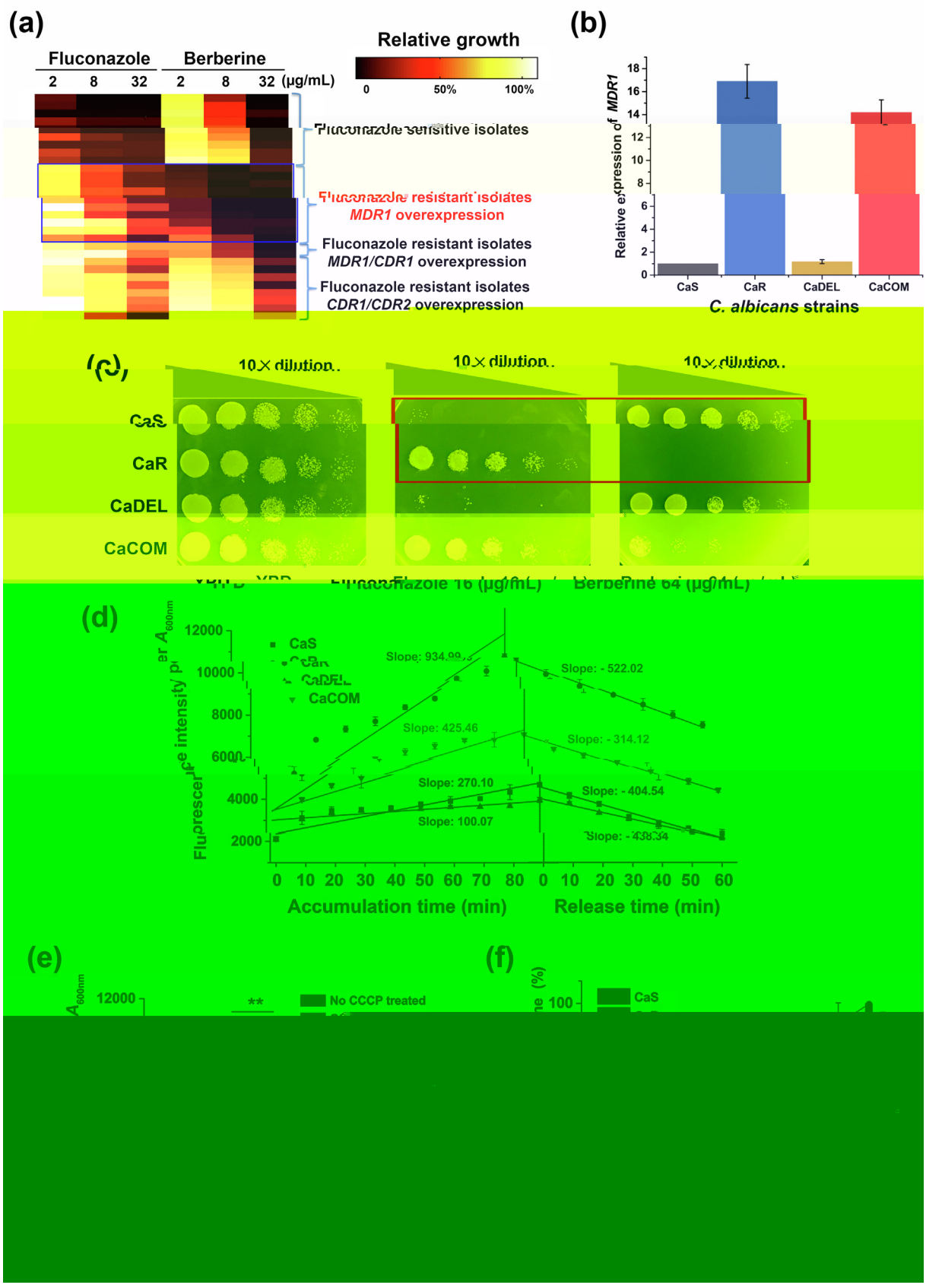
To confirm this possibility, we further tested whether the accumulation of berberine was dependent on a proton gradient, as Mdr1p utilizes the proton gradient across the cytoplasmic membrane as its energy source for transportation [4,7]. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) was used to uncouple the proton gradient. Berberine accumulation indeed decreased in Mdr1p-(over)expressed strains after CCCP treatment (Fig. 1e). This data not only confirmed the proton gradient played an important role in berberine accumulation but also indicated that the effects of Mdr1p-overexpression in berberine accumulation.

