Antibacterial Mechanisms of Berberine and Reasons for Little Resistance of Bacteria

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Abstract: **Objective** To study the antibacterial mechanisms of berberine and try to understand the reasons why bacteria cells difficultly resisted to it. **Methods** Detecting the minimal inhibitory concentration (MIC) of bacterial cultures incubated under sub-MIC concentration of berberine, *Huanglian*, and Neomycin for more than 200 generations, in order to analyze the bacteria resistance. Detecting the binding kinetics of berberine to DNA, RNA, and proteins. Observing the changes in bacterial cell surface structure with scanning electron microscopy. Detecting the Ca^{2+} and K^{+} released from berberine-treated bacterial cells with atomic absorption spectrum. Detection the absorption of methyl-^{3}H-thymine (^{3}H-dT), ^{3}H-uridine (^{3}H-U), and ^{3}H-tyrosine (^{3}H-Tyr) into berberine-treated bacterial cells. **Results** MICs of bacterial cultures, growing more than 200 generations in MH medium with 1/2 MIC of berberine (BA200) or *Huanglian* (HA200), did not increase compared to the control, while remarkably increased in MH medium with 1/2 MIC of Neomycin (NA200). In addition, from the culture NA200 it was easy to isolate resistant mutant strains which could grow in MH medium with more than four times MIC Neomycin, but from the culture BA200 and HA200 it was difficult to isolate berberine or *Huanglian* mutant strains could grow in MH medium with more than four times MIC berberine or *Huanglian*. The binding kinetics of berberine to DNA, RNA, and proteins illustrated that berberine could easily and tightly bind to DNA and RNA, and hardly dis-bind from DNA- and RNA-berberine complexes. Berberine could easily bind to protein too, but also easily dis-bind from berberine-protein complex. The bacterial cells treated with berberine sharply decreased the absorption of ^{3}H-dT, ^{3}H-U, and ^{3}H-Tyr, as the radioactive precursors of DNA, RNA, and protein biosynthesis. Berberine could damage bacterial cell surface structure, especially for Gram-negative bacteria. Ca^{2+} and K^{+} released from berberine-treated cells increased significantly compared to the control. **Conclusion** All of above results indicate that bacterial cells could not easily become resistant mutants to berberine. The mechanisms for the bactericidal effect of berberine include: inhibiting DNA duplication, RNA transcription, and protein biosynthesis; influencing or inhibiting enzyme activities; destructing the bacterial cell surface structure and resulting in Ca^{2+} and K^{+} released from cells. All of the berberine bactericidal mechanisms are the most essential physiological functions for a live cell, if influenced any one such function, the mutation would be lethal mutation, so that it is difficult to get berberine resistant cells. The results in this paper also prefigure that berberine and its related Chinese medicines would provide a feasible way to control antibiotic resistance problem.

Key words: antibacterial effect; antibiotic resistance; berberine; binding kinetics; Neomycin

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Introduction

It is well known that antibiotics have been regarded as miracle drugs to control infectious diseases caused by bacteria for more than half a century, and this status will be continued. However, the bacterial cells resist to not only single but also usually multiple antibiotics, which has become a knotty problem for the microbiologists and physicians in the world. Therefore, searching for new remedies to which bacteria difficultly resist is strongly expected.

Coptidis chinensis Franch. (*Huanglian*), which is a famous Chinese traditional herbal medicine, has been used to treat intestinal diseases and other infected diseases for more than 2000 years (Wang, Shi, and Zeng,

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2003). Berberine is the main functional component of *Huanglian*, derived from its rhizomes. Berberine is a quaternary alkaloid, and it has been proved that berberine has antibacterial (Eduardo and Groisman, 1996; Wu and Wen, 2000; Yi *et al*, 2007; Dai *et al*, 2010), antifungal (Yu *et al*, 2005; Park *et al*, 2006; Zhao, Zhou, and Zhang, 2006), antiprotozoal (Vennerstrom *et al*, 1990), anti-inflammatory (Choi *et al*, 2006; Li *et al*, 2006; Lee *et al*, 2003), and antitumor (Fukuda *et al*, 1999; Tanabe *et al*, 2005, Issat, Jakóbisiak, and Golab, 2006) activities, *etc*. For antibacterial activity, most studies have focused on the bacteriostatical or/and bactericidal activities of berberine and its derivatives to different bacterial species (Kim *et al*, 2004; Grippa *et al*, 1999), and few on the mechanisms (Kapp and Whiteley, 1991; Islam, Sinha, and Kumar, 2007; Chen *et al*, 2005; Chang, 1991).

