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Toxic effects of protoberberine alkaloids from *Rhizoma Coptidis* on *Tetrahymena thermophila* BF_5 growth based on microcalorimetry

A reliable evaluation method of structure–function relationship

Yumei Han · Dan Yan · Yanling Zhao · Cheng Peng · Xiaohe Xiao

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Abstract *Rhizoma Coptidis* consists mainly of protoberberine alkaloids (PAs), and has been used for many years as a traditional medicine. Recent research revealed the toxicity of *Rhizoma Coptidis*, but studies focusing on the relationships between the structures of PAs and their toxicities are lacking. The toxic effects of four PAs from *Rhizoma Coptidis* on the growth of *Tetrahymena thermophila* BF_5 were investigated by microcalorimetry. The power-time curves of *T. thermophila* BF_5 with and without PAs were obtained; the extent and duration of toxic effects on the metabolism of this organism were evaluated by studying thermokinetic parameters and the half-inhibitory ratio (IC_{50}). All the thermokinetic parameters showed regular variations with alteration of PA concentrations. The magnitude of the toxic effects of PAs was ascertained from IC_{50} values: palmatine > jateorhizine > berberine ≈ coptisine. The structure–function relationship of PAs indicated that the C2 and C3 positions contributed more to the toxic effect of PAs. That is, when substituted with methoxyl groups, the toxic effect would be increased.

Y. Han · D. Yan (✉) · Y. Zhao · X. Xiao (✉)
China Military Institute of Chinese Materia Medica, 302 Military Hospital of China, Beijing 100039, People's Republic of China
e-mail: yd277@126.com

X. Xiao
e-mail: pharmsci@126.com

Y. Han · C. Peng
Pharmacy College, Chengdu University of Traditional Chinese Medicine, Chengdu 611137, People's Republic of China

Y. Han
College of Chemistry and Biotechnology, Chongqing University of Technology, Chongqing 400050, People's Republic of China

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Introduction

In any living system, the various metabolic events occurred within the cells involving heat-production reactions. Therefore, by monitoring the heat effects with sufficiently sensitive calorimeters, the metabolic curves of living cells are obtained, which reflects the information of the metabolic processes [1, 2]. Microcalorimetry can be employed to directly determine the biological activity of a living system and provide continuous measurement of heat production, thereby giving qualitative and quantitative data [3, 4]. In recent years, the application of microcalorimetry in biochemistry, biophysics, and environmental sciences has received increasing attention [5–9].

The ciliate *Tetrahymena thermophila* BF_5 is a eukaryotic unicellular organism that has many useful features for toxicological research [10–15]. It is sensitive to toxic substances in the environment, and has also been selected as a standard living for the toxic detection, apoptosis, and water examination [16, 17]. The growth of *T. thermophila* might indicate the status of the aquatic environment. This feature has allowed this organism to be used as a pharmacological tool in different bioassay techniques to detect toxicants [14, 18, 19].

Rhizoma Coptidis contains protoberberine alkaloids (PAs, e.g., berberine, coptisine, palmatine, and jateorhizine), and has been used for thousands of years for the treatment of dysentery, arrhythmia, diabetes mellitus, and inflammation-related diseases in India and China [20–22]. It has been associated with zero toxicity and thought to

have superior effects for the treatment of many diseases. However, recent research has demonstrated the toxic effect of the PAs in *Rhizoma Coptidis* [22]. But the relationships between the structure and toxic effect of PAs investigated by microcalorimetry have not been reported from then on.

In the present study, we applied this precise method to investigate the toxic effects of PAs on the growth of *T. thermophila* BF_5 by microcalorimetry, which focuses on the energy change in the growth of organism. Moreover, combined with previous studies [19], we discussed the actions of PAs on *T. thermophila* BF_5 and *Bacillus shigae*, and their structure–function relationship.

Materials and methods

Instrument

A TAM air isothermal microcalorimeter (Thermometric AB, Stockholm, Sweden) was used to determine the metabolic power-time curves of *T. thermophila* BF_5. This microcalorimeter is an eight-channel twin instrument; it is thermostated at 5–60 °C and has a limit of detection of 2 μW [23].