Due to the drug/H^+ antiporter property of Mdr1p, we hypothesized that an alkaline extracellular environment might switch Mdr1p from a drug efflux protein into a drug importer. Thus, we evaluated the extracellular pH effect on berberine accumulation. We observed that an increased extracellular pH (reduced extracellular H^+ concentration) promoted berberine accumulation in strains with a high level of Mdr1p (Fig. 1f).

3.3. Intracellular berberine causes mitochondrial dysfunctions

Then we asked how intracellular berberine inhibits the growth of *C. albicans*. We reasoned that a comparison of the intrinsic differences of response-to-berberine-treatment between CaS and CaR might reveal the targets of berberine. We analyzed the transcriptomes of CaS and CaR with and without berberine treatment, finding that a total of 182 genes were upregulated in CaR (cut-off $\geq$ 2.0-fold, $P < 0.05$ and FDR < 0.2) (Fig. 2a), especially genes encoding oxidoreductases (21.7%, GOID: 16491, P value 5.41×10^{-7}) such as the aldo-keto reductase family, *IFD6* and *CSH1*. To further narrow down the cellular pathways affected by berberine in *C. albicans*, we performed Gene Set Enrichment Analysis (GSEA), and the

Fig. 1. The anti-*C. albicans* activity of berberine is inversely correlated with MDR1 overexpression. (a) The antifungal activity of berberine is compared to fluconazole in 29 *C. albicans* isolates, many of which are fluconazole resistant. Susceptibility profiles are indicated as color changes from no growth (black) to growth (white) for each inhibitor (average of three independent experiments). MDR1 overexpression isolates are hypersusceptible to berberine while resistant to fluconazole. The right panel shows the isolates are clustered according to their susceptibility, source, and/or resistance mechanisms. (b) MDR1 expression level of CaS, CaR, CaDEL, and CaCOM, CaS is the original azole-susceptible strain [25], while CaR, isolated from the same patient after the long time treatment with fluconazole, is resistant to fluconazole [7,26], CaDEL is a MDR1 deletion strain derived from CaR [26], and CaCOM is the CaDEL strain with reintroduced MDR1 through expressing the PADH1-MDR1 fusion [7]. Quantitative real-time PCR analysis of MDR1 expression was performed in triplicate. Mean values from three independent experiments are shown. Error bars indicate standard deviation. (c) Drug susceptibility tested by spot assay. Growth of 10-fold serial dilution of CaS, CaR, CaDEL, and CaCOM on YPD or YPD containing berberine (64 μg /mL) and fluconazole (16 μg / mL). (d) A 90-min berberine accumulation time course followed by a 60-min release time course of CaS, CaR, CaDEL, and CaCOM. Lines represent the linear fittings, the slope for each line is displayed. (e) Endpoint accumulation of berberine in CaS, CaR, CaDEL, and CaCOM, after a 90-min incubation of berberine, with and without CCCP treatment, respectively. A two-tailed test is used for statistical analysis, ** $P < 0.01$. (f) Extracellular pH affected the berberine accumulation in CaS, CaR, CaDEL, and CaCOM.


(a)
Fluconazole
Berberine
2 8 32 2 8 32 (μg/mL)
Relative growth
0 50% 100%
Fluconazole sensitive isolates
Fluconazole resistant isolates
MDR1 overexpression
Fluconazole resistant isolates
MDR1/CDR1 overexpression
Fluconazole resistant isolates
CDR1/CDR2 overexpression
(b)
Relative expression of MDR1
CaS
CaR
CaDEL
CaCOM
C. albicans strains
(c)
10× dilution
CaS
CaR
CaDEL
CaCOM
Fluconazole 16 (μg/mL)
Berberine 64 (μg/mL)
(d)
Fluorescence intensity per A600nm
CaS
CaR
CaDEL
CaCOM
Slope: 934.99
Slope: 425.46
Slope: 270.10
Slope: 100.07
Slope: - 522.02
Slope: - 314.12
Slope: - 404.54
Slope: - 438.34
Accumulation time (min)
Release time (min)
(e)
A600nm
**
No CCCP treated
(f)
CaS