When comparing the resistant property of bacteria to berberine, *Huanglian*, and antibiotics Neomycin, we found that bacterial cells easily mutated into resistant cells to Neomycin, whereas hardly mutated into resistant cells to berberine and *Huanglian*. In order to know the reason for this phenomenon, the possible antibacterial mechanisms of berberine were studied in this paper.

Materials and methods

Bacterial strains

Escherichia coli ATCC31343, ATCC 25922, and *Staphylococcus aereus* ATCC 25923 were purchased from the American Type Culture Collection, *Bacillus subtilis* As.1.398, *Proteus vulgaris* As1.491, *Salmonella typhimurium* As1.1174, and *Pseudomonos aeruginasa* As1.50 were purchased from China General Microbiological Culture Collection Center.

Materials

Berberine chloride was purchased from Sigma. Kanamycin and Chloramphenicol were purchased from Gibco; Gentamicin and Cefotaxine were purchased from Sangon (Shanghai), Mueller-Hinton (MH) medium from Landbridge Company (Beijing), *Huanglian* was from Jianlian Chinese Traditional Medicine Pharmacy (Jinan). Radioactive (methyl-^{3}H)-thymine (^{3}H-T), ^{3}H-uridine (^{3}H-U), and ^{3}H-tyrosine (^{3}H-Tyr) were purchased from Institute of Atomic Energy, Chinese Academy of Sciences (Beijing). Salmon sperm DNA was purchased from Takara, *Sacchromyces revivesiae* RNA from Sangon, and bovine serum albumin (BSA) from Promega.

Media

Unless otherwise stated all bacterial cells cultured in MH medium at 37 ℃ with rotary shaking (220 r/min), and on MH medium plates with or without berberine, *Huanglian*, and other antibiotics.

Minimal inhibitory concentration (MIC) determination of bacteria to berberine, *Huanglian*, and antibiotics

The antimicrobial activities of berberine chloride, *Huanglian*, and antibitotics were determined in triplicate by serial two-fold dilution of test compounds, following the recommendations of the National Committee for Clinical Laboratory Standards. Cells (10^5 CFU/mL) were inoculated into test tubes which had 2 mL MH broth. The MIC was defined as the minimum concentration of berberine that completely inhibited the cell growth in test tube during 18 h incubation at 37 ℃ with shaking. The bacteria included bacterial strains (Table 1) and bacterial cultures, such as culture of *E. coli* 31343 grown in MH + Neomycin medium for 200 generations (NA200), culture of *E. coli* 31343 grown in MH + Berberine medium for 200 generations (BA200), and culture of *E. coli* 31343 grown in MH + *Huanglian* medium for 200 generations (HA200). The MIC of NA200 to antibiotics included Neomycin (Neo), Chloromycetin (Clm), Gentamicin (Gen), and Cefotaxime (Cef).

Growth inhibitory activities of berberine to bacteria

In order to investigate the effect of berberine on the growth of bacteria, a loopful of bacterial cells was transferred into a flask containing 20 mL liquid MH medium and incubated on shaker for 12 h at 37 ℃. The optical density at 600 nm (A_{600}) of this culture was adjusted to about 0.5. And the culture (0.5 mL) and berberine solution (5 mL, 0.5 mg/mL) were transferred into test tubes which contained 44.5 mL liquid MH broth. Growth was monitored by A_{600}.

Generation calculation

According to the generation calculation formula, $g = t \times \ln 2 / (\ln N_2 - \ln N_1)$ (Jin *et al*, 2009). Here, t is culturing time, N_2 is the number of cells at time t, N_1 is the number of cells at time t_0.

Binding kinetics of berberine to DNA, RNA, and protein, and the dis-binding characteristics of these complexes

Each 50 mg of Salmon sperm DNA, yeast RNA, and BSA was dissolved into a 2 mL centrifuge tube with 0.4 mL PBS buffer (0.02 mol/L, pH 7.0), then added 0.1 mL berberine solution to make the final berberine concentrations be 25, 50, 75, 100, 150, 200, 250, 300, 400, and 500 μg/mL, respectively. The control group contained only berberine for each concentration. After resting the mixture at room temperature for 1 h, 1.2 volumes of isopropanol were added into DNA and RNA tube, 1.2 volumes of 80% $(NH_4)_2SO_4$ were added into the BSA tube, mixed, and stored at 4 ℃ for 1 h, then centrifuged at 13 000 r/min for 30 min. The supernatants were transferred into new tubes, and the deposits were collected and dried. Afterwards, the A_{370} of the supernatants was tested by UV-Vis-NIR Recording Spectrophotometer (UV—3100 Shimadzu), and defined as A_d, which meant the remainder berberine. The A_{370} of the control group was defined as A_c. The amount of bound berberine (A_b) was calculated by the equation: $A_b = A_c - A_d$. The binding kinetic curves were plotted using the software GraphPad Prism 4 (1992—2003 GraphPad Software, Inc.), with the *X* axis of berberine concentration and *Y* axis of the A_b value. The kinetic parameters were given by one site binding (hyperbola) equation of the same software. The dried deposits were used to investigate the dis-binding characteristics of these DNA-, RNA-, and protein-berberine complexes.

