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Screening for novel antibacterial agents based on the activities of compounds on metabolism of *Escherichia coli*: A microcalorimetric study

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ABSTRACT

The emergence and prevalence of resistance to antibacterial agents is a pressing threaten for human health. Screening for novel antibacterial agents targeting not only multiplying but also non-multiplying bacteria using appropriate approach is in great demand. In this study, the microcalorimetric method was used to measure the metabolic curves of *E. coli* growth affected by chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA). By analyzing the metabolic curves and thermo- kinetic/dynamic parameters, the antibacterial activities of CDCA and UDCA on multiplying and non-multiplying bacteria of *Escherichia coli* (*E. coli*) were evaluated. The results illustrated that, for the multiplying metabolism of *E. coli*, the two compounds controlled the anaerobic fermentative, with $IC_{50(1)}$ (half-inhibitory concentration) of 566.2 μg/mL for CDCA and 573.6 μg/mL for UDCA, respectively, but had no effective action on aerobic metabolism of the bacteria. The action of the two compounds on the non-multiplying metabolism was studied by taking the heat output of *E. coli* in the stationary phase as the additive guideline of the activity. The values of $IC_{50(2)}$ were 543.4 and 547.5 μg/mL, and MSC_{50} (minimum stationary-cidal concentration 50) were 532.6 and 537.3 μg/mL for CDCA and UDCA, respectively. So, CDCA had more powerful antibacterial activity on *E. coli* than UDCA either for multiplying bacteria or non-multiplying metabolism, and they both showed stronger activities on non-multiplying metabolism than on multiplying metabolism of the bacteria. The microcalorimetric method should be strongly suggested in screening novel antibacterial agents for fighting against multidrug-resistant bacteria.

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1. Introduction

Although there are many antibiotics in the marketplace, bacterial resistance has been developed for all. The emergence and increasing prevalence of multidrug-resistant bacteria in human pathogens to antibacterial agents has been a pressing threaten for human health and has challenged our ability to treat bacterial infections and diseases over the past decades [1–5]. The development of novel antibacterial agents with high efficacy and long activity and new approaches for screening and evaluating the antibacterial activities of these agents are therefore in great demand to combat this problem [4,6–9].

It is reported that there are four commonly used approaches for the development of new systemic antibacterial agents: classic screening; structural changes to existing agents; genome hunting and a new route that targets non-multiplying latent bacteria ('non-multipliers'). The first three approaches resulted in the majority of antibiotics used today. But there are still some disadvantages, for example, the overall problem of the classic screening approach, which only based on the inhibition of actively multiplying bacteria, is that bacterial resistance arises soon after the new antibacterial agent is widely used in the community; the new one from structural changes to existing antibacterial classes will also lead to a high likelihood of cross resistance, which further reduces the effectiveness of related antibacterial agents; the 'genome hunting' approach results in few agents with good antibacterial activities [10–13]. Recently the fourth new approach development-namely, targeting non-multiplying latent bacteria has got more and more attention [10,14,15]. It is of great significance to test the antibacterial activities of compounds from nature or synthesis targeting non-multiplying bacteria, so as to find novel antibacterial agents that will reduce the resistance rate.

In a clinical infection, multiplying bacteria and non-multiplying bacteria exist side by side [10]. Antibiotics kill multiplying bacteria, but they are very inefficient at killing non-multipliers [15] leading to slow, partial death of the total target population in an infected

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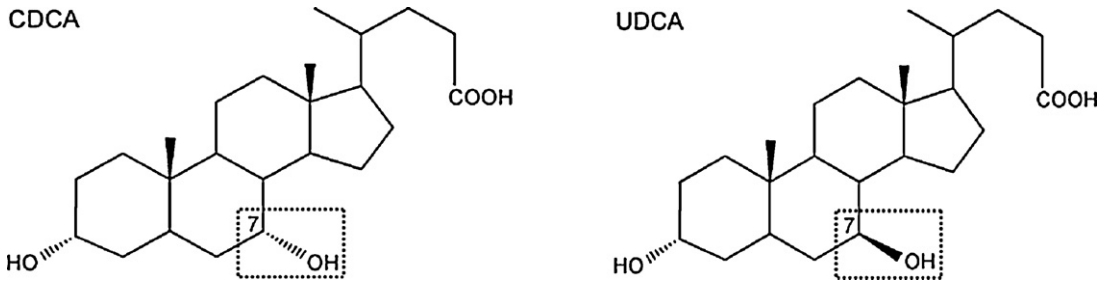

Fig. 1. Chemical structure of the two bile acid derivatives. The two compounds are a pair of epimerides with different conformations of hydroxy at C-7 position of steroid skeleton.

tissue and resulting in the administration of repeated doses of antibiotics. Non-multipliers might be truly non-multiplying or they might be slowly multiplying, and they probably exist in a spectrum of growth states within a bacterial population. Although non-multipliers do not cause overt disease, they act as a pool from which multiplying bacteria emerge to cause recurrent disease. Approximately 60% of all clinical bacterial infections, such as tuberculosis, sore throat and infected eczema, were associated with non-multipliers. People have realized these and found that non-multiplying latent bacteria are one of the main sources of resistant bacteria and these bacteria can prolong the treatment period and resist to most of the existing antibacterial agents, further, they have put forward a new strategy targeting non-multiplying latent bacteria [14,16,17].

