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Activity of berberine on *Shigella dysenteriae* investigated by microcalorimetry and multivariate analysis

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Abstract In this study, the microcalorimetric method was applied to investigate the activity of berberine on *Shigella dysenteriae* (*S. dysenteriae*). Heat flow power (HFP)–time curves of the growth metabolism of *S. dysenteriae* affected by berberine were determined using the thermal activity monitor (TAM) air isothermal microcalorimeter, ampoule mode, at 37 °C. By analyzing these curves and some quantitative parameters using multivariate analytical methods, similarity analysis (SA) and principal component analysis (PCA), the antibacterial activity of berberine on *S. dysenteriae* could be accurately evaluated from the change of the two main parameters, the maximum heat flow power P_m^2 and total heat output Q_t: berberine at low concentration (25 μg mL^{-1}) began to inhibit the growth of *S. dysenteriae*, high concentrations (50–200 μg mL^{-1}) of berberine had strong antibacterial activity on *S. dysenteriae*, when the concentration of berberine was higher (250–300 μg mL^{-1}), this antibacterial activity was stronger. All these illustrated that the antibacterial activity of berberine on *S. dysenteriae* was enhanced with the increase of the concentration of this compound. Berberine can be used as potential novel antibacterial agent for treating multidrug-resistant *Shigella*. This work provided a useful idea of the combination of microcalorimetry and multivariate analysis for studying the activity of other compounds or drugs on organisms.

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Introduction

Shigellosis is a highly contagious enteric bacterial infection caused by the enteroinvasive bacteria *Shigella*, characterized by fever, diarrhea, and bloody mucoid stools. Among *Shigella* species, *Shigella dysenteriae* (*S. dysenteriae*) has been associated with epidemic outbreaks of bacillary dysentery that pose major public health problems in many countries [1–3] and accounts for brisk and deadly epidemics in the poorest populations. It is a Gram-negative, facultative intracellular bacterium with high contagiousness because as few as 10–100 bacteria can cause the disease in man [4]. Though a few agents are now available in clinical use for these diseases, some problems existed, for example, resistance mutations and/or side effects [5–8]. And the emergence of multidrug resistance in *Shigellae* has necessitated a search for new antibacterial drugs using appropriate approach.

Drugs that can either inhibit the growth of pathogens or kill them without harming host cells are considered candidates for developing new antibacterial agents. In recent years, berberine, an isoquinoline alkaloid with extensive antimicrobial effect on *Escherichia coli*, *Bifidobacterium adolescentis*, *Candida albicans* [9–12], etc., has got more and more interests and studies. However, we believed that whether berberine could be used as a new antibacterial agent should include the assay of antibacterial activity of it on more microbes rather than above mentioned species. As far as we know, the investigation of the antibacterial activity of berberine on *S. dysenteriae* by microcalorimetric technique has not been reported.

As an established procedure, microcalorimetry has been extensively applied as a useful method for rapid bioassay of drugs on organism. This technique allows analysis to be performed directly on a test substance, regardless of its homogeneous or heterogeneous nature. It permits quantitative measurements on very different and heterogeneous substrates such as drug, soil, metal, and foodstuffs [13–16]. Besides the good sensitivity, accuracy, and reproducibility, it has some peculiar advantages in new drug discovery and response for the antibacterial challenge, for example, continuous, real-time, quantitative detecting can be realized so as to obtain abundant thermo-kinetic/dynamic information or quantitative structure–activity relationship, even the information about the mechanism of action of the antimicrobial drug [17, 18].

In view of the potential benefits of this microcalorimetric method, microcalorimetry was used to investigate the activity of berberine against the causative pathogens, *S. dysenteriae*, in this study. The aim is to provide a useful idea of the combination of microcalorimetry and multivariate analysis for investigating the activity of drug on microbes and searching for new antibacterial agents.