When 50 mg dried DNA-, RNA-, and protein-berberine complexes suspended into 2 mL ethanol, whirled for 10 s, stored at room temperature for 10 min, and then centrifuged, the A_{370} of the supernatants was tested and defined as A_d. The percentage of re-disbind berberine was calculated by the fomulation: $P = A_d/A_b \times 100\%$.

Release of K^+ and Ca^{2+} from *B. subtilis* and *E. coli* cells treated by berberine

The cells of *B. subtilis* and *E. coli* were inoculated in a flask with 10 mL MH medium at 37 ℃, shaking at 220 r/min overnight; Then 5 mL culture was transferred into a flask with 50 mL fresh MH medium and incubation at 37 ℃ was continued for 3 h, to make the cells in exponential phase. Bacterial cells were harvested by centrifugation at 8000 r/min for 3 min, washed three times with 0.02 mol/L PBS (pH 7.2) and then suspended in the same buffer. Berberine solution was added to a final concentration of 0.2 mg/mL (1 × MIC) for *B. subtilis* cells and 2 mg/mL (1 × MIC) for *E. coli* cells. Blank was generated similarly except PBS instead of berberine solution. The mixture was incubated for 1 h at 37 ℃, shaking at 120 r/min. Then bacterial cells were collected by a centrifuge and washed three times with super-purified water (for HPLC). Then bacterial cells were weighed and 100 mg cells (wet weight) were re-suspended in 1 mL water (for HPLC). After 1 h at room temperature, the mixtures were filtered. The filtrates passing through the membrane filter (0.45 μm, Milipore) were used to test the concentrations of K^+ and Ca^{2+} with Polarized Atomic Absorption Spectrophotometer (180—80, Zeaman) according to the instructions of the instrument (Taylor *et al*, 1999).

Incorporation of precursors of ^{3}H-T, ^{3}H-U, and ^{3}H-Tyr into DNA, RNA, and protein biosynthesis progresses

^{3}H-dT, ^{3}H-U, and ^{3}H-Tyr were used as precursors for the biosynthesis of DNA, RNA, and protein, respectively. We demonstrated that berberine inhibited the incorporation of these precursors into DNA, RNA and protein biosynthesis progresses. The cells of *B. subtilis* were inoculated in a flask with 10 mL MH medium at 37 ℃, and shaking at 220 r/min overnight; Then 1 mL culture was transferred into a flask with 50 mL fresh MH medium, and incubation at 37 ℃ was continued for 3 h to make the cells in exponential phase. Then A_{600} was adjusted to 0.1 by using fresh pre-warmed MH medium. Each 0.9 mL of such adjusted culture was transferred into an Eppendorf tube, added 0.1 mL solution contained the radioactive precursors at a final concentration of 0.5 μCi/mL and berberine at various concentrations (1/4 MIC = 0.05 mg/mL) or Neomycin (MIC = 1 μg/mL) into the Eppendorf tube, too. After incubating at 37 ℃ for 20 min (for DNA and RNA) and 2 h (for protein), the cells were collected and washed for three times with PBS (pH 7.2), and the precipitate cells in Eppendorf tubes were torrefied at 75 ℃ overnight. Then the liquid scintillation was added into Eppendorf tubes directly. The radioactivity of the cells was counted with a liquid scintillation spectrometer (LS3801, Beckman) (Miko and Devunsky, 1993).

Results

Gram-positive bacteria were more sensitive to berberine and *Huanglian* than Gram-negative ones

The susceptibility of bacterial strains to berberine was tested, with the MICs among 0.1－2.0 mg/mL (Table 1). The data suggested that Gram-positive bacteria were more sensitive to berberine and *Huanglian* than Gram-negative ones.