However, almost all of these existing screening methods are not suitable for this new strategy. It is very necessary to find a more appropriate screening approach.

An alternative to these techniques that is non-invasive and non-destructive is microcalorimetry. This technique allows analysis to be performed directly on a test substance, regardless of its homogeneous or heterogeneous nature. Microcalorimetry is an established procedure that has been extensively used to study the antimicrobial activities of many materials on microorganisms, since it permits the online tests of bioactivity screening and can obtain a lot of important information about the process of cell growth [18–23]. It can provide a continuous measurement of heat production and supply the metabolic curves to describe the growth process without disturbing the normal activity of the bio-system. It also has some peculiar advantages in new drug discovery and evaluating the antibacterial activities of these new drugs with good sensitivity and reproducibility. The homogeneous system is not necessary and continuous, *in situ*, real time, quantitative detecting can be realized so as to obtain abundant thermo-kinetic/dynamic information about the microbial metabolism progress including multiplying and non-multiplying.

In view of the potential benefits of microcalorimetry in quantitative microbiology, the microcalorimetric technique has been used, in this study, to investigate the activities of two bile acid derivatives (chenodeoxycholic acid, CDCA and ursodeoxycholic acid, UDCA) on *Escherichia coli* (*E. coli*) growth, trying to obtain the activities of them on both multiplying and non-multiplying bacteria. Results showed that CDCA and UDCA had certain activities both on the multiplying and non-multiplying bacteria of *E. coli* and the microcalorimetric method was an effective method to accommodate the new antibacterial screening strategy and screen for novel antibacterial agents.

2. Materials and methods

2.1. Materials

Chenodeoxycholic acid (CDCA, 3α, 7α-dihydroxy-5β-chloanic acid) and ursodeoxycholic acid (UDCA, 3α, 7β-dihydroxy-5β-chloanic acid) were purchased from the National Institute for the Control of Pharmaceutical and Biological Compounds, Beijing, PR China and their structures were given in Fig. 1. Methanol (MeOH) was used as a solvent for preparing the original solution of the two compounds and the concentrations of them were both 50 mg/mL. All other chemicals used were of analytical grade and available locally.

2.2. E. coli culture

E. coli (CCTCC AB91112) was provided by China Center for Type Culture Collection, Wuhan University. Briefly the broth culture medium was composed of peptone (10 g), beef extract (6 g) and NaCl (5 g) dissolving in 1000 mL deionized water (pH 7.0–7.2) and sterilized in high-pressure steam at 121 °C for 30 min. *E. coli* were grown in 25 mL broth culture medium in a 100 mL container and incubated in the shaker for 8 h at 37 °C. The rotation speed of incubator shaker is 110 rpm. For this microcalorimetric measurement, the bacterial suspension of *E. coli* was inoculated in Luria-Bertani (LB) culture medium, which was prepared from peptone (10 g), yeast extract (5 g) and NaCl (5 g) dissolving in 1000 mL deionized water (pH 7.0–7.2) and sterilized by autoclaving at 0.1 MPa and 121 °C for 30 min.

2.3. Microcalorimetric experiment

All microcalorimetric experiments were performed at 37 °C using a 3114/3236 thermal activity monitor (TAM) air isothermal calorimeter (Thermometric AB, Sweden) with ampoule method in batch-mode. The calorimeter was brought to equilibrium temperature over night in advance. Each sterilized 20 mL glass ampoule was filled with 5 mL LB culture medium containing *E. coli* at a cell density of 1×10^6 colony forming units (CFU)/mL and each sample had a reference containing the same volume of uninoculated medium. Then, each ampoule containing the cell suspension of *E. coli* and CDCA or UDCA was sealed up and put into the eight-channel calorimeter block. After about 30 min (the temperature of ampoules reached 37 °C), the heat-flow power (HFP)-time curves were recorded until the recorder returned to the baseline. All data were collected continuously using the dedicated software package.

3. Results and discussion

3.1. Choosing the appropriate volume of the solvent

Because of the poor water solubility of the two bile acid derivatives, we got their solutions dissolved in MeOH. Firstly, the influence of MeOH on *E. coli* growth was investigated. Different volumes of MeOH were added into the glass ampoule containing *E. coli* suspension and the HFP–time curves were shown in Fig. 2.

