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Anti-fungal effect of berberine on *Candida albicans* by microcalorimetry with correspondence analysis

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Abstract Using a LKB-2277 bioactivity monitor, stop-flow mode, the power–time curves of *Candida albicans* growth at 37 °C affected by berberine were measured. The check experiments were studied based on agar cup method to observe the inhibitory diameter and serial dilution method to determine the minimal inhibitory concentration (MIC) of berberine on *C. albicans* growth. By analyzing the quantitative thermogenic parameters taken from the power–time curves using correspondence analysis (CA), we could find that berberine at a low concentration (5.0 μg mL^{-1}) began to inhibit the growth of *C. albicans* and at a high concentration (75.0 μg mL^{-1}) completely inhibited *C. albicans* growth. The anti-fungal activity of berberine could also be expressed as half-inhibitory concentration IC_{50}, i.e., 50% effective in this inhibition. The value of IC_{50} of berberine on *C. albicans* was 34.52 μg mL^{-1}. The inhibitory diameters all exceeded 10 mm in test range and the MIC was 500 μg mL^{-1}. Berberine had strong anti-fungal effect on *C. albicans* growth. This work provided an important idea of the combination of microcalorimetry and CA for the study on anti-fungal effect of berberine and other compounds. Compared with the agar cup method and serial dilution method, microcalorimetry not only offered a useful way for evaluating the bioactivity of drugs, but also provides more information about the microbial growth and all this information was significant for the synthesis and searching of antibiotics.

Keywords Berberine · Anti-fungal activity · Microcalorimetry · Correspondence analysis

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Introduction

Berberine, an isoquinoline alkaloid originally isolated from *Hydrastis canadensis*, *Rhizoma coptidis*, *Berberis aquifolium*, and *Berberis vulgaris*, had extensive pharmacological actions including anti-microbial [1], anti-cancer [2], anti-oxidative [3], and anti-inflammation effect [4]. It has also been shown that berberine interacted with nucleic acids, especially DNA in vitro and inhibited DNA, RNA, and protein synthesis [5]. Moreover, the effects of berberine on promotion of apoptosis [6, 7], heart failure [8], and cyclooxygenase-2/Ca^{2+} pathway in human colon cell line [9] have also been reported. Though the antibacterial activities of berberine on *Helicobacter pylori* growth by slip diffusion method [10], *Escherichia coli*, *Staphylococcus aureus*, *Shigella dysenteriae* growth, etc. by anti-microbial circle method [1] has been reported, the anti-fungal activity of berberine on *Candida albicans* growth investigated by microcalorimetry has not been reported.

Candida albicans is the major human pathogen giving rise to opportunistic oral and vaginal infections. It is a leading cause of invasive fungal disease in premature infants, diabetics, surgical, AIDS, and other immunocompromised patients [11]. In these cases, the intensive prophylactic use of anti-fungal drugs, such as azoles [12] and plagiochin E [13], leads to emergence of resistant strains of *C. albicans*. This causes a great concern for finding suitable new therapeutics and anti-fungal compounds and drugs [14]. Also, the study on the metabolic process of *C. albicans* affected by drugs using suitable method is important for researching the mode of action of *C. albicans*, the anti-fungal compounds and drugs and the anti-fungal activities of these compounds and drugs.

Biological investigations are very important for the assessment of anti-fungal properties of compounds and

drugs. In any living systems including fungi, all the metabolic processes occur within the cells produce heat. Thus, the metabolic processes of living cells can be studied by monitoring the heat change with a sufficiently sensitive microcalorimeter. Because of high sensitivity and accuracy of the monitor and the fact that the whole metabolism of the living system can be examined automatically and continuously, the microcalorimetric method could reveal more qualitative and quantitative information about the metabolism of the living system [15–20] than the existing methods, for example, serial dilution, micro-dilution, antimicrobial circle and agar cup method [11, 13, 21]. It allows the study of biology at the molecular and cellular levels with the power–time curves generating a lot of kinetic information. By the analysis of the power–time curves, kinetic parameters, such as rate constant for bacterial growth, peak power for microbial activity can be obtained and the actions of compounds and drugs on the microbes can be evaluated. We have been studying microbial metabolism and the effect of compounds and drugs on microbes in our laboratory using microcalorimetry [22–24], so as to understand the influence of drugs on the environment and human health.