Experimental

Materials

Strain *S. dysenteriae* (*Shigella dysenteriae* AB210562) was provided by the National Institute for the Control of Pharmaceutical and Biological Products, Beijing 100051, P. R. China. It was grown in Luria–Bertani (LB) culture medium, which was a solution containing 10 g peptone, 5 g NaCl, and 5 g yeast extract per 1,000 mL (pH 7.2–7.4) 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. Its purities exceeded 98% and structure was given in Fig. 1. Water was purified using a Milli-Q water purification system (Milipore, Bedford, MA). All other chemicals were of analytical purity.

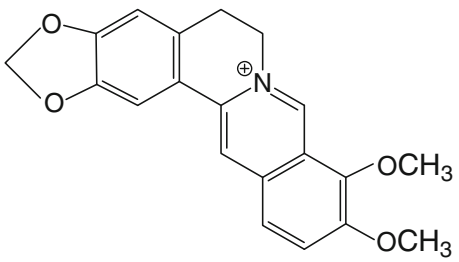

Fig. 1 Chemical structure of berberine

Instrument

A 3114/3236 thermal activity monitor (TAM) air isothermal microcalorimeter (Thermometric AB, Sweden) was used to determine the metabolic heat flow power (HFP)–time curves of *S. dysenteriae*. This microcalorimeter was an eight-channel twin instrument and thermostated at the range of 5–60 °C with a limit of detectability of 2 μW. The software supplied to TAM air was used to monitor and record the heat flow power over the Peltier module when the baseline drift was less than 20 μW over 24 h. For more details of the instrument, see the report of Wadsö [19].

Experimental procedure

This microcalorimetric measurement was performed using ampoule method at 37 °C. Eight reference ampoules containing purified water, together with another eight ampoules containing the cell suspension of *S. dysenteriae* and berberine solutions of different concentrations were all sealed up and put into the 8-channel calorimeter block. After about 30 min (the temperature of ampoules reached 37 °C), the HFP–time curves were recorded. When the curves returned to the baseline, the experiments were finished. All data were collected continuously using the dedicated software package.

Multivariate analysis

Similarity analysis (SA)

To draw the idea from SA on the chemical HPLC/UPLC chromatographic fingerprints of traditional Chinese medicine [20, 21], the HFP–time curves of *S. dysenteriae* growth affected by berberine were first studied by their similarities to intuitively and quickly find the influence of this compound on the bacterial growth. In this part, the similarities between the HFP–time curves of *S. dysenteriae* growth without (the control) and with different concentrations of berberine were calculated on the correlative coefficient of original data from these curves by cosin method using software of Microsoft Excel 2003 [22].

Principal component analysis (PCA)

From the HFP–time curves of *S. dysenteriae* growth, many quantitative parameters could be obtained to represent the activity of berberine on these bacteria. But the different or disordered change trends of these parameters resulted in many difficulties for efficiently and definitely evaluating the activity of this compound. In order to reduce the parameters and easily find the change trend of this activity, the main parameters should be got. The PCA [23, 24] transforms the correlated data set to a smaller set of variables, the principal

components (PCs), which are uncorrelated and contain nearly all of the original information. From the score plots, the concentration cluster can be found, and from the loading plot, the main parameter(s), which is/are this/these furthest away from the main cluster of variables, can be identified. Here, multivariate PCA statistics in unsupervised mode was performed by the software Statistica 6.0 (StatSoft, Tulsa, OK) on unit variance scaled data of these quantitative thermokinetic parameters.

Results and discussions

Metabolic HFP–time curves of *S. dysenteriae*

Figure 2 showed the metabolic curve of *S. dysenteriae* growth at 37 °C without any substance. It was a typical HFP–time curve of *S. dysenteriae* and could be divided into six phases: a lag phase (A–B), the first exponential growth phase (B–C), a transition phase (C–D), the second exponential growth phase (D–E), a stationary phase (E–F), and a decline phase (F–G).

Correspondingly, Fig. 3 showed the HFP–time curves of *S. dysenteriae* growth at 37 °C affected by different concentrations of berberine. As could be seen from the profiles of these curves, the growth of *S. dysenteriae* was influenced by this compound.