Table 1 MICs of berberine and *Huanglian* to bacteria (n = 3)

Groups	MICs / ($mg \cdot mL^{-1}$)						
	E. coli ATCC31343	*E. coli* ATCC 25922	*P. vulgaris* *As1.491*	*S. typhimurium* As1.1174	*P. aeruginasa* *As1.50*	*B. subtilis* *AsC1.398*	*S. aereus* *ATCC25923*
berberine	2.0	2.0	1.0	2.0	2.0	0.2	0.1
Huanglian	31.25	31.25	62.5	31.25	31.25	3.9	3.9

MICs not increasing after the bacterial cells growing 200 generations in MH medium with 1/2 MIC of berberine and *Huanglian*, while antibiotics increasing remarkably

In order to make sure the bacterial cells in exponential phase, the growth curves of bacteria in different sub-MIC concentrations of berberine, *Huanglian,* and Neomycin (as an antibiotic control) were tested (just the curve for berberine shown in Fig. 1, the others omitted). The optimum concentrations at which the bacterial population could be kept in exponential phase at least for 12 h were shown in Table 2. In order to make sure that the bacterial cells were in exponential phase during 12 h at the selected concentration of berberine, the optimum number of bacterial cells (CFU) transferred into a new culturing flask was tested, too (Fig. 1B). The optimum CFU and the growth conditions were summarized in Table 2.

The MICs of the cultures, in which the bacterial cells had grown 200 generations, were shown in Fig. 2. The data in Fig. 2 exhibited that the MICs of BA200 and HA200 did not remarkably change, while the MIC of NA200 increased significantly.

Bacteria hardly becoming resistant mutants to berberine and *Huanglian*, but easily to Neomycin

Resistant cells denoted the bacterial cells which could grow on four MIC MH plates or higher. It was easy to select Neomycin resistant cells, either from cultures NA200 or MH medium with no Neomycin. But we could not get berberie- or *Huanglian*-resistant cells, neither from BA200 or HA200 nor from the cultures in MH medium with no berberine or *Huanglian*. The proportions of resistant cells, defined as the ratios of the resistant cells to the total viable cells, were tested and shown in Table 3.

Release of K^+ and Ca^{2+} from bacterial cells

It is known that some membrane-active anti-bacterial agents can produce rapid loss of cations from the metabolic pool of the cell. In order to know whether berberine had membrane-active antibacterial function, we examined the K^+ and Ca^{2+} release from berberine-treated bacterial cells. The data were shown in Fig. 3. The results indicated that the amounts of K^+ and Ca^{2+}

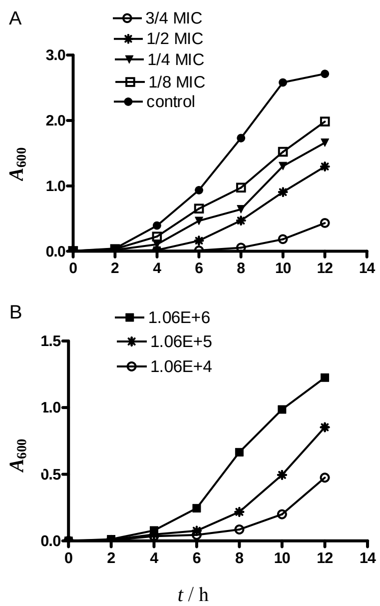

Fig. 1 Growth curves of *E. coli* ATCC31343 in medium contained berberine

MIC of berberine to *E. coli* 31343 was 2 $mg \cdot mL^{-1}$

A: Number of cells at the beginning time was 1.06×10^5 $CFU \cdot mL^{-1}$

B: Berberine concentration was 1/2 MIC = 1 $mg \cdot mL^{-1}$

Table 2 Conditions for culturing *E. coli* ATCC 31343 in MH medium under 1/2-MIC for 200 generations

Experiental groups	CFU at start time	CFU 12 h after	Concentration
control	about 10^3	about 10^9	—
neomycin	about 10^4	about 10^8	1 $\mu g \cdot mL^{-1}$
berberine	about 10^5	about 10^8	1 $mg \cdot mL^{-1}$
Huanglian	about 10^5	about 10^8	25 $mg \cdot mL^{-1}$