In this study, microcalorimetry coupled with correspondence analysis (CA) was applied to investigate the anti-*C. albicans* effect of berberine. Meanwhile, the agar cup method used to observe the inhibitory zone diameter and serial dilution method used to determine the minimal inhibitory concentration (MIC) of berberine on *C. albicans* growth were regarded as the check experiments [11]. Our objective is to provide a simple, quick, useful and sensitive method, and an important idea of the combination of microcalorimetry and CA for the study of anti-fungal activity of berberine and other compounds.

Experimental

Instrument

A microcalorimeter, LKB-2277 bioactivity monitor manufactured by LKB corporation (Bromma, Sweden) was used to obtain the metabolic power–time curves of *C. albicans* growth. The microcalorimeter was thermostated at 37 °C. To minimize the systematic error and disturbance effect, a differential or twin detector system was used. This system is very sensitive; the detection limit is 0.15 μW and the baseline stability (over a period of 24 h) is ±0.2 μW. In the monitoring system, two precision resistors for electrical calibration are built into each measuring cylinder, one for each detector. When a known electronic current is passed through the appropriate resistor, the detector can be calibrated easily. Other methods for calibration include suitable internally calibrated radioactive sources and chemical reactions. Using one of these techniques, a calorimetric constant can be evaluated. The time constant (ζ) of this instrument is about 120 s. For details of the performance and structure of the instrument, the instruction and report of Xie et al. [25] can be referred.

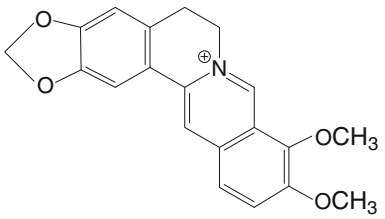

Fig. 1 Chemical structure of berberine

Materials

Strain *C. albicans* (64550) was provided by China Center of Type Culture Collection (Wuhan University, Wuhan 430072, P.R. China). It was grown in Lactose Broth (LB) culture medium which was a solution per 1,000 mL (pH 7.2–7.4) containing: NaCl 5 g, peptone 10 g, beef extract 6 g and was sterilized in high pressure steam at 121 °C for 30 min. Berberine was purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing 100051, P.R. China. The purities of berberine exceeded 98%, and its structure was given in Fig. 1. Water was purified using a Milli-Q water purification system (Milipore, Bedford, MA). All other chemicals are of analytical purity.

Methods

Microcalorimetric study

The power–time curves of *C. albicans* growth at 37 °C affected by berberine were measured by this microcalorimeter using stop-flow mode. In all of the microcalorimetric experiments, the equipment was thermostated at 37 °C and the flow-mixed cell was completely cleaned and sterilized. The clean procedure was as: sterilized distilled water, 0.1 mol L^{-1} HCl, 0.1 mol L^{-1} NaOH and sterilized distilled water were pumped into this microcalorimeter in sequence by an LKB-2132 microperpex peristaltic pump through the cell, each for 15 min at a flow rate of 50 mL h^{-1}. After the system was cleaned and sterilized and the baseline had been stabilized, the bacterial suspension, initially containing 2×10^6 cells/mL and berberine solution at different concentrations was pumped into this microcalorimeter through the calorimetric cell with a LKB-2132 perpex peristaltic pump at a flow rate of 50 mL h^{-1}.

When the flow cell (volume 0.6 mL) was filled, the pump was stopped and the monitor was used to record the power–time curves of *C. albicans* growth.

In this experiment, the *C. albicans* were suspended in the LB culture medium. Berberine solution was added at the beginning of the experiment, i.e., it was introduced as soon as the *C. albicans* were inoculated in the culture medium. The solutions of berberine were prepared in sterilized distilled water at different concentrations, and were prepared freshly each time.