Metabolic thermo-kinetic parameters for *S. dysenteriae*

As depicted in Figs. 2 and 3, cell growth was exponential during the two exponential growth phases. If P_0 and P_t are the heat flow powers at time = 0 and time = t, respectively, then $P_t = P_0 \exp(kt)$ or $\ln P_t = \ln P_0 + kt$. The growth rate constant (k_1 and k_2) for the first and second exponential growth phase can be obtained from the raised part of the first and second peak by plotting a linear curve of $\ln P_t$ against t. Then, other metabolic thermokinetic parameters, such as the maximum heat flow powers for the first and second exponential phase P_m^1, P_m^2, the appearance time t_m^1, t_m^2 for P_m^1, P_m^2, the heat output Q_1, Q_2 for the first and second exponential phase, and total heat output Q_t for all the metabolic progress were obtained from the HFP–time curves of *S. dysenteriae* growth affected by berberine and shown in Table 1.

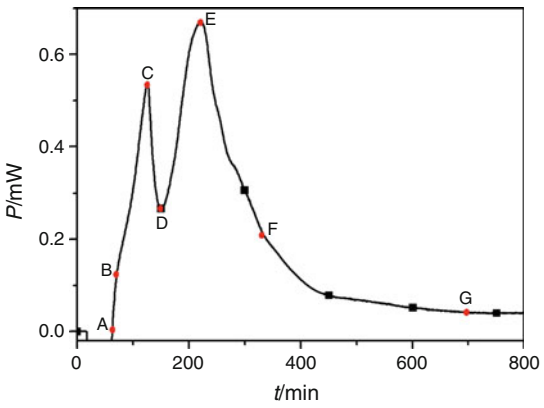

Fig. 2 HFP–time curves of *S. dysenteriae* growth at 37 °C without any substance. It was a typical metabolic profile of *S. dysenteriae* culturing in LB culture medium supplemented without any substance and monitored by the microcalorimeter at 37 °C, and could be divided into six phases: a lag phase (*A–B*), the first exponential growth phase (*B–C*), a transition phase (*C–D*), the second exponential growth phase (*D–E*), a stationary phase (*E–F*), and a decline phase (*F–G*)

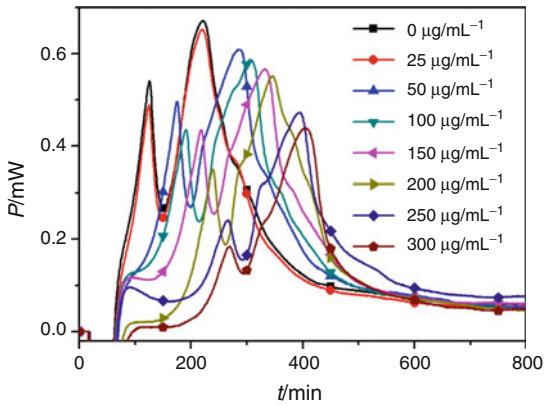

Fig. 3 HFP–time curves of *S. dysenteriae* growth at 37 °C affected by different concentrations of berberine. The concentrations of berberine were shown in this figure. The peak height and appearance time of the two peaks changed with increasing the concentration of berberine, showing that berberine of different concentrations had varied effects on *S. dysenteriae*

Relationships between metabolic parameters and concentration, *c*, of berberine

The three-dimensional histograms in Fig. 4 clearly showed the relationships between the nine quantitative metabolic parameters and concentration, c, of berberine. It could be found from these histograms that the values of k_1, P_m^2, Q_1, and Q_t decreased with the increase of the concentration of berberine, while the values of t_m^1, P_m^1, t_m^2, k_2, and Q_2 had irregular change trends (increasing or decreasing). This phenomenon brought many difficulties to objectively and exactly evaluate the activity of berberine on *S. dysenteriae*. So, it was necessary to introduce multivariate analytical methods for this evaluation.