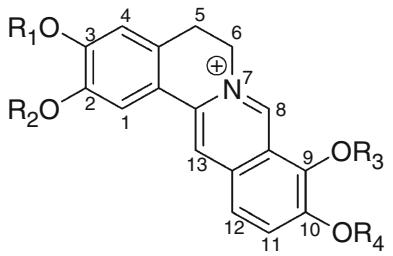
Materials

Tetrahymena thermophila BF_5 (mononuclear) was provided by the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, P.R. China). The PGYE culture medium was a solution (pH 7.2–7.4) containing peptone (15 g), yeast extract (5 g), and glucose (1 g). It was sterilized in high-pressure steam at 121 °C for 30 min.

All the chemicals used in the experiments were of analytical grade. Doubled-distilled water was used to prepare all solutions. Berberine, coptisine, palmatine, and jateorhizine were supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, P.R. China). Their structures are given in Fig. 1.

Methods

Initially, *T. thermophila* BF_5 cells were cultivated in the incubator at 28 °C. They were then inoculated in the prepared 10 mL PGYE culture medium in a 20 mL glass ampoule; the initial density was 4.5×10^3 cells/mL. PAs at different concentrations were added into each glass ampoule. The glass ampoules were then sealed with a cap and put into the TAM air isothermal microcalorimeter. Growth was continuously monitored, and their power-time curves plotted. The experiments were carried out using aseptic techniques.



	R_1	R_2	R_3	R_4
berberine	—CH_2—		CH_3	CH_3
coptisine	—CH_2—		—CH_2—	
palmatine	CH_3	CH_3	CH_3	CH_3
jateorrhizine	H	CH_3	CH_3	CH_3

Fig. 1 Chemical structures of the four tested PAs

Results and discussion

Growth rate constant (k) and generation time (t_G) of *T. thermophila* BF_5 with and without PAs

Figure 2 shows the power-time curve of *T. thermophila* BF_5 without PAs at 28 °C. The growth curve of *T. thermophila* BF_5 could be divided into four phases: lag phase, log phase, stationary phase, and decline phase [24]. In the log phase of growth, the cell number and heat-output power grows exponentially, and the kinetic equation is:

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

where P_0 represents the heat-output power at the beginning and P_t represents that at time t. The power-time curves of the log phase of growth correspond to the equation. Using the data ln P_t and t taken from the curves to fit a linear equation, the growth rate constant (k) of the growth of *T. thermophila* BF_5 and the correlation coefficient were obtained (Table 1). They indicated good reproducibility and correlation. By obtaining k, we readily calculated the generation time (t_G) of the corresponding cells using the following equation [25]:

$$t_G = (\ln 2)/k$$

shown in Table 2.

The power-time curves of *T. thermophila* BF_5 with PAs were monitored by the microcalorimeter. The power-time curves of *T. thermophila* BF_5 at different concentrations of PAs showed that the peak time of maximum heat-output power (t_p) and generation time (t_G) were prolonged, and that k decreased with the increase in PA concentration. All correlation coefficients were >0.9, indicating a good linear correlation between k and the corresponding concentration.

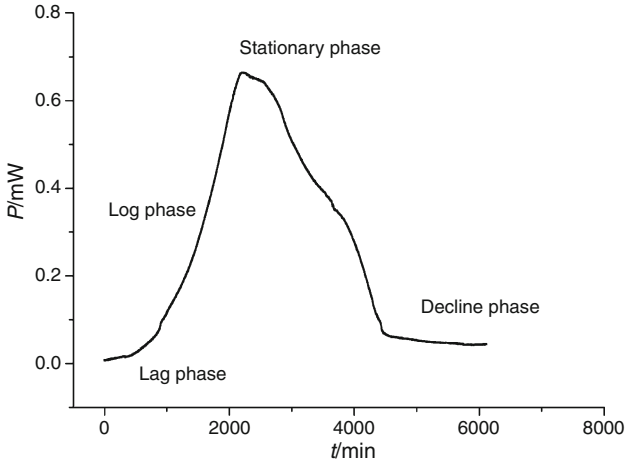


Fig. 2 Power-time curve of *T. thermophila* BF_5 cultured in PGYE culture medium and monitored by the TAM air isothermal microcalorimeter, ampoule mode, at 28 °C