Fig. 2 showed that the addition of MeOH influenced the metabolism of *E. coli*. Within the volume range (20–80 μL) of MeOH,

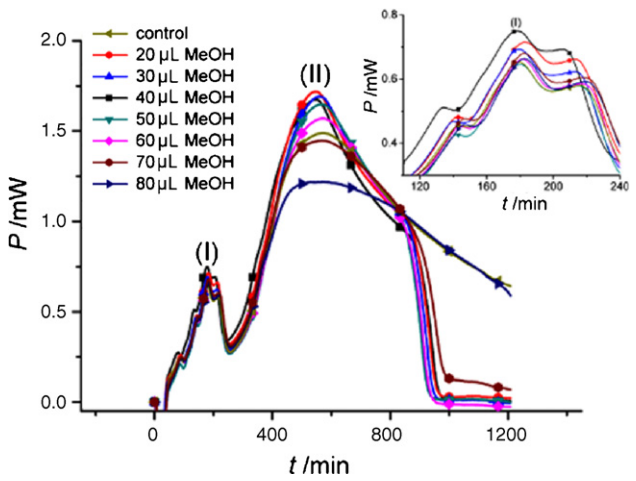

Fig. 2. Influence of the solvent (MeOH) on metabolism of *E. coli*. The small figure in top right corner is the amplificatory results of the first peak (I).

the lower (first) peak (I) changed irregularly, giving expression to the increase or decrease of the heat-flow power. When the volumes of MeOH increased, especially above 70 μL (1.4%, V/V), the higher (second) one (II) of the metabolic HFP–time curves were all inhibited gradually. And when the volumes of MeOH were less than 70 μL (1.4%, V/V), the influence could be neglected. By repeat experiments, the volume of MeOH was controlled within 70 μL (1.4%, V/V) during all the experiments. So, the maximum added volume of the solutions of the two compounds into the ampoule was 70 μL.

3.2. Metabolic HFP–time curve of E. coli: multiplying and non-multiplying metabolism

Using this microcalorimeter, the metabolic HFP–time curves of *E. coli* in the absence of any substance (the control condition) were measured as shown in Fig. 3.

There are two curves in Fig. 3, the *HFP–t* curve (a) and ln *P–t* curve (b) of the control. The former shows the total metabolic profile of *E. coli* and the latter indicates the changing character of the metabolic heat-flow output power, which can be used to divide the

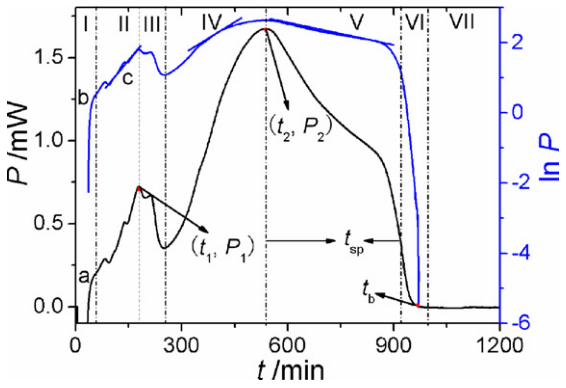

Fig. 3. The metabolic HFP–time curves of *E. coli* in the absence of any substance. (a) *HFP–t* curve; (b) ln *P–t* curve. It is a typical metabolic profile of *E. coli* culturing in LB culture medium supplemented without any substance monitored by the microcalorimeter at 37 °C and can be divided into seven phases: (I)-the lag phase; (II)-the first exponential phase; (III)-the transition phase; (IV)-the second exponential phase; (V)-the stationary phase; (VI)-the decline phase; (VII)-the dormant phase. The straight line (*c*) in curve *b* is the fit linear results of the corresponding phases. P_1, P_2 (mW) are the heat-flow output powers of the first and second highest peak and t_1, t_2 (min) are the corresponding appearance times. t_{sp} (min) is the time of stationary phase and t_b (min) is the time when the heat-flow output power returns to the baseline.

growth phases of the bacteria. P_1, P_2 (mW) are the heat-flow output powers of the first and second highest peaks and t_1, t_2 (min) are the corresponding appearance times. t_{sp} (min) is the time of the stationary phase and t_b (min) is the time when the heat-flow output power returns to the baseline.

In order to veritably depict the metabolic profile including multiplying and non-multiplying metabolism of *E. coli*, based on the reports of Xie et al. [24], the HFP–time curve of *E. coli* growth is divided into seven phases: the lag phase (I), the first exponential phase (II), the transition phase (III), the second exponential phase (IV), the stationary phase (V), a decline phase (VI) and the dormant phase (VII). The peaks and troughs in the HFP–time curves were thought as the characteristics of organism growth in complex medium (LB medium) with restricted (only the head space within the ampoule) oxygen availability [25].