Multivariate analysis

SA

The correlative coefficients for SA between the HFP–time curves of *S. dysenteriae* growth without and with different

Table 1 Metabolic thermo-kinetic parameters for *S. dysenteriae* growth at 37 °C affected by different concentrations of berberine

c/μg mL^{-1}	k_1/min^{-1}	t_m^1/min	P_m^1/mW	k_2/min^{-1}	t_m^2/min^{-1}	P_m^2/mW	Q_1/J	Q_2/J	Q_t/J
0	0.02619	125.7	0.542	0.01708	220.7	0.671	1.99	2.81	8.51
25	0.02266	123.7	0.484	0.01613	218.3	0.652	1.87	2.51	8.27
50	0.01993	175.3	0.496	0.00714	285.0	0.608	1.06	2.41	8.17
100	0.01835	190.3	0.434	0.00511	305.7	0.588	1.04	2.37	7.92
150	0.01827	218.0	0.435	0.00545	330.3	0.566	0.98	2.29	7.60
200	0.01117	237.7	0.347	0.00480	345.3	0.551	0.67	1.95	6.71
250	0.01083	265.3	0.241	0.00715	394.7	0.473	0.58	2.06	6.14
300	0.01037	267.7	0.184	0.00934	402.3	0.439	0.35	1.86	4.16

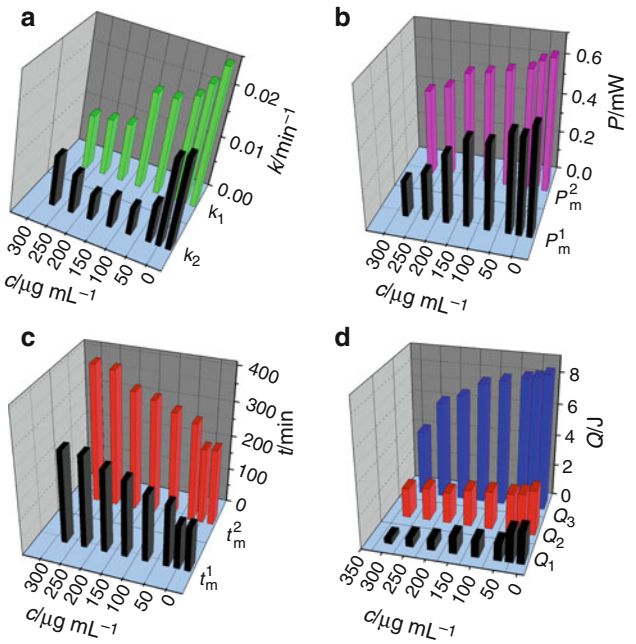

Fig. 4 Relationships between metabolic parameters and concentration c of berberine The column maps of the nine quantitative metabolic parameters **a** the growth rate constants k_1 and k_2; **b** the maximum heat flow power P_m^1 and P_m^2; **c** the appearance times of the maximum heat flow power t_m^1 and t_m^2; **d** the heat output Q_1, Q_2, and Q_t and concentration, c, were performed using the Software Origin 8.0

concentrations of berberine were listed as 1.0000, 0.9995, 0.9474, 0.9304, 0.9276, 0.8532, 0.8231, and 0.7507. The decline of correlative coefficients illustrated that the metabolic curves of *S. dysenteriae* growth affected by berberine changed and berberine of different concentrations had varied activities on *S. dysenteriae* growth.

PCA

PCA is a popular method in applied statistical work and data analysis, and it allows visualizing the information of the data set in a few PCs and reducing the computation burden. So, PCA was performed on nine quantitative parameters k_1, t_m^1, P_m^1, k_2, t_m^2, P_m^2, Q_1, Q_2, and Q_t. The parameters in nine-dimensional space were projected to the two-dimensional plane, which accounted for 97.49% of the total variance. The loading plot (Fig. 5a) indicated that parameters P_m^2 and Q_t might be the main two parameters, which would play more important role in evaluating the activity of berberine on *S. dysenteriae*. The score plot in Fig. 5b showed the distribution of concentration of berberine. Berberine of low concentration (25 μg mL^{-1}) began to inhibit the growth of *S. dysenteriae*, high concentrations (50–200 μg mL^{-1}) of berberine had strong antibacterial activity on *S. dysenteriae*, when the concentration of berberine was higher (250–300 μg mL^{-1}), this antibacterial activity was stronger. All these illustrated that the antibacterial activity of berberine against *S. dysenteriae* was enhanced with increasing the concentration of this compound. On the other hand, the values of P_m^2 and Q_t in Table 1 both decreased gradually with the concentration range (25–300 μg mL^{-1}), further showing that the anti-*S. dysenteriae* activity of berberine was strengthened with the increase of the concentration of this compound. So, from PCA on the nine parameters, we could find the main parameters, based on which, we could quickly and clearly find the antibacterial activity and action trend of this compound on *S. dysenteriae*.