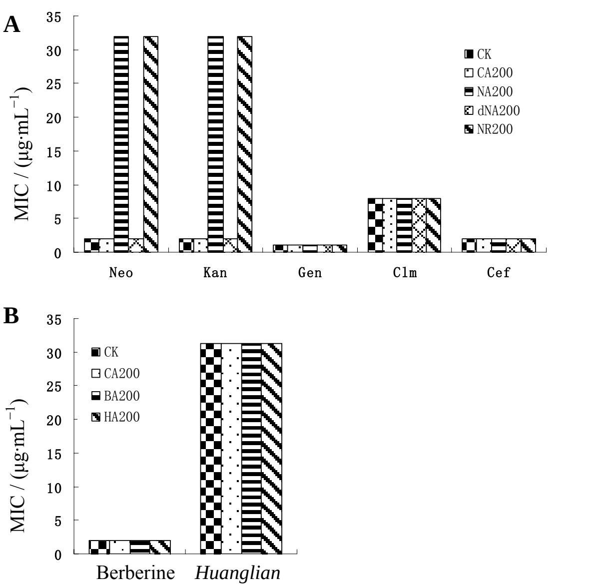

Fig. 2 MICs changes of NA200, BA200, and HA200

A: MICs changes of NA200 to Neomycin and other antibiotics

B: MICs changes of BA200, and HA200 to berberine and *Huanglian*

Table 3 Resistant cell proportions of *E. coli* ATCC31343 in different cultures ($\overline{X} \pm s$)

Cultures	Resistant cell proportion **
ATCC31343*	$3.6883 \pm 2.4561 \times 10^{-9}$ (n = 24)
CA100	$3.9017 \pm 0.5291 \times 10^{-8}$ (n = 5)
CA200	$7.0185 \pm 2.3524 \times 10^{-7}$ (n = 5)
NA100	$2.6833 \pm 0.9866 \times 10^{-6}$ (n = 3)
NA200	$1.1947 \pm 1.2129 \times 10^{-5}$ (n = 3)
NA250	$2.2376 \pm 1.3713 \times 10^{-5}$ (n = 3)

* Cultures of *E. coli* ATCC 31343 in MH medium at 37 ℃ for 6—24 h

** Resistant cell proportion was defined as the ratio of the resistant cells to the total viable cells

released from cells increased if cells were pretreated by berberine.

Surface morphology and structure changes of bacterial cells treated by berberine

The results in Fig. 3 indicated that K^+ and Ca^{2+} released from bacterial cells increased if cells were treated by berberine. It was expected to know whether bacterial cell surface morphology and structures changed after berberine treatment. The morphological changes were demonstrated using electric scanning microscope and detailed in Fig. 4. The photos showed that *E. coli* cells treated with berberine (Fig. 4D) had more rough surface than those control cells which treated by PBS (Fig. 4E); whereas for *B. subtilis* cells such changes were not so notable (Fig. 4A and Fig. 4B). Both *B. subtilis* and *E. coli* cells treated by polymyxin B could cracked into pieces, whereas treated by berberine could not.

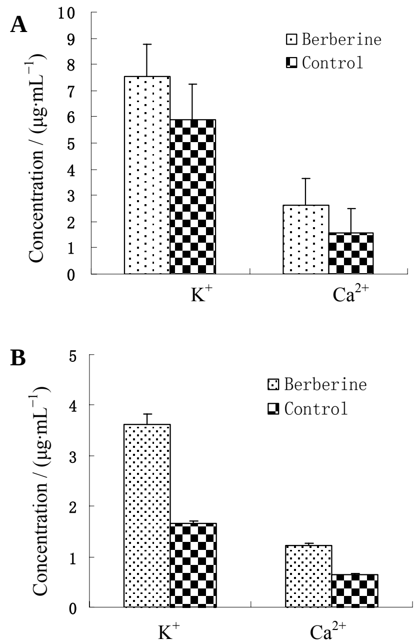

Fig. 3 Release of K^+ and Ca^{2+} from the bacterial cells treated with berberine ($\overline{X} \pm s$, n = 3)

A: 0.2 $mg \cdot mL^{-1}$ berberine (MIC) for *B. subtilis* cells

B: 2 $mg \cdot mL^{-1}$ berberine (MIC) for *E. coli* cells. Control was PBS instead of berberine

Binding kinetics of berberine to DNA, RNA, and protein, and the dis-binding characters of DNA-, RNA-, and protein-berberine complexes

In this study, we tested the binding kinetic curves of berberine to double helix DNA, single strand RNA, and protein BSA, and the details were shown in Fig. 5. The binding kinetic parameters were computed using Graphpad Prism 4 (GraphPad Software, Inc.) and shown in Table 4. The binding kinetic parameters of berberine to DNA and RNA were very similar, i.e., higher amount berberine binded to DNA or RNA than BSA.

It was very interesting that berberine binded to RNA and DNA tightly, but binded to protein loosely. The dis-binding percentage values in Table 4 exhibited that berberine could not easily dis-bind from DNA- and RNA-berberine complexes, but easily dis-bind from the protein-berberine complex.