These results demonstrated that all the PAs inhibited the growth of *T. thermophila* BF_5, and that the toxic activities increased with increasing concentration of PAs. Thus, *k* decreased gradually, indicating the different toxic effects of PAs. The different toxic effects of PAs are demonstrated in Fig. 3. We could also extract more information by carefully analyzing the power-time curve of each alkaloid. For example, for the power-time curve of palmatine, steady delay was shown at high concentrations (100–150 mg/L), which is called a "characteristic variation", whereas it disappeared at low concentrations (25–75 mg/L). For coptisine, the power-time curve had no significant change at low concentrations (50–150 mg/L), but showed significant toxic effects on the growth and metabolism of *T. thermophila* BF_5 at >300 mg/L. Therefore, these characteristic features of power-time curves enable assessment of the growth and metabolism of biosystems using microcalorimetry.

Table 1 Growth rate constant (*k*) of *Tetrahymena thermophila* BF_5 cells cultured in PGYE culture medium and monitored by a microcalorimeter at 28 °C

Experiment number	1	2	3	4	5	RSD[a]/%
$k/min^{-1} \times 10^{-3}$	2.49	2.64	2.61	2.58	2.54	2.29
r[b]	0.9752	0.9684	0.9805	0.9785	0.9822	0.56

[a] *RSD* relative standard deviation; [b] correlation coefficient

Table 2 The effects of different concentrations of PAs on the values of *k*, and on the growth of *Tetrahymena thermophila* BF_5 at 28 °C ($n = 6$)

Sample	$C/mg/L^{-1}$	$k/min^{-1} \times 10^{-3}$	r	t_p/min	I/%	$P_{max}/mW \times 10^{-1}$	t_G/min
Control	0	2.57	0.9770	2191	–	6.575	270
Berberine	25	1.53	0.9904	3241	40.47	5.999	453
	50	1.48	0.9758	3384	42.41	5.529	468
	75	1.43	0.9686	3460	44.36	5.499	485
	100	1.31	0.9666	4139	49.03	4.877	529
	250	0.85	0.9782	5052	66.93	4.22	815
Coptisine	50	1.51	0.9773	3277	41.25	5.352	459
	75	1.43	0.9792	3679	44.36	4.962	485
	100	1.33	0.9771	3883	48.25	4.932	521
	150	1.18	0.9770	3919	54.09	4.659	587
	300	0.73	0.9941	4559	71.60	4.058	949
Plmatine	25	1.44	0.9950	3130	43.97	6.458	481
	50	1.32	0.9831	3752	48.64	5.66	525
	75	1.24	0.9748	4230	51.75	5.331	559
	100	0.95	0.9872	5783	63.04	4.487	730
	150	0.64	0.9763	6460	75.10	3.391	1083
Jateorhizine	25	1.53	0.9878	3131	40.47	5.988	453
	50	1.39	0.9832	3570	45.91	5.387	499
	100	1.21	0.9835	4340	52.92	5.125	573
	125	1.02	0.9893	4961	60.31	4.781	680
	200	0.55	0.9819	5783	78.60	4.008	1260

The data are all mean value, relative standard deviation (RSD) is not more than 5%

C concentration; *k* growth rate constant; *r* correlation coefficient; t_p peak time of maximum heat-output power; *I* growth inhibitory ratio; P_{max} maximum heat-output power; and t_G generation time