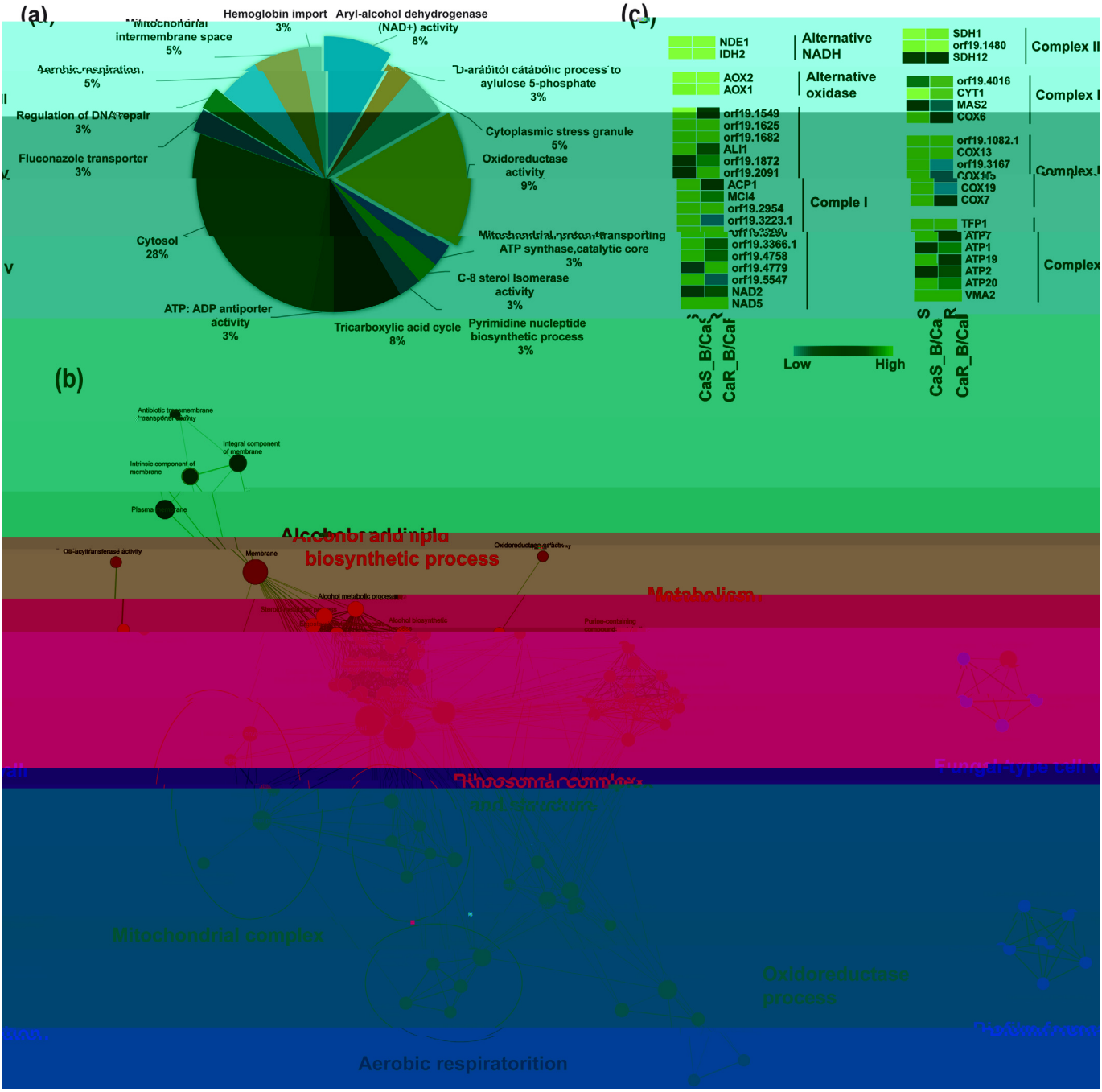


Fig. 2. Differential expression analysis of the transcriptome after berberine treatment in CaS and CaR. (a) RNA-seq analysis of berberine-treated CaR/CaS. Data were presented as a pie chart of functional gene categories (Gene Ontology Term analysis) of upregulated genes in CaR compared to CaS. A total of 182 genes were upregulated in CaR (cut-off of 2.0-fold, $P < 0.05$ and FDR < 0.2). (b) The RNA sequencing of the genes differentially expressed after berberine treatment in CaS and CaR; mitochondrial genes were significantly altered. The network of functional groups of genes regulated by berberine was constructed with Cytoscape. Blue circles represent downregulated gene sets, while red circles represent upregulated genes. The size of the circle reflects the number of modulated genes in each functional group. (c) Electron Transport Chain (ETC) genes were induced by berberine treatment in CaR and CaS. Data were indicated by a blue (downregulation) to yellow (upregulation) growth scale by comparing treated cells with untreated cells of three independent experiments. Genes are clustered by respiratory functional groups. Gene names are taken from the Candida Genome Database (www.candidagenome.org) and their orf19 names are given. Red and blue represents up-regulation and down-regulation, respectively. The dot size represents gene numbers.

ranked gene lists from the transcript profiles were compiled according to the change in their expression to a predefined database of 8123 gene sets [28]. Significantly enriched gene sets were further visualized using Cytoscape. We observed that the expression of mitochondrial function/aerobic respiration related genes was significantly upregulated after berberine treatment (Fig. 2b, c). These results suggested that berberine impacted mitochondrial function.

To test this hypothesis, we first confirmed that berberine can accumulate in the mitochondria and as expected, mitochondria of CaR accumulate a higher level of berberine than that of CaS (Fig. 3a). Berberine also exhibited a greater inhibition on non-