Inhibition on the incorporation of biosynthesis precursors of ^{3}H-dT, ^{3}H-U, and ^{3}H-Tyr into macromolecular biosynthesis progress

Berberine effectively inhibited ^{3}H-dT, ^{3}H-U, and ^{3}H-Tyr as precursors in DNA, RNA, and protein macromolecular biosynthesis, and incorporated into *B. subtilis* cells. The results were shown in Fig. 6. These data indicated that berberine strongly and rapidly inhibited

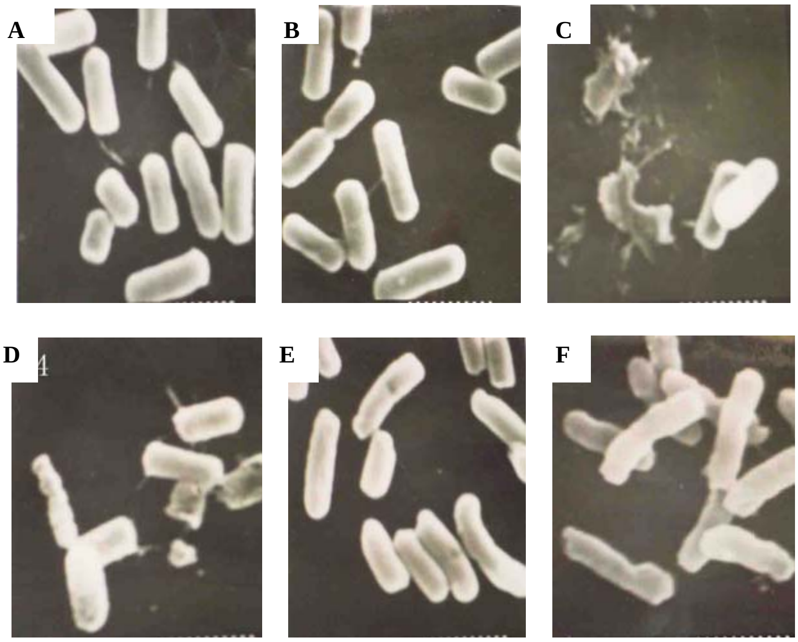

Fig. 4 Morphology and structure of bacterial cells under scanning electron microscope

A: *B. subtilis*, berberine (0.2 mg·mL^{-1}) B: *B. subtilis*, physiological saline C: *B. subtilis*, polymyxin B (20 U·mL^{-1}) D: *E. coli*, berberine (2 mg·mL^{-1}) E: *E. coli*, physiological saline F: *E. coli*, polymyxin B (20 U·mL^{-1}).

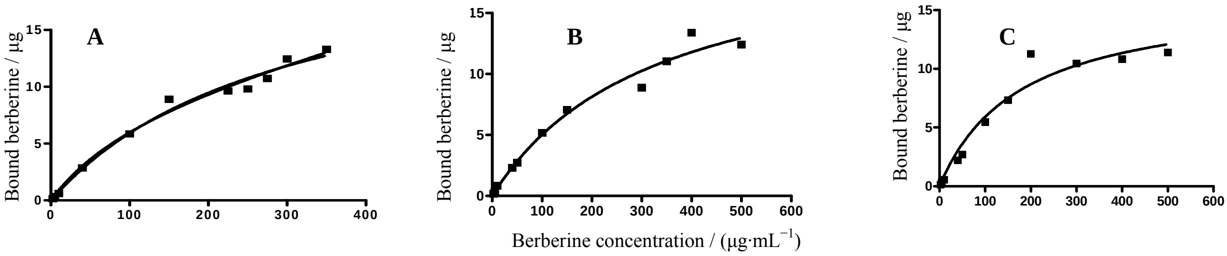

Fig. 5 Binding kinetic curves of berberine to DNA (A), RNA (B), and protein (C)

Table 4 Binding kinetic parameters of berberine to DNA, RNA, and protein

Parameters	DNA	RNA	Protein (BSA)
B_{max}	23.65 ± 3.508	21.47 ± 2.910	16.38 ± 2.077
KD	299.6 ± 81.16	328.2 ± 97.79	177.4 ± 53.99
R^2	0.9869	0.9803	0.9581
dis-binding percent /%	11.286 ± 1.364	9.813 ± 2.582	78.736 ± 13.427

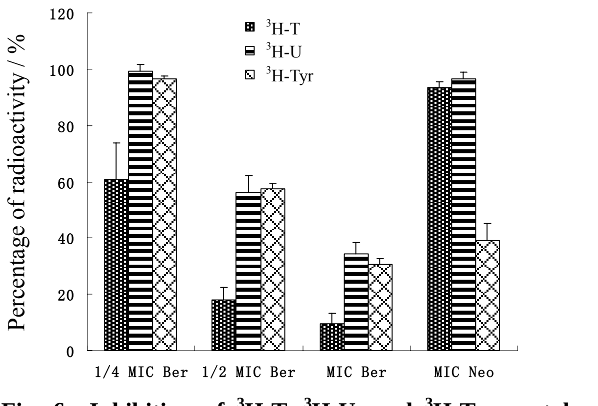

Fig. 6 Inhibition of ^{3}H-T, ^{3}H-U, and ^{3}H-Tyr up-taken into cells in *B. subtilis* ($\overline{X}$ ± *s*, *n* = 3)