Determination of the diameter of inhibitory zones

Two hundred microliter suspension of the fungus (10^7 cells mL^{-1}) was plated on agar layer in Petri dishes (10 cm in diameter). The sample solution of berberine was diluted, respectively, with LB culture medium to get the diluted solution of 2,000, 1,000, 500, 250, 125, and 62.5 μg mL^{-1}. Five wells per dish were prepared, each 10 mm in diameter. One hundred microliter of each diluted solution was added to the appropriate well. For pre-diffusion the Petri dishes were placed at 4 °C for 2 h. Positive control experiments were carried out with microbial suspension no berberine, while blank control group with berberine no microbial suspension in the pure culture medium. The anti-*C. albicans* effect was estimated by the diameter of inhibitory zones in the agar layer after incubation at 37 °C for 48 h [26].

Measurement of MIC

MIC was determined by serial dilution of each berberine sample solution (0–2,000 μg mL^{-1}) in test tubes using fresh LB culture medium. Each test tube was inoculated with fungal suspension containing 10^5 cells mL^{-1} and incubated at 37 °C for 24 h. The lowest dilution that visibly showed no growth compared to drug-free culture medium inoculated with microbial suspension was considered the MIC. For more precise detection, tubes that showed no visible growth were streaked on fresh LB agar plates, incubated at 37 °C for 24 h and checked for growth [27].

Results

Power–time curves of *C. albicans* growth

The power–time curve of *C. albicans* growth in the absence of drug was shown in Fig. 2. It was a typical power–time curve of *C. albicans* and could be divided into the following four phases: lag phase (a–b), stationary phase (b–c), exponential phase (c–d), and decline phase (d–e).

Next, the corresponding power–time curves of *C. albicans* growth affected by different concentrations of berberine solutions were shown in Fig. 3. As could be seen from the profiles of these curves, the growth of *C. albicans* was significantly influenced by berberine solutions.

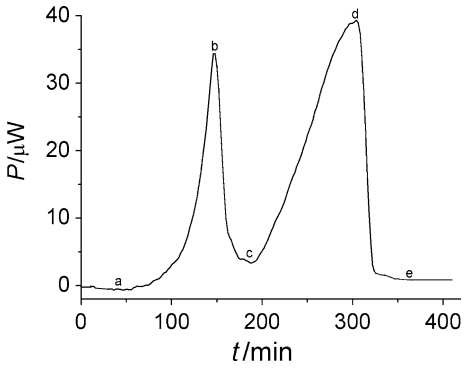

Fig. 2 The power–time curves of *C. albicans* growth at 37 °C without drug. It was a typical metabolic profile of *C. albicans* culturing in LB culture medium supplemented without any substance monitored by the LKB-2277 microcalorimeter at 37 °C, and could be divided lag phase (a–b), stationary phase (b–c), exponential phase (c–d), and decline phase (d–e)

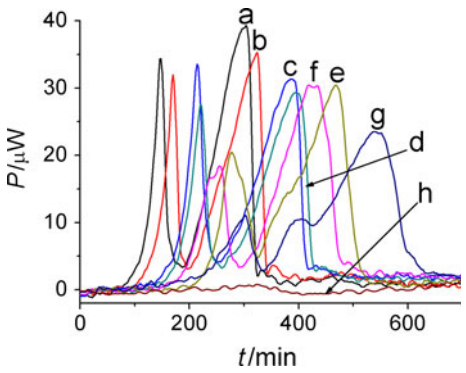

Fig. 3 The power–time curves of *C. albicans* growth affected by berberine. The concentrations of berberine were (a) 0 μg mL^{-1}, (b) 5.0 μg mL^{-1}, (c) 7.5 μg mL^{-1}, (d) 10.0 μg mL^{-1}, (e) 12.5 μg mL^{-1}, (f) 25.0 μg mL^{-1}, (g) 50.0 μg mL^{-1}, and (h) 75.0 μg mL^{-1}, respectively. These curves changed with the increase of concentration of berberine, showing that berberine had various anti-fungal effects on *C. albicans* growth