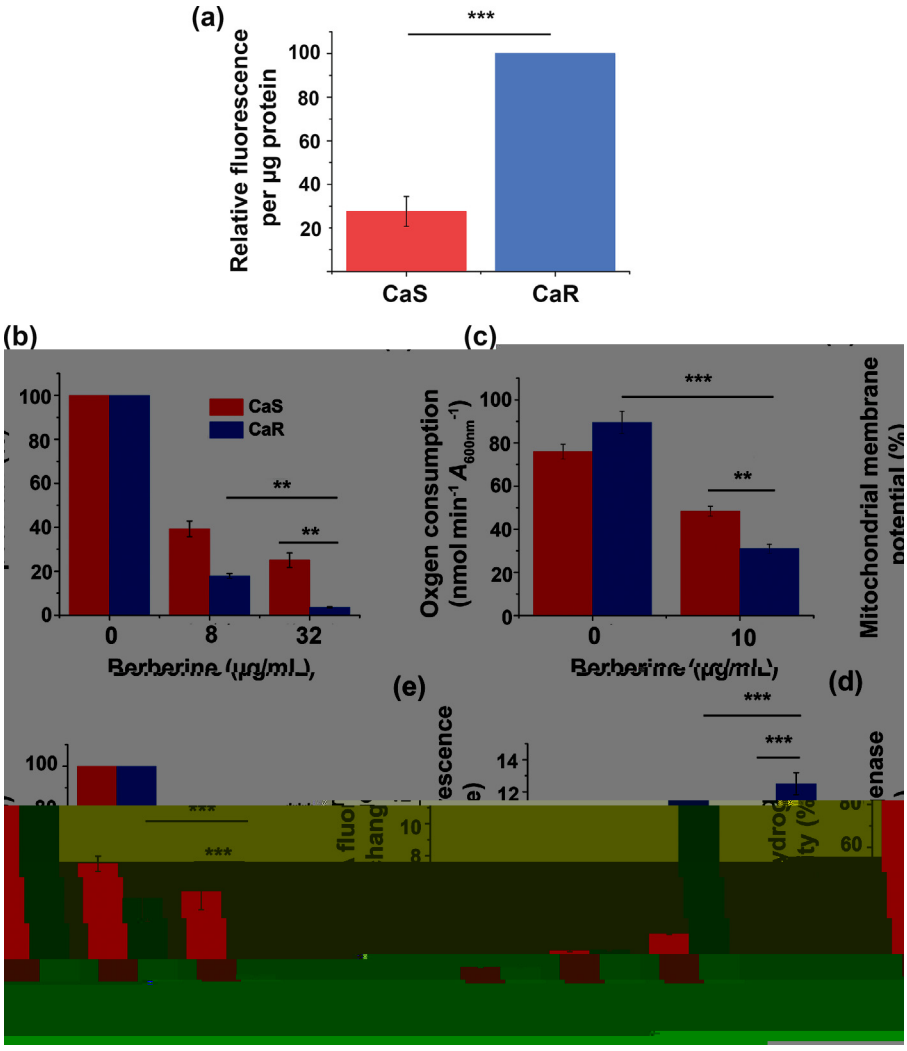


Fig. 3. Berberine accumulates in mitochondria and causes mitochondrial dysfunction. (a) Berberine accumulates in mitochondria. Berberine accumulation in percoll gradient purified mitochondria is shown. Fluorescence was measured and normalized to protein concentrations (μg/mL) (mean ± SD, n = 3). The value of CaR was presented as 100%. (b) Berberine treatment reduces mitochondrial membrane potential (MMP) of CaS and CaR cells. MMP was measured using rhodamine 123 in both strains treated with berberine at indicated concentrations. Fluorescence intensity was monitored with flow cytometry and normalized with control samples. Data are presented as the percentage of control cells (mean ± s.d., n=3). (c) Berberine inhibits oxygen consumption in *C. albicans*. Respiratory activity of untreated or treated cultures with berberine was measured and normalized to A_{600nm} value of cells (mean ± SD, n = 3). (d) Mitochondrial Complex I (NADH dehydrogenase) activity is inhibited by berberine treatment. NADH dehydrogenase enzymatic activity is normalized to the protein content of mitochondria. Data are presented as the percentage of untreated cells (mean ± SD, n = 3). (e) Berberine induces ROS production. Cells were treated with berberine at the indicated concentrations. ROS levels were measured using 2′,7′-Dichlorodihydrofluorescein diacetate (DCFDA) by flow cytometry and shown as fold-changes (mean ± SD, n = 3). A two-tailed test is used for statistical analysis, ** P < 0.01; *** P < 0.001