^{3}H-dT up-taken into cells. Subsequently, berberine inhibited ^{3}H-U and ^{3}H-Tyr up-taken into cells, too. As control, Neomycin, an amido-indican antibiotic, inhibited ^{3}H-Tyr up-taken into cells only.

Discussion

Greater MICs of berberine and *Huanglian* on Gram-negative bacteria than Gram-positive ones

The data in Table 1 showed that there were higher MICs of berberine to Gram-negative bacteria than Gram-positive ones. These data were well agreed with the results by Kim *et al* (2004). They found that berberine chloride had MICs 50—400 mg/mL to Gram-positive bacteria and the tested Gram-negative bacteria strains had MICs over 400 mg/mL.

The reason for the apparent ineffectiveness of berberine to Gram-negative bacteria may be mainly due to their permeability barrier. Because disabling of the multi-drug resist system strongly increases the level of berberine penetration into the cells of Gram-negative bacteria (Tegos *et al*, 2002), berberine was penetrating cations and substrates of an MDR pump of *S. aureus* cells proved by Severina *et al* (2001).

Reason for higher MIC of NA200 to Neomycin than that of CA200, but similar MIC of BA200 to berberine

In order to know why the MIC of NA200 was higher than that of CA200, the resistant cell proportion was tested and shown in Table 3. These data indicated that the Neomycin resistant cell proportion of NA200 was about 3—4 orders of magnitude higher than that of the control CA200. This result gave an explanation that it was possibly due to that a few parts of resistant cells were introduced into the test tubes and gave a very high MIC of NA200 to Neomycin. This explanation was proved by the following experiments. When the initial number of cells which were introduced into Neomycin MIC test tubes reduced from the usual 10^5 to 10^3 CFU per tube, it mostly got the similar MICs of NA200 to that of CA200.

In order to get more evidence for this explanation, the MICs of NA200 to other antibiotics were also tested (Fig. 2A). The results indicated that bacteria cells in NA200 had similar MIC of Clm, Gen, and Cef to the control CA200, but higher MIC of Kanamycin than that of CA200. Because only Kanamycin had similar antibacterial mechanism to Neomycin, the resistant cells were likely to be resistant to both Neomycin and Kanamycin via cross-resistance pathway. Further, when the NA200 culturing mixture was cultured in fresh MH medium for another 200 generations (named dNA200), their MICs to Neomycin and Kanamycin decreased to the level of the control group CA200, while the MIC of NR200 (the Neomycin resistant cells growing in MH medium for 200 generations) did not decrease anymore. All these results together proved that the resistant cell proportion increased during the long time culturing in the medium containing 1/2 MIC Neomycin, and small amount of resistant cells were introduced into the MIC testing tubes thus resulted in higher MIC of NA200 to Neomycin.

The reason for MIC of BA200 to berberine was similar to that of control CA200; Our viewpoint was that: berberine has multi-antibacterial pathways including inhibition of DNA duplication, RNA transcription, and protein biosynthesis; interfering enzymes' activities; changing cell surface structures, *etc*. Any mutant included in one of these pathways would be lethal, so in all experiments it was hard for us to isolate resistant mutant bacteria strains which could grow in medium with berberine (the berberine concentration was more than four MICs).

Berberine inhibition on DNA duplication, RNA transcription, and protein biosynthesis

It was well known that berberine had multi-phenyl cycles and a positive electrical charge (Fig. 7). It was easy to be customarily regarded that the plane multi-phenyl cycles of berberine molecule could help it intercalate to the DNA or RNA molecules, and the positive electrical charge could also help berberine bind itself to any molecules with negative electrical charges, such as DNA, RNA, proteins, and others.

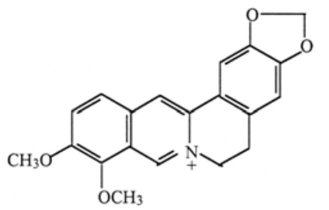

Fig. 7 Molecule structure of berberine

Several papers revealed that berberine could non-cooperatively bind to various form (double and triple) helical DNAs and RNAs (Bhadra *et al*, 2005; Chen *et al*, 2005; Das *et al*, 2003; Kumar *et al*, 2003; Maiti and Kumar, 2007). They suggested that the binding of berberine to tRNA appeared mostly by partial intercalation. They reported a surprising component of the non-electrostatic contribution to the binding of the charged berberine to tRNA (Islam *et al*, 2007).