Thermokinetic data for *C. albicans* growth

In the exponential phase of growth, the cell growth is exponential. If the cell number is n_0 at time 0, and n_t at time t, then

$$n_t = n_0 \exp(kt) \tag{1}$$

If the power-output of each cell is w, then

$$n_t w = n_0 w \exp(kt) \tag{2}$$

$P_0 = n_0 w$ and $P_t = n_t w$, giving

$$P_t = P_0 \exp(kt) \quad \text{or} \quad \ln P_t = \ln P_0 + kt \tag{3}$$

Table 1 Rate constant k for the growth of *C. albicans* at 37 °C

Experiment	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	Mean values	RSD/%[a]
k/min^{-1}	0.05559	0.05692	0.05891	0.05666	0.05701	0.05691	0.05700 ± 0.00332	1.88
R[b]	0.9976	0.9978	0.9981	0.9972	0.9969	0.9957	0.9972 ± 0.0024	0.09

[a] Relative standard deviation

[b] Correlation coefficient

Table 2 Thermokinetic data for *C. albicans* growth at 37 °C in the presence of berberine

c/μg mL^{-1}	k/min^{-1}	R[a]	I/%	t_G/min	Q_t/J	t_{max}/min	P_{max}/μW
0	0.05692	0.9978	0	12.18	0.14	146	34.40
5.0	0.04901	0.9994	13.89	14.14	0.13	170	31.98
7.5	0.04684	0.9996	17.71	14.80	0.11	214	33.54
10.0	0.04595	0.9986	19.27	15.08	0.09	220	27.61
12.5	0.04305	0.9964	24.37	16.10	0.08	278	20.46
25.0	0.03663	0.9974	35.64	18.92	0.07	254	18.43
50.0	0.01562	0.9965	72.56	44.38	0.04	302	11.11
75.0	0	–	100.00	–	0	–	0

[a] Correlation coefficient

where P_0 represents the power-output at time 0 and P_t represents the power at time t. The exponential phase of *C. albicans* growth corresponded to Eq. 3. Using this equation, the growth rate constant k (listed in Table 1) of all experiments could be calculated by analyzing the data of the highest peak in these curves. The relative standard deviation (RSD) for k was 1.88%, showing that good reproducibility was obtained under identical experimental conditions. The generation time (t_G), which was equal to (ln 2)/k, was also obtained and shown in Table 2.

The growth inhibition ratio I is calculated on the basis of the growth rate constant. Inhibitory ration can be defined as:

$$I = [(k_0 - k_c)/k_0] \times 100\% \quad (4)$$

where k_0 is the growth rate constant of *C. albicans* in the absence of berberine, k_c is the growth rate constant of the log phase for *C. albicans* growth inhibited by berberine at inhibitor concentration c. When the inhibitory ratio I is 50%, the corresponding concentration of inhibitor is called half-inhibitory concentration, IC_{50}. The values of I, IC_{50}, the maximum power-output in the exponential phase P_{max}, the appearance time of the maximum power-output in the exponential phase t_{max} and total heat-output obtained from the power–time curve of *C. albicans* growth affected by berberine were also shown in Table 2.

Relationship between k, P_{max}, t_{max}, I, Q_t and the concentration c

To evaluate the effect of berberine on *C. albicans* growth swiftly, the relationship between k, P_{max}, Q_t, and c was studied and shown in Fig. 4. From $I - c$ equation, $I = 1.281c + 5.79129$ (0–75 μg mL^{-1}) ($R = -0.9960$), IC_{50} (34.52 μg mL^{-1}) of berberine on *C. albicans* were obtained. As could been seen from Table 2 and Fig. 4, an increase in concentration c of berberine caused a decrease in k, Q_t and an increase of I. However, the values of P_{max} and t_{max} changed irregularly. These complicated and inconsistent phenomenon gave some difficult for evaluating the effect of berberine on *C. albicans* growth. So, CA was inlet in the next part.