fermentable carbon sources compared to that on glucose, which possibly indicated that respiration was compromised during berberine treatment (Fig. S3 online). Besides, important parameters reflecting fungal mitochondrial function were examined. Berberine was found to significantly impair mitochondrial membrane potential (Figs. 3b and S4 online), and oxygen consumption (Fig. 3c). Also, after berberine treatment, the activity of the Complex I (NADH dehydrogenase) was sharply reduced (Fig. 3d). In addition, *NDH51*, encoding the mitochondrial Complex I 51-*k*D subunit of the NADH dehydrogenase protein Ndh51p, was downregulated by 29.9 folds after berberine treatment. To validate Ndh51p as one potential target of berberine, haploinsufficiency (HI) was examined, since the organism is diploid and heterozygote strains lacking one allele usually demonstrate HI. In this regard, the heterozygote *NDH51* mutant demonstrated an HI phenotype that was more susceptible to berberine whereas *ndh*51Δ was more tolerant compared with the wild type due to the lack of target gene (Fig. S5 online). All of these results indicate that berberine interacts with and causes mitochondria dysfunction, which typically stimulates ROS production.

Consistent with this hypothesis, we found that berberine treatment indeed induced ROS production (Figs. 3e and S6 online). In contrast, the addition of antioxidant agents such as ascorbic acid and N-acetyl cysteine (NAC) abrogated the inhibitory effects of berberine (Fig. S7 online). Collectively, this data reveals that berberine activity is related to mitochondrial dysfunction in *C. albicans*. However, we cannot exclude the possibility that berberine, like many other drugs, has multiple targets, given the fact that berberine exhibits a wide spectrum of biological activities.