The internal environment of *E. coli* growth in the glass ampoule is a containing system and the oxygen in which is limited. The presence and absence of oxygen is vital for the growth of the bacteria as it can affect the metabolic ways of these bacteria. The volume of glass ampoule is 20 mL and there is 15 mL air for *E. coli* to use under the condition of ampoule method. So, when the bacteria are inoculated into the fresh culture medium, the bacteria begin to adapt to new conditions and exist a lag phase (phase (I) in Fig. 3). Regrettably it cannot be exactly displayed for the thermal balance in the ampoule method. Following phase (II) is the first exponential growth phase, which represents the aerobic multiplying metabolism of *E. coli*. It corresponds well to a linear relationship in the logarithmic curve b. When the oxygen is exhausted, the bacteria will adapt to the anaerobic condition and go into a transition phase (III), displaying a small peak in this phase. Sufficient nutrients remain in this containing system, the bacteria will adjust themselves to adopt another way of fermentation metabolism and begin the second exponential growth phase (IV), which also has a very good linear relationship in curve b. In this phase, the bacteria all sufficiently utilize the nutrients to grow actively and multiply swiftly. So, the metabolic heat-flow output power is slower and higher (representing the highest peak) than the first exponential phase (judging from curve a), which maybe the result from various metabolic pathways. When the limited nutrients, especially the nitrogen source, are exhausted, these bacteria go into the stationary phase (V), which is used to describe the non-multiplying bacteria. In this phase the bacteria take 'non-multiplying metabolism'. So many metabolites are produced that most of the bacteria take the endogenous metabolism, resulting in the slow linear decrease of ln *P* as shown in curve b of Fig. 3. With running out of the culture medium and producing of many metabolites even some toxic materials, the metabolic heat-output power *P* (mW) decreases quickly, giving expression to the quick diminish of ln *P*. And following the decline phase (VI) appears. When no further bacterial metabolic activity is measurable, *P* gets to the baseline and the bacteria go into the dormant phase (VII). So, the experiment is finished.

Analyzing the *HFP–t* curves of *E. coli*, some useful information of the multiplying metabolism (phase (II) and (IV)) and the non-multiplying metabolism (phase (V)) can be simultaneously obtained, which can be used to objectively depict the metabolic progress of *E. coli* growth, and further to evaluate the antibacterial activities of compounds. Based on this information, two screening methods would be established.

3.3. Influence of compounds on multiplying metabolism of E. coli

When the two bile acid derivatives (CDCA and UDCA) were added into the internal system of *E. coli* growth in the glass ampoule, the metabolism (multiplying and non-multiplying metabolism) of the bacteria would be influenced. These influences were firstly shown from the multiplying metabolism, especially phase (IV) and

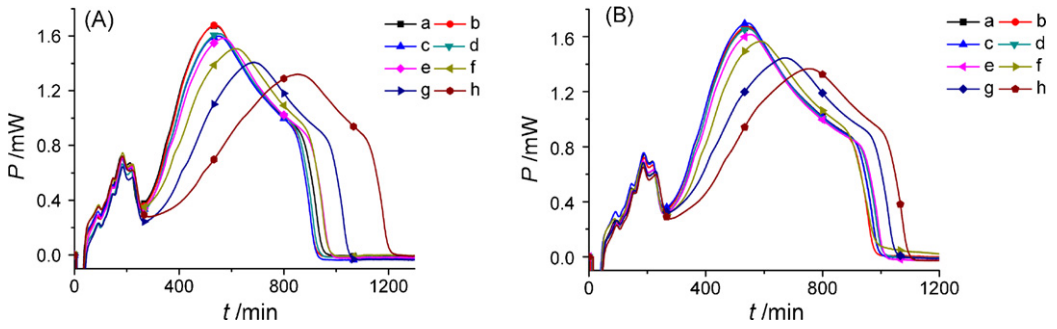

Fig. 4. The HFP–time curves of *E. coli* growth in the presence of (A) CDCA and (B) UDCA. The concentrations of the two compounds are both (a) 0, (b) 100, (c) 200, (d) 300, (e) 400, (f) 500, (g) 600 and (h) 700 μg/mL, respectively. With addition of the compounds, the basic shapes of the HFP–time curves are changed compared with that of the control (without compound in the system), showing that the two compounds have various activities on *E. coli*.

Table 1
Quantitative thermo-kinetic parameters from the HFP–time curves for multiplying metabolism of *E. coli* affected by CDCA and UDCA.