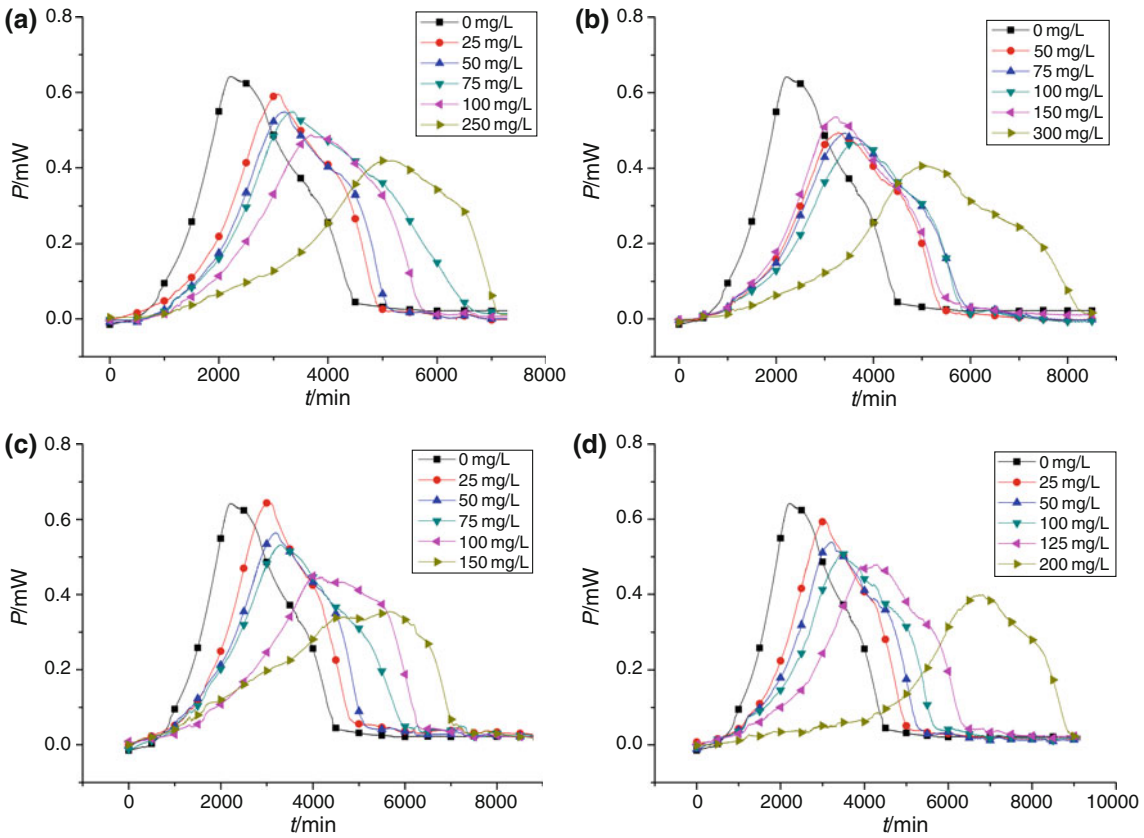


Fig. 3 Power-time curves of *T. thermophila* BF_5 growth in the presence of berberine (**a**) jateorhizine, (**b**) palmatine, (**c**) coptisine, and (**d**) monitored by the TAM air isothermal microcalorimeter, ampoule mode, at 28 °C. (**d**) concentrations of jateorhizine (mg/L): 25 (*filled square*), 50 (*filled circle*), 100 (*filled triangle*), 125 (*inverted triangle*), and 200 (*filled diamond*)

Peak time (t_p) and maximum heat-output power (P_{max})

The growth of *T. thermophila* BF_5 was significantly influenced by PAs (Fig. 3). In comparison with the control, Fig. 3 shows that the peak time (t_p) was prolonged and maximum heat-output power (P_{max}) decreased gradually with the increase in PA concentration. All these data illustrated the toxic actions of PAs on the growth of *T. thermophila* BF_5. Moreover, there were regular variations in toxic effect among different PAs. Table 2 shows the toxic effects of PAs.

Inhibitory ratio (I) and half-inhibitory concentration (IC_{50})

The growth inhibitory ratio (I) is calculated on the basis of the growth rate constant. Inhibitory ratio could be defined as:

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

where k_0 is the rate constant of the control in the log phase; k_c is the rate constant in the log phase of *T. thermophila* BF_5 growth inhibited by PA at a concentration c. When the inhibitory ratio I is 50%, the corresponding concentration of inhibitor is called half-inhibitory concentration, IC_{50}. To demonstrate the toxic effects of various PAs on *T. thermophila* BF_5, I values are also shown in Table 2. Hence, the IC_{50} value for: berberine was 112 mg/L; coptisine was 119.9 mg/L; palmatine was 54.9 mg/L; jateorhizine was 73.9 mg/L. Considering the values of k and IC_{50}, we can conclude that the magnitude of toxic effect was: palmatine > jateorhizine > berberine ≈ coptisine.