3.4. Berberine has a high potential to be an antifungal agent against multidrug-resistant invasive fungal pathogens

Besides the novel observation of SEMOC property. We next extensively evaluated the antifungal activity of berberine against several other wild types of common fungal pathogens. We saw that

Table 1
Berberine is active against various fungal pathogens with low toxicity to human cells.

Species	MIC (μg/mL)[a]		
	Fluconazole	Itraconazole	Berberine
C. albicans (SC5314)	0.25		8
C. guilliermondii	2		16
C. glabrata	2		1
C. tropicalis	0.5		2
C. parapsilosis	1		16
C. lusitaniae	2		4
C. apicola	0.25		4
C. krusei	32		4
C. neoformans (H99)	4		4
C. neoformans (JEC-21)	2		2
A. fumigatus (H11-20)		0.5	4
A. fumigatus (AF293)		0.5	4
MDR *A. fumigatus* RIT2		>100	4
MDR *A. fumigatus* RIT3		>100	4
MDR *A. fumigatus* RIT5		>100	4
MDR *A. fumigatus* RIT8		>100	8
MDR *A. fumigatus* RIT10		>100	4
MDR *A. fumigatus* RIT11		>100	4
MDR *A. fumigatus* RIT14		>100	4
HepG2 liver cell			>90
NIH/3T3 fibroblast cell			>100
293T kidney cell			>80

[a] MIC: minimum inhibitory concentration.

berberine was active against most of those fungal pathogens at relatively low concentrations (from 1 to 16 μg/mL) (Table 1). Remarkably, we also observed that berberine strongly inhibited MDR *Aspergillus fumigatus* (Table 1), the chief cause of invasive aspergillosis (IA). Patients with IA have a mortality rate higher than 90% [29]. However, berberine only showed negligible cytotoxicity against several human cell lines (Table 1).

To further evaluate berberine's potential as an anti-MDR *C. albicans* agent, we tested the antifungal activity of berberine on a set of clinical *C. albicans* isolates, 1#, 4#, 7#, and 11#. They were sequentially isolated from an HIV patient who was given an increasing dose of fluconazole during a two years period [30]. As a result, strains 4#, 7#, and 11# displayed fluconazole resistance. Interestingly, like CaS and CaR strains, 4#, 7#, and 11# strains were also found to have *MDR1* overexpression (by quantitative real-time PCR) (Fig. 4a). Consistent with our previous observation, these naturally acquired drug-resistant strains 4#, 7#, and 11# showed enhanced susceptibility to berberine over strain 1# (the parental strain) (Fig. 4b). Again, our results demonstrate that the drug-resistant *C. albicans* due to Mdr1p overexpression could be specifically inhibited by berberine.

Candidiasis is often fatal in immunocompromised patients. For this reason, we tested the efficacy of berberine in an immunocompromised animal model of candidiasis. For this purpose, we established an immunocompromised mouse model by i.p. injection of cyclophosphamide (CY) at a dose of 100 mg/kg (body weight) once a day for three consecutive days to specific pathogen-free female ICR mice as described previously [15]. An inoculum (0.1 mL) of 5×10^4 CFU/mL cells of *C. albicans* strain 11# (fluconazole-resistant, while berberine sensitive, *MDR1* overexpression) per mouse killed all mice within 7–8 d (mean survival time, MST was 4.1 ± 0.5 d). Berberine and fluconazole (as a control) were administered by i.p. 6 h post-infection and once a day thereafter for 3 d. A control group of 20 mice received 0.1 mL of diluent DPBS by the same route as the placebo regimens. We saw that berberine treatment dramatically prolonged the MST of those infected mice ($P < 0.01$), while due to the *MDR1* overexpression causing multidrug resistance, fluconazole failed to save the infected mice (Fig. 4c). From this data, we could conclude that berberine has a very good potential being an antifungal agent against multidrug-resistant *C. albicans* due to *MDR1* overexpression.