Correspondence analysis

Correspondence analysis [28, 29] is an important multivariate statistical method for studying the relationship between investigated factor and some variables. This method can compress some multivariate variables to two principal component (PC) variables Z_1 and Z_2, which have the same contribution to the total data set. Then, the PC variable aggregations [Z_1, Z_2] of every valid point are calculated, the loads of investigated factor and some variables are reflected on the same factor axis, and the relationship between them can be easily analyzed and explained. Then, the internal change regularity of investigated factor can be directly represented. Therefore, in order to assess this tendency of anti-fungal effect of berberine and the internal change regularity of concentration on the effect, CA for the four quantitative parameters k, t_{max}, P_{max} and Q_t were taken from the power–time curves, and the different concentrations of berberine was performed using software of Windows SAS 8.0 (SAS, USA).

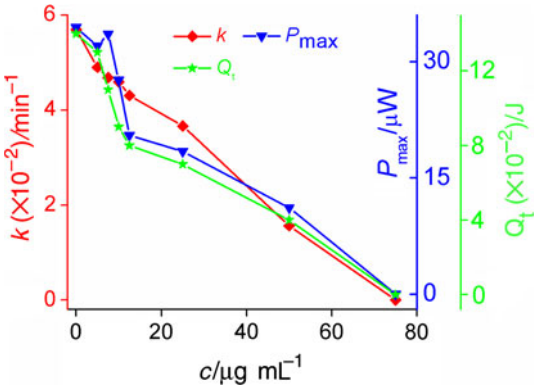

Fig. 4 Relationship between the growth rate constant (k), maximum power-output (P_{max}), total heat-output (Q_t), and c

The results of CA showed that the first two PCs (Z_1 and Z_2) contained 97.86% of the information of the original four variables/parameters and the equation of Z_1 and Z_2 were:

$$Z_1 = -0.1406k + 0.0258t_{max} - 0.0652P_{max} + 0.1972Q_t$$
$$Z_2 = -0.1413k + 0.0557t_{max} - 0.0718P_{max} + 0.1809Q_t$$

The absolute values of the coefficient before these parameters represented the contribution rate of the parameter to Z_1 and Z_2. It could be concluded from the two equations that k and Q_t contributed more to the two PCs among the four parameters, and they were the main parameters to evaluate the effect of berberine on *C. albicans* growth. From the values of these two parameters, we could quickly and accurately conclude the anti-*C. albicans* effects of berberine: a low concentration (5 μg mL^{-1}) of this compound began to inhibit the growth of *C. albicans* and a high concentration (75 μg mL^{-1}) completely inhibited *C. albicans* growth.

From the scatter plot for CA of the first two PCs (Z_1 and Z_2) shown in Fig. 5, it could be seen that the distance between each data point and original point was lengthened with the increase of the concentration of berberine,

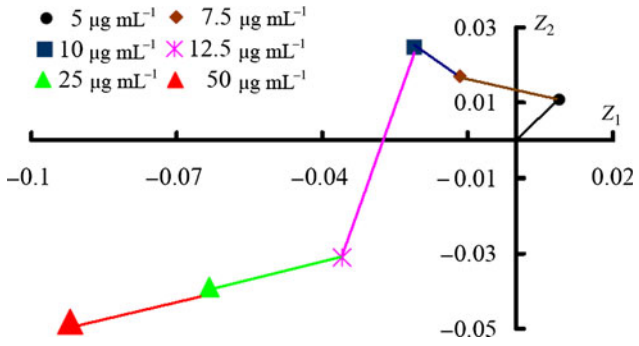

Fig. 5 Scatter plot for CA. This scatter plot was obtained by CA on the relationship between concentration of berberine and the four parameters from the power–time curves of *C. albicans* growth using software of Windows SAS 8.0 (SAS)