Comparison of actions of PAs on the growth of *T. thermophila* BF_5 and *Bacillus shigae*

The power-time curves of the growth of *T. thermophila* BF_5 indicated that all the PAs tested had toxic effects on this organism. In combination with our previous studies [19], the IC_{50} of PAs on *Bacillus shigae* (living model related efficacy) and *T. thermophila* BF_5 (living model related toxicity) are analyzed in Fig. 4. This produced a sequence of magnitude of antimicrobial activity of PAs on *B. shigae* was: berberine > coptisine > palmatine > jateorhizine. Alternatively, the magnitude of toxic effects of PAs was in the sequence: palmatine > jateorhizine > berberine ≈ coptisine. At an identical concentration, berberine and coptisine showed significant antibacterial activities, but low toxic

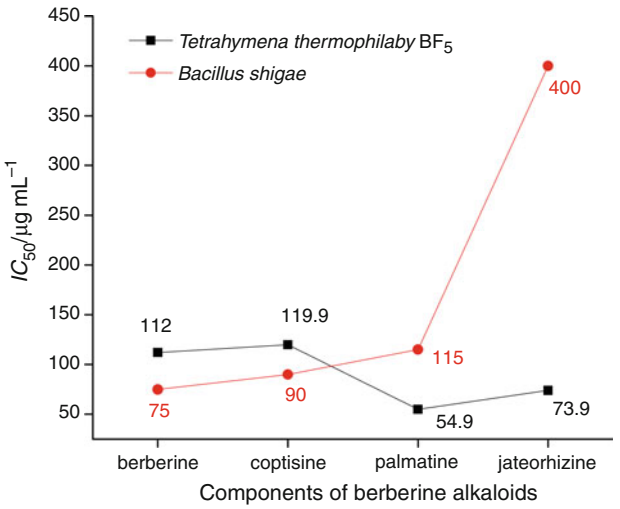


Fig. 4 Comparison of the effects of PAs on *T. thermophila* BF_5 and *B. shigae* with respect to IC_{50}

effects. Hence, when compared with jateorhizine, berberine and coptisine are suitable to be developed as new agents against infectious diseases (e.g., dysentery).

Structure–function relationship of PAs on *T. thermophila* BF_5

All four PAs were berberine alkaloids with different substituents at C2, C3, C9, and C10 of the benzene ring (Fig. 1). Figure 4 is indicated that the functional group methoxyl at C2, C3, C9, and C10 of the benzene ring possibly improves toxic effect of PAs on *T. thermophila* BF_5. Furthermore, the methoxyl group at C2 and C3 could better enhance the toxic effect of PAs than that at C9 and C10. The methoxyl groups are all located at a six-membered ring, and the spatial structure also shows specificity, which corresponds to the work of Su (Su XY [26], Master's thesis, Chinese Academy of Sciences, Dalian, China). He shows that it is the different biomacromolecules of *T. thermophila* BF_5 that could be inserted into the spatial structure of a specific molecule. With respect to antibacterial activity, the methylenedioxy group at C2 and C3 could significantly enhance the antibacterial activity of PAs [19]. Hence, the functional groups at C2 and C3 are more important for the antibacterial activity and toxic effect of PAs.

Conclusions

Tetrahymena thermophila BF_5 was a kind of eukaryotic unicellular microbe and used as a reference test species in toxicological tests of protozoan. And, new methods and approaches are needed for the development of toxicity test systems and studies. Our experiments showed that microcalorimetry was a powerful tool for monitoring the growth process of *T. thermophila* BF_5. It provided more growth kinetic and thermodynamic information of microbe than conventional techniques and all this information was significant and useful in understanding the biological processes and studying the toxic action of drug and other toxic substances.

The power-time curves of *T. thermophila* BF_5 growth under the actions of PAs showed that with increasing concentrations of PAs, the lag phase became longer, k and P_{max} decreased gradually, t_p and t_G were delayed, which indicated that the normal living environment was changed with addition of PAs and the mononuclear microbes took longer time to produce a sufficient number of cells for a detectable signal and that excess PAs inhibited the growth of *T. thermophila* BF_5 or killed the microbes.

The structure–function relationship of PAs indicated that the C2 and C3 positions contributed more to the toxic effect or antibacterial activity of PAs. That is, when substituted with methylenedioxy groups, the antibacterial activity would be enhanced; when substituted with methoxyl groups, the toxic effect would be increased. Hence, berberine and coptisine are suitable to be developed as new agents against infectious diseases (e.g., dysentery). Also, berberine has a wider distribution and more content in plants compared with coptisine, which could make berberine a more frequently used medicine for the treatment of diarrhea.

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