Then, to describe the extent of the inhibition of berberine against *S. dysenteriae* growth, we defined the inhibitory ratio (I/%) as: $I = [(Q_{t(0)} - Q_{t(c)})/Q_{t(0)}] \times 100\%$, where, $Q_{t(0)}$ and $Q_{t(c)}$ represent the total heat production in the whole metabolic progress of the bacteria under the condition of control and final concentration, c, of the two drugs. And we can also obtain the I–c curves, from which the value of IC_{50} (the half-inhibitory concentration) of 294.8 μg mL^{-1} for berberine can be obtained, further showing the stronger antibacterial activity of berberine on *S. dysenteriae* growth.

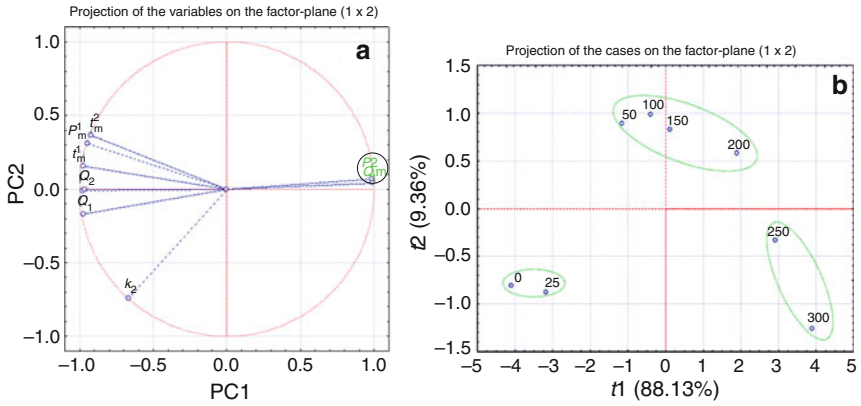

Fig. 5 a Loading plot representing the contribution of the original variables (parameters) for the first two PCs PC1 and PC2. The main two parameters were marked with a circle. b Score plots showing the distribution of concentration of berberine. In the plot, we see two largest components (PC1, *horizontal*, and PC2, *vertical*), and the scores *t*1 and *t*2 are new variables aiming at describing as much of the original variation as possible without losing information. Numbers indicate the value of concentration of berberine

Conclusions

Emergence and prevalence of multidrug resistance in pathogenic *Shigella* as well as undesirable side effects of certain antibiotics has triggered immense interest in the search for novel antibacterial drugs to combat these organisms. The antibacterial investigation using appropriate approach should be important index for these drugs.

In this paper, the HFP–time curves of *S. dysenteriae* growth affected by berberine were determined by microcalorimetry. By analyzing these curves and some quantitative parameters using SA and PCA, we could find that the anti-*S. dysenteriae* activity of berberine was strengthened with the increase of concentration of this compound. Parameters P_m^2 and Q_t were the main two parameters, which could be used for fast and accurately evaluating the antibacterial activity of berberine on *S. dysenteriae*. These results would provide some references for the use of berberine as potential novel antibacterial drugs. Further study should be focused on investigating the activity of this compound on more microbes and the mechanism of action. This work also provided an important idea of the combination of microcalorimetry and multivariate analysis for studying the activity of other compounds or drugs on organisms. In conclusion, microcalorimetric investigations on microorganisms are possible and promising. We believe that microcalorimetry is a useful and accurate system for studying the detailed mechanism of microorganisms and can provide important information for screening out new antibacterial drugs.

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