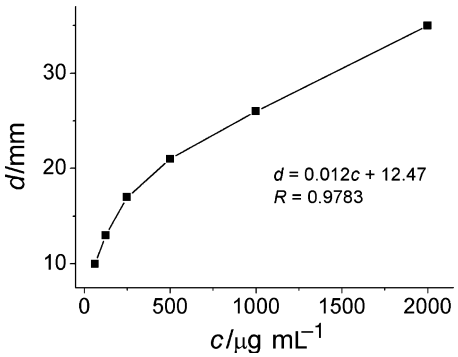

Fig. 6 Relationship between inhibitory zone diameter (d) and c

showing that the effect of this compound on *C. albicans* growth was enhanced and this enhancing potency was definitely dose-related. The scatter plot in Fig. 5 illustrated that berberine at different concentrations had various anti-*C. albicans* effects: a low concentration (5 μg mL^{-1}) of this compound began to inhibit the growth of *C. albicans* and a high concentration (75 μg mL^{-1}) completely inhibited *C. albicans* growth. The anti-fungal effect of berberine was further illustrated and authenticated.

The inhibitory zone diameter (d) and MIC

The results showed that the blank control group had no growth of microbes, but the microbes grew significantly in the diluted solution and positive control group. The diameter (d) of inhibitory zones in the agar layer of berberine on *C. albicans* growth was measured with a sliding caliper and the relationship between d and c was shown in Fig. 6. From the d to c equation, it could be seen that berberine gave inhibitory zones of diameters between 10 and 35 mm within test range.

The growth condition of the microbes in liquid media (LB culture medium) showed that berberine was highly active with MIC of 500 μg mL^{-1}.

Discussion

The power–time curves of *C. albicans* growth under the action of berberine showed that with increasing concentrations of berberine, the lag phase became longer, the time of the maximum power-output in the exponential phase t_{max} increased and the generation time t_G delayed, which indicated that the fungal culture took longer time to produce a sufficient number of cells for a detectable signal and that excess berberine inhibited the growth of *C. albicans* or killed the fungus. The results of microcalorimetric study coupled with CA showed that berberine had strong capacity to inhibit the growth metabolism of *C. albicans* to

different extents and the inhibitory capacity was concentration-dependent: the anti-*C. albicans* activities strengthened with the increasing concentration of berberine. When the concentration of berberine reached 75.0 μg mL^{-1}, *C. albicans* would not grow: the growth was inhibited completely. The values of IC_{50} of 34.52 μg mL^{-1}, MIC of 500 μg mL^{-1} and inhibitory zones of diameters between 10 and 35 mm of berberine all further illustrated the strong anti-fungal effect of this compound on *C. albicans* growth.

This phenomenon probably resulted from the fact that the quaternary nitrogen atom and the methylenedioxy group at the aromatic ring played a significant role in the anti-fungal effect of berberine. The action of the drug on microbe depended on its structure. Factors that determine the characteristics of a dose–response curve were the mode of action of the compound at cells, the number of its target sites, and its affinity to those target sites, etc. The quaternary nitrogen atom and the methylenedioxy group at the aromatic ring of berberine had a higher affinity to the fungal cell, which made berberine easily enter into the fungal cell. At the cellular level, the possible action mechanism was that berberine integrated with the cell wall of *C. albicans* and the cell membrane penetrability was changed and the transport of nutrients and wastes crossing the membrane was disrupted, then berberine diffused into the cell and further combined with the cell membrane and phospholipids of the cell nucleus resulting in the disappearance of the cell organ and the damage of the structure and functions of cells [1, 30–32].

Though microcalorimetry with high sensitivity, high accuracy, and possibility for automation has been used in many fields, such as life sciences [33, 34], pharmacological analysis [17, 19], and biotechnology [20], the study using it for the anti-fungal activity of berberine on *C. albicans* growth has not been reported from then on. Our experiments showed that microcalorimetry was a powerful tool for monitoring and controlling the growth process of *C. albicans*. Compared with the agar cup method and serial dilution method, it not only offered a useful way for the evaluation of bioactivity of drugs, but also provides more information about the microbial growth and all this information was significant for the synthesis and searching of antibiotics. The results were important for evaluating the anti-fungal activities of berberine and gave new method and idea of the combination of this method and CA for studying the anti-fungal effects of other compounds and materials.

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