3.5. Investigation of acquired berberine resistance from the berberine sensitive strain CaR

To investigate if berberine resistant phenotype would be developed in the berberine sensitive strain CaR over the usage of berberine, we used a rapid assay by plating large numbers of CaR cells onto YPD plates containing a high concentration of berberine (100 μg/mL). After 3–5 d of incubation at 30 °C, some colonies could be seen from the plate. As described previously, only colonies with sizes ≥1.6 mm^2 had acquired robust, reproducible resistance [31]. Therefore, we randomly picked 10 clones with sizes larger than ≥1.6 mm^2, upon MIC test, we found that the MIC of berberine had increased about 50-fold (100 μg/mL) in the 8 out 10 picked colonies (Fig. S8 online). We named this group of berberine resistant strains CaRm. As anticipated, CaRm strains become susceptible to fluconazole again with a MIC of 0.39 μg/mL (Fig. S8 online). We also investigated the *MDR1* expression levels of CaRm with qRT-PCR. Results showed that *MDR1* expression in CaRm decreased more than 12-fold compared to CaR. It is consistent with our findings that the susceptibility of *C. albicans* strains to berberine is inversely correlated with *MDR1* expression levels. As a consequence of less Mdr1p, this acquired berberine resistance in CaR results in fluconazole sensitization, which we believe could be a good sign for the clinic.

4. Discussion and conclusion

Multidrug resistance is a worldwide problem that is exacerbated by the shrinking pipeline of new antimicrobial agents. Fungal pathogens adopt intricate strategies to avoid the lethal effects of antibiotics [2,32], one of which is overexpression of efflux pump Mdr1p [7]. Here we investigated whether Mdr1p, instead of promoting resistance, could be co-opted to promote selective killing of resistant *C. albicans* instead. The enhanced antifungal activity results from the increased intracellular accumulation of berberine in *MDR1* overexpressed *C. albicans*. Intriguingly, berberine is reported to accumulate in the rhizome of *Coptis japonica via* an ABC transporter (Cjmdr1p) [33], and it can be excreted by some bacterial multidrug excretion transporters, thereby rendering it relatively ineffective as a therapeutic antibacterial agent [34]. Though Mdr1p in *C. albicans,* Cjmdr1p in *Coptis japonica* and NorA [34,35] in bacteria do not share structure similarities, they can serve as the channels for berberine, either importing or exporting into cells, which indicated that berberine could be transported by many different types of transporters. The findings reported here may represent a novel strategy to overcome MDR, not only in fungal pathogens but also in bacterial pathogens or even other human diseases such as drug-resistant cancers. Moreover, we suggest the potential of mitochondria to be a target for new antifungal drug discovery, given that fungal mitochondria have proteins that differ from human mitochondria. Supporting this hypothesis, the deletion of genes encoding these proteins causes cell dysfunction [21,36,37]. Given the significant differences between human cell mitochondria and *C. albican* mitochondria [38], targeting mitochondria might be one of the reasons that berberine showed selective fungal cytotoxicity over the selected human cells of HepG2, NIH/3T3, and 293T.

Conflict of interest

The authors declare that they have no conflict of interest.