Compound	c (μg/mL)	k_1(mim^{-1})	r_1[a]	I_1 (%)	t_1 (min)	P_1 (mW)	k_2(mim^{-1})	r_2	I_2 (%)	t_2 (min)	P_2 (mW)
CDCA	0	0.01352	0.9901	0	178.3	0.723	0.00996	0.9960	0	539.7	1.680
	100	0.01130	0.9891	16.4	181.7	0.717	0.00895	0.9939	10.1	539.0	1.599
	200	0.01123	0.9907	16.9	181.7	0.713	0.00882	0.9959	11.4	548.0	1.617
	300	0.01133	0.9878	16.2	181.7	0.665	0.00874	0.9946	12.2	552.0	1.584
	400	0.01108	0.9886	18.0	185.7	0.710	0.00747	0.9931	25.0	569.3	1.508
	500	0.00897	0.9915	33.7	181.7	0.740	0.00623	0.9960	37.4	613.0	1.407
	600	0.00626	0.9879	53.7	185.7	0.643	0.00427	0.9951	57.1	682.7	1.320
	700	0.00492	0.9886	63.6	181.7	0.719	0.00319	0.9911	68.0	852.7	1.671
UDCA	0	0.01368	0.9870	0	190.3	0.741	0.00977	0.9954	0	552.0	1.671
	100	0.01155	0.9859	15.6	190.3	0.721	0.00880	0.9962	9.9	546.0	1.698
	200	0.01163	0.9870	14.9	186.0	0.753	0.00796	0.9932	18.5	546.0	1.656
	300	0.01116	0.9885	18.4	186.0	0.688	0.00721	0.9921	26.2	546.0	1.616
	400	0.01105	0.9889	19.2	186.0	0.696	0.00695	0.9965	28.9	550.3	1.566
	500	0.00941	0.9915	31.2	181.7	0.681	0.00644	0.9971	34.1	587.7	1.445
	600	0.00638	0.9925	53.4	186.0	0.683	0.00426	0.9961	56.2	670.3	1.366
	700	0.00413	0.9896	69.8	186.3	0.651	0.00389	0.9967	60.2	758.0	1.680

[a] Correlation coefficient.

the second highest peak (II), as shown in Fig. 4, which could be reflected from the changes of the metabolism rate constant (k_1 and k_2), t_1, P_1 for the first highest peak and t_2, P_2 for the second highest peak. As described by Xie et al. [24], in the exponential phase, the heat-flow output power P obeys the following equation:

$$P_t = P_0 \ \ exp(kt) \quad \text{or} \quad \ln P_t = \ln P_0 + kt \tag{1}$$

where, P_0 and P_t are the heat-flow output powers of the bacterial at time 0 and t (min), respectively. Using this equation, k_1 and k_2 of the first and second exponential phase for the growth of *E. coli* at 37 °C in the absence or presence of the compounds were calculated by analyzing the data of the first and second highest peak, which all had a good correlation coefficient r (Table 1). The addition of compounds into the system affected the multiplying metabolism of the bacteria, resulting in the changes of quantitative thermo-kinetic parameters k_1, k_2, t_1, P_1 and t_2, P_2. So the activities of the compounds on the multiplying metabolism of *E. coli* could be studied by analyzing these parameters.

Furthermore, the growth inhibition ratio I is calculated on the basis of the growth rate constant k and can be defined as [24]:

$$I = \frac{k_0 - k_c}{k_0} \times 100\% \tag{2}$$

where k_0 and k_c (min^{-1}) are the growth rate constants of *E. coli* in the absence of the compound (the control condition) and that inhibited by an inhibitor at a certain concentration c. I can show the inhibitory extent of some concentration of antibacterial agent on the bacteria's metabolism. The relationship between the activity and the concentration of the compound can be directly reflected from I-c curve. When the inhibitory ratio I is 50%, the corresponding concentration of inhibitor is called half-inhibitory concentration IC_{50} (μg/mL). The value of IC_{50} can quantitatively represent the activity of the antibacterial agent.

The bigger the values of t_1, t_2, I, and the smaller the values of k_1, k_2, P_1, P_2, IC_{50} are, the stronger the inhibitory effect of the compound has. So, the antibacterial activities of different compounds could be quantitatively compared by analyzing the change of these quantitative thermo-kinetic parameters, which were listed in Table 1 and shown in Fig. 5. $IC_{50(1)}$ (μg/mL) was the concentration of CDCA or UDCA with 50% inhibitory ratio on *E. coli* in the second exponential phase for the multiplying metabolism, which could be obtained from the I_2-c curve.