Taking together the results of Table 4, they showed that berberine bind to DNA and RNA tightly. So if DNA and RNA molecules were binded by berberine, they may possibly change their structures (Bhadra *et al*, 2005), thus making the strand break of DNA and RNA become easy (Letasiová *et al*, 2006). These data suggested that the structure changes and/or strand damages of DNA and RNA may possibly not be normal template during DNA duplication, RNA transcription, and protein biosynthesis. The data in Fig. 6

revealed that amount of ^{3}H-dT absorbed by berberine-treated bacterial cells quickly and remarkably decreased, while ^{3}H-U and ^{3}H-Tyr decreased subsequently. These data agreed well with the viewpoint that DNA duplication (Letasiová *et al*, 2006; Sethi, 1983; Iizuka *et al*, 2000; Kuo, Chou, and Yung, 1995), RNA transcription (Fukuda *et al*, 1999; Choi *et al*, 2006; Chang, 1991), and protein biosynthesis (Tanabe *et al*, 2005; Lin *et al*, 1999) were inhibited by berberine.

Berberine inhibition or/and interfering enzyme activities

There were numerous researches focusing on the enzyme activity inhibition by berberine (Kapp and Whiteley, 1991; Gudima *et al*, 1994; Grippa *et al*, 1999; Ro, Lee, and Lee, 2001; Sriwilaijareon *et al*, 2002). The explanation for berberine inhibition enzymes activity may be: berberine could inhibit mRNA transcription and therefore inhibit enzyme protein biosynthesis. The binding kinetic result of berberine to protein in this paper gave another possible explanation: Enzymes activity of berberine inhibition may be due to berberine loosely binding to enzyme protein molecules and therefore immediately influencing the enzyme activities.

Berberine increased K^+ and Ca^{2+} released from bacterial cells

The data in Fig. 3 showed that higher ratio of K^+ than Ca^{2+} released from *E. coli* cells, but similar K^+ to Ca^{2+} released from *B. subtilis* cells. Comparing with *E. coli* and *B. subtilis*, higher ratio of K^+ released from *E. coli* cells than that from *B. subtilis* cells, but similar Ca^{2+} ratio released from *E. coli* to *B. subtilis* cells. The data in Fig. 4 showed that there were greater morphology or/and membrane changes in Gram-negative *E. coli* cells than that in Gram-positive *B. subtilis* cells. Whether there is any relationship between the released and the cell surface morphological changes needs further investigation.

Meyerson *et al* (2004) discovered that berberine exhibited the most effective inhibitory actions on cation-dependent ATP-phosphohydrolases *in vitro*. In most cases the Na^+, K^+-ATPase was more sensitive than Mg^{2+}-ATPase in inhibition by berberine. The correlation between the data of K^+ released more easily from berberine treated *E. coli* cells than Ca^{2+} (Fig. 3) and K^+-ATPase was more sensitive than Mg^{2+}-ATPase to inhibition by berberine also needs further investigation.

Conclusion

From all the results and discussion above, several conclusions can be drawn. Firstly, the antibacterial mechanisms of berberine included: 1) Berberine inhibition on DNA duplication, RNA transcription, and protein biosynthesis in bacterial cells could possibly be due to that berberine tightly binded to DNA and RNA, changed their structures or/and damaged their strands, and therefore made them not act as the normal templets in DNA duplication, RNA transcription, and protein biosynthesis progresses; 2) Enzyme activities maybe interfered by berberine because berberine could bind to proteins; 3) The amount of ions such as K^+ and Ca^{2+} leaked from cells could be increased if cells were treated by berberine, and partially due to the surface morphological changes of berberine-treated bacterial cells. Secondly, all the DNA duplication, RNA transcription, protein synthesis, and maintenance of the integrity of cell surface structure would be the most essential physiological functions for a live cell, so if a mutation influences one of these functions, the mutation would be lethal. Therefore, no mutant cells could survive and be selected. Thus, it is difficult to select berberine or *Huanglian* resistant cells, whereas it is easy to select antibiotic resistant cells. Thirdly, berberine and *Huanglian* have notable antibacterial activities, and bacterial cells do not or at least difficultly become resistant to them, so they will be widely used to help antibiotics control the diseases caused by bacteria. In addition, berberine and its related Chinese materia medica would provide a feasible way for controlling antibiotic resistance problems.

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