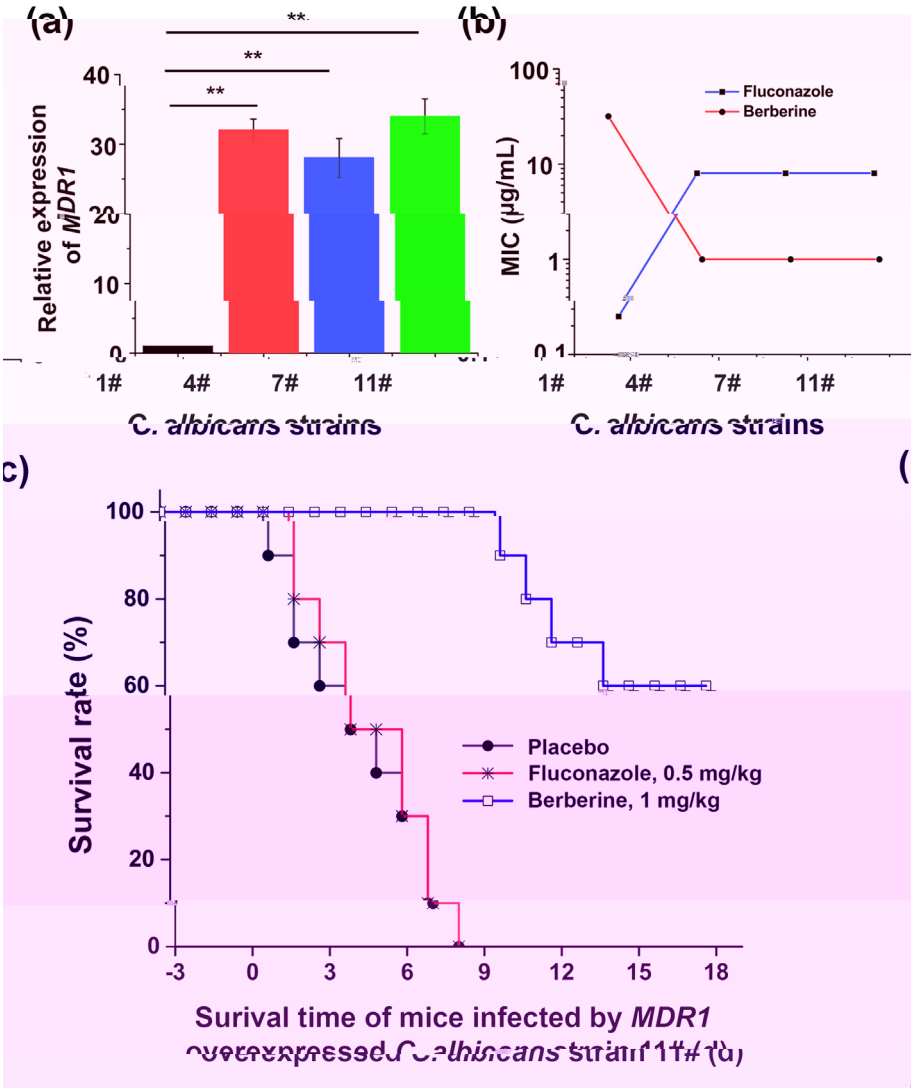


Fig. 4. Selective killing of drug-resistant clinical isolates by berberine, both *in vitro* and *in vivo*. (a) MDR1 expression level in *C. albicans* clinical isolates 1# and 4#, 7#, 11# from an HIV patient [30]. Quantitative real time PCR analysis of MDR1 expression was performed in triplicate. Mean values from three independent experiments are shown. Error bars indicate standard deviation. A two-tailed test is used for statistical analysis, ** $P < 0.01$. (b) *In vitro* susceptibilities of *C. albicans* isolates 1#, 4#, 7#, and 11# to fluconazole and berberine. (c) Berberine-treatment dramatically prolonged the life span of the mice that were infected by an Mdr1p overexpressed *C. albicans* isolate 11#. The total observation period is 21 days.

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Author contributions

Yaojun Tong, Nuo Sun, Fan Bai, and Lixin Zhang conceived and designed the experiments. Yaojun Tong, Jingyu Zhang, Nuo Sun, Xiang-Ming Wang, Qi Wei, Yu Zhang, Yingying Pu, Huanqin Dai, Biao Ren, Gang Pei, Fuhang Song, Guoliang Zhu, Xinye Wang, Xuekui Xia, Xiangyin Chen, and Lan Jiang performed all experiments. Nuo Sun, Yaojun Tong, Lixin Zhang, Richard Calderone, Buchang Zhang, Ren Huang, Xueting Liu, Yuanying Jiang, Gil Alterovitz, and Fan Bai analyzed the data. Yaojun Tong, Nuo Sun, Fan Bai, and Lixin Zhang wrote the manuscript. Yaojun Tong, Xiang-Ming Wang, Fan Bai, Lixin Zhang, Liming Ouyang, Shenlin Wang, and Ning Xie revised the manuscript.

Appendix A. Supplementary materials

Supplementary materials to this article can be found online at https://doi.org/10.1016/j.scib.2020.12.035.

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