It could be found from the parameters in Table 1: (1) for the first exponential growth phase (aerobic multiplying metabolism

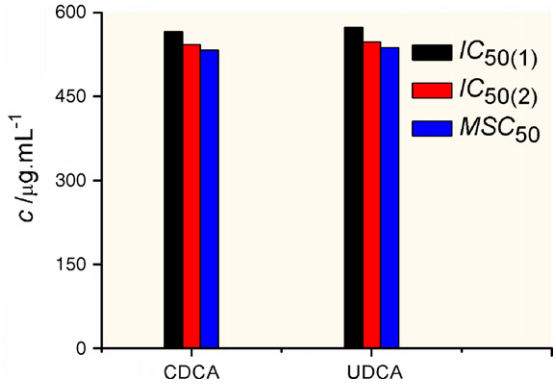

Fig. 5. IC_{50} and MSC_{50} for CDCA and UDCA on multiplying and non-multiplying metabolism of *E. coli*.

Table 2
Quantitative thermo-dynamic parameters from the HFP–time curves for non-multiplying metabolism of *E. coli* affected by CDCA and UDCA.

Compound	c (μg/mL)	k_1 (min^{-1})	r_3[a]	I_3 (%)	t_{sp} (min)	t_b (min)	Q_{sp}	I_{sp}
CDCA	0	0.00281	−0.9966	0	378.0	982.3	27.5	0
	100	0.00204	−0.9970	27.4	353.0	947.0	26.3	4.4
	200	0.00203	−0.9991	27.8	349.0	955.3	25.1	8.7
	300	0.00201	−0.9987	28.1	348.3	963.7	24.0	12.7
	400	0.00171	−0.9984	39.1	375.4	1005.3	21.3	11.6
	500	0.00148	−0.9994	47.3	336.7	1009.7	16.4	40.4
	600	0.00116	−0.9989	58.7	326.0	1079.3	10.4	62.1
	700	0.00117	−0.9983	58.4	318.6	1236.3	6.7	75.6
UDCA	0	0.00293	−0.9974	0	407.3	1018.3	27.7	0
	100	0.00198	−0.9972	32.4	400.7	1002.3	26.4	4.7
	200	0.00197	−0.9970	32.8	414.7	1026.0	26.1	5.8
	300	0.00195	−0.9977	33.5	428.7	1040.0	25.7	7.2
	400	0.00183	−0.9961	37.5	426.7	1044.3	19.3	30.3
	500	0.00151	−0.9981	48.5	354.6	1080.7	16.3	41.2
	600	0.00118	−0.9979	59.7	350.4	1090.3	10.9	60.6
	700	0.00130	−0.9994	55.6	304.3	1126.7	7.5	72.9

[a] Correlation coefficient.

phase (II)), the values of k_1 for *E. coli* growth affect by different concentrations of the two compounds were all smaller than those for *E. coli* growth without compound. Within the concentration range of 100–700 μg/mL, k_1 increased and then decreased, resulting in the irregular change of I_1; also, the values of t_1 and P_1 both had some fluctuation within tested concentration range. So the actions of compounds on the aerobic multiplying metabolism of *E. coli* were complicated. The possible reason was that within the beginning time (about 250 min) (Fig. 5) the bacteria grew and multiplied slowly (low P) in the aerobic new environment and the compound did not begin to play its marked effect on the bacteria. This phenomenon was not helpful for evaluating the antibacterial activities of the compounds and IC_{50} in this growth phase could not be obtained. (2) For the second exponential growth phase (anaerobic fermentation metabolism phase (IV)), the values of k_2 and P_2 were decreased (decline of the second highest peak), the values of I were increased and t_2 were prolonged with the increase of the concentrations of CDCA and UDCA, showing that the antibacterial activities of the two compounds were enhanced. The two compounds both showed strong activities on the anaerobic multiplying metabolism (phase (IV)) of *E. coli*, with $IC_{50(1)}$ of 566.2 μg/mL for CDCA and 573.6 μg/mL for UDCA, respectively. CDCA had more powerful activity than UDCA on the multiplying metabolism of *E. coli*. (3) For the whole multiplying metabolism of *E. coli*, both of the two compounds mainly acted on the anaerobic fermentation metabolism of *E. coli* without effective action on aerobic multiplying metabolism of the bacteria. The microcalorimetric method could be conveniently used to characterize the antibacterial activities of two bile acid derivatives based on inhibiting the multiplying metabolism of the bacteria in detail.

3.4. Influence of compounds on non-multiplying metabolism of E. coli

In the stationary phase (phase (V) in Fig. 3), the bacteria undertake non-multiplying metabolism. In humans and animals, multiplying bacteria can be killed quickly by antibacterial agents, whereas, non-multiplying or slowly multiplying bacteria tolerate repeated doses of them, which lead to the need for a conventional prolonged course of these agents. It is significant to investigate the activity of novel antibacterial agents on the non-multiplying bacteria [10,14–16,26].

As shown in Fig. 4, with addition of different concentrations of compounds into the bacterial suspension, the stationary phases of the HFP–time curves were influenced and the heat-flow output powers for this non-multiplying metabolism were declined slowly. This influence could be reflected from the results shown in Table 2. By analyzing the changes of these quantitative thermo-dynamic parameters, the actions of the compounds on non-multiplying metabolism of bacteria could be obtained. Therefore new antibacterial screening methods could be established.

It could be seen from the data in Table 2 that the non-multiplying metabolism of *E. coli* showed two kinds of different behaviors under the effect of CDCA and UDCA. At low concentrations (almost smaller than 400 μg/mL for both), the time of stationary phase (t_{sp}, min) became longer gradually with increasing the concentration, and the decline velocity of the metabolism (k_3) became slower than those of the control (without drug). With further increasing the concentration (bigger than 400 μg/mL for both), t_{sp} became shorter gradually and the decline velocity of the metabolism became a little much faster than those of the previous concentrations. This phenomenon might be attributed to the stress behavior of *E. coli* to the influence of exotica [27,28]. Within the concentration range of 100–700 μg/mL, I_3 was increased with the increase of concentration of the two compounds. From the relationships of I_3 and c, the values of $IC_{50(2)}$ of 543.4 μg/mL for CDCA and 547.5 μg/mL for UDCA (Fig. 5) were calculated, respectively, showing that the two compounds both had good activities on the non-multiplying metabolism of *E. coli*, and the activity of CDCA was much stronger than that of UDCA in this phase.

Because most bacteria take the endogenous metabolism in the stationary phase, it can be supposed that the heat output in the stationary phase (Q_{sp}, J) is directly relative to the activity of the bacteria. Integrating the peak areas under the curves of the stationary phase, Q_{sp} can be obtained as the activity guideline for these non-multiplying bacteria so as to quantificationally characterize the activity of the antibacterial agents on the non-multiplying bacteria. Under all the experimental conditions for CDCA and UDCA, Q_{sp} was decreased and t_b of decline phase was increased gradually with the increase of the concentration of the two compounds, further showing the inhibitory activities of them on these non-multiplying bacteria.

In this phase, the bacteria did not multiply and began to go into the dormant phase, while the metabolites were augmented. So Q_{sp} was decreased and the decreasing tendency was speeded up with the increase of the concentration of the two compounds. To describe the inhibitory extent of some concentration of the compounds on the bacteria's non-multiplying metabolism, the inhibitory ratio (I_{sp}, %) in the stationary phase based on the Q_{sp} is defined as:

$$I_{sp} = \frac{Q_{sp(0)} - Q_{sp(c)}}{Q_{sp(0)}} \times 100\% \quad (3)$$

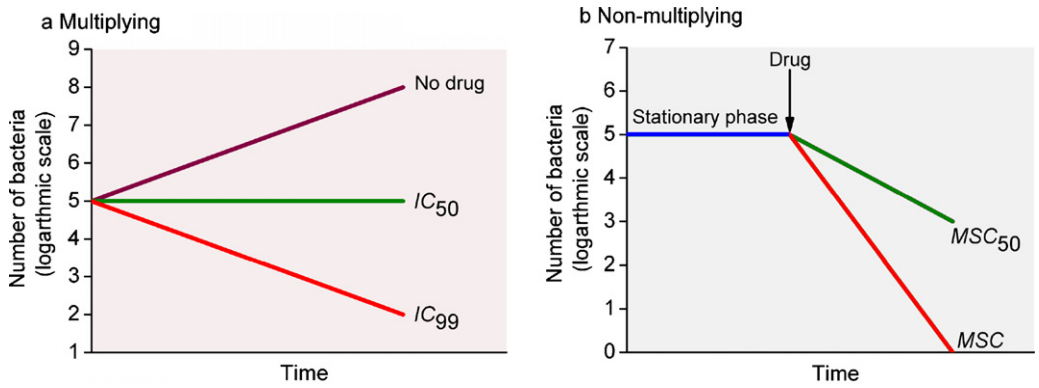

Fig. 6. Strategy of screening for novel antibacterial agents targeting on (a) multiplying and (b) non-multiplying bacteria. This strategy is based on the microcalorimetric method. Using this method to screening for novel antibacterial agents, the concentration of inhibitor with 50% and 99% inhibitory ratio (IC_{50} and IC_{99}) on the multiplying bacteria, and the minimum stationary-cidal concentration 50 (MSC_{50}) and the minimum stationary-cidal concentration (MSC) on the non-multiplying bacteria should be calculated and obtained.

where, $Q_{sp(0)}$ and $Q_{sp(c)}$ (J) are the heat outputs in the stationary phase of *E. coli* under the condition of control (no compound) and final concentration c of the compound. I_{sp} could also describe the inhibitory extent of some concentration of the compound on the non-multiplying metabolism of bacteria. The higher the I_{sp} is, the stronger the inhibitory activity of the compound has. The minimum stationary-cidal concentration 50 MSC_{50} (μg/mL) of the compound, which was defined by Coates et al. [10] as the concentration of the compound that led to a reduction of half of the initial log bacterial count, could also be obtained from the I_{sp}-c curve. Here, MSC_{50} (μg/mL) was the effective concentration of the compound that could inhibit 50% of the activity of *E. coli* in the stationary phase. So the activity of different compounds on the non-multiplying bacteria could also be compared from MSC_{50}. The MSC_{50} in Table 2 indicated that CDCA and UDCA both had some activities on the non-multiplying metabolism of *E. coli* and the activity of CDCA was better than that of UDCA, with MSC_{50} of 532.6 and 537.3 μg/mL, respectively.

As seen from Fig. 5, the values of MSC_{50} of the two compounds were smaller than those of $IC_{50(2)}$ obtained from the non-multiplying metabolism. The possible reason was that, in the stationary phase, the bacteria were relative stable and some of them were dormant, so the two bile acid derivatives of low concentration had inhibitory effect on the non-multiplying metabolism of the bacteria. Also, the values of $IC_{50(2)}$ and MSC_{50} of the two compounds for non-multiplying metabolism were smaller than those of $IC_{50(1)}$ for multiplying metabolism of *E. coli*, illustrating that the compounds had stronger activities on non-multiplying metabolism than on multiplying metabolism of the bacteria. From the integrative comparison of the values of $IC_{50(1)}$, $IC_{50(2)}$ and MSC_{50}, it could be concluded that the antibacterial activity of CDCA was much stronger than that of UDCA. And the future work will focus on the mechanism of action of them on *E. coli*.

3.5. Strategy for screening novel antibacterial agents

In most bacterial infections or diseases, the microbes consist of at least two populations that exist simultaneously: multiplying and non-multiplying. Multiplying bacteria are killed quickly by antibacterial agents, whereas non-multiplying bacteria tolerate repeated doses of antibacterial agents or resistant to these agents and survive. The survival numbers of non-multiplying bacteria depend on the species of bacteria, the dose, the duration and the type of antibacterial agents, etc. But no antibacterial agents have been specifically developed against non-multiplying bacteria from then on. One possible solution to this problem might be to shorten the duration of chemotherapy by targeting non-multiplying bacteria with novel antibacterial agents [10]. So it is of great significant to search for novel antibacterial agents and evaluate their activities targeting not only the multiplying bacteria but also the non-multiplying bacteria through a more appropriate approach than the existing screening methods.

This study showed that the microcalorimetric method could be used for screening novel antibacterial agents based on the new strategy of targeting non-multiplying bacteria. The two bile acid derivatives both had powerful antibacterial activities on the metabolism of *E. coli*, and the activities of them on non-multiplying bacteria were much stronger than those of them on multiplying bacteria, proving that it was of significant to investigate the activities of compounds on non-multiplying metabolism but not merely the multiplying metabolism of the bacteria. Compound CDCA and UDCA were a pair of epimerides (different conformations of hydroxy at C-7 position of steroid skeleton as shown in Fig. 1), but CDCA had much stronger antibacterial activity than UDCA, showing that the structure of compounds had large influences on their antibacterial activities, which gave us some hints that the activities of compounds should be veritably and objectively investigated to avoid some false-positive or negative results in screening for novel antibacterial agents. In this study, the whole metabolic progress (including multiplying and non-multiplying metabolism) of *E. coli* growth affected by compound CDCA and UDCA was obtained using the microcalorimetric method. By analyzing the HFP–time cures and some quantitative parameters from these curves, the activities of the two compounds were effectively evaluated. Of course, the normal and wide use of these two compounds as new antibacterial agents need some more work and clinical study based on more validated models. So, based on the theory of Coates et al. [10], we recommend the microcalorimetric method as a useful approach to screen for novel antibacterial agents. Some important quantitative parameters shown in Fig. 6, such as IC_{50}, IC_{99} for the compounds on multiplying bacteria and MSC_{50}, MSC for them on non-multiplying bacteria should be obtained to evaluate their antibacterial activities.

4. Conclusions

In summary, the present study selected the complex culture medium (LB medium) to cultivate *E. coli* and used microcalorimetry to characterize the antibacterial activities of two bile acid derivatives (CDCA and UDCA) on *E. coli*. The results showed that using the microcalorimetric method, which did not need special equipments, in the novel drug screening field could breakthrough the limitation of classic existing methods, as well as could expand the range of the drug discovery and obtain the action of them on both the multiply-

ing bacteria and non-multiplying bacteria in one microcalorimetric experiment. So, it is a potential powerful tool in searching for novel antibacterial agents to combat the multidrug-resistant bacteria, with many benefits of decreasing the potential for resistance development, reducing product costs and increasing efficacy, ultimately extending the life of these mew antibacterial agents.

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