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RESEARCH ARTICLE

Effect of the microbial lipopeptide on tumor cell lines: apoptosis induced by disturbing the fatty acid composition of cell membrane

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ABSTRACT

Microbial lipopeptides play an important role in apoptosis induction of tumor cells. However, there is little knowledge about the relationship between apoptosis induction and membrane fatty acids. The present study focused on the effects of lipopeptides produced by *Bacillus subtilis* HSO121 on Bcap-37 cell lines. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl (MTT) colorimetric assay and surface tension measurements, showed that the critical micelle concentration (CMC) was a critical level for the inhibitory activity of lipopeptides on the growth of Bcap-37 cells. Under the CMC, the order of least to greatest cytotoxicity effect on cancer cell lines by lipopeptides is C_{13}-lipopeptide < C_{14}-lipopepitde < C_{15}-lipopeptide. Above CMC, all lipopeptides directly exert cytolytic activity. The flow cytometric analysis and Hoechst33258 staining experiments confirmed the apoptosis of Bcap-37 cell lines induced by lipopeptides in a dose-dependent manner. This apoptosis was associated with a significant decrease of the unsaturated degree of the cellular fatty acids of Bcap-37 cell lines due to the changes in the cellular fatty acids composition induced by the lipopeptide treatment. These results indicated that disturbance of the cellular fatty acid composition of breast cancer cell lines were related to in the cell apoptosis. Furthermore, significant difference in IC_{50} values of tumor cells and normal cell showed that the lipopeptide exerted selective cytotoxicity on the cancer cells. Thus HSO121 lipopeptides may have potential applications as an anticancer leads.

KEYWORDS *Bacillus subtilis*, lipopeptide, antitumor activity, membrane fatty acid, apoptosis

INTRODUCTION

Breast cancer has become a global concern (Singletary, 2008). Efforts in the field of chemotherapeutic treatments have led to the discovery of tamoxifen, cytotoxics paclitaxel and docetaxel, all of which show severe side effects (Marsh and McLeod, 2007). Furthermore, the emergence of resistant tumor cells calls for novel anticancer compounds with unique modes of action. These threats have prompted investigation into new targets for cancer action including: cellular efflux pumps, lipopolysaccharide synthesis, mRNA and membrane fatty acid synthesis (Menendez et al., 2005; Dai et al., 2008; Kuo, 2009). Cellular membranes constitute a particularly appealing target because resistance to membrane-active anticancer agents requires major changes in membrane structure, of which most structural elements are conserved (Vaara, 1992; Savage et al., 2002; Michel and Bakovic, 2007). Therefore, investigations on membrane-active natural products play an important role in dissecting fundamental biologic processes of tumor cells and, therefore, the further development of chemotherapeutic antitumor drugs.

Microbial lipopeptides are a class of amphiphilic molecules with fatty acid moieties covalently connected with peptide moieties (Bonmatin et al., 2003). Many of these molecules with membrane active properties play an important role in the apoptosis induction process of tumor cells. For example, surfactin, a cyclic lipopeptide produced by *Bacillus subtilis* (Arima et al., 1968), is well-known for its antitumor activity on Ehrlich carcinoma tumor cells and LoVo cells (Kameda et al., 1974; Kim et al., 2007). In addition, surfactin can cause membrane leakage through pore formation (Grau et al., 1999), ion channel formation (Sheppard et al., 1991), action as a cation carrier (Sheppard et al., 1991) and detergent like effects (Heerklotz and Seelig, 2001), all of which eventually

lead to cytotoxic effects. A recent study suggests that cyclic lipopeptides have anti-proliferative effect on K562 cells by inducing apoptosis (Wang et al., 2007). Characterization of lipopeptide structures has revealed surprising diversity and, along with the unique functional groups they possess, point to an encouraging area of anticancer research. However, how the membrane active properties of the lipopeptides contribute to the induced apoptosis of tumor cells is still unknown.

We have found a group of surfactin-like lipopeptides produced by *Bacillus subtilis* HSO121 (Liu et al., 2007). To explore the anticancer activity of these lipopeptides, we tested their cytotoxicity against Bcap-37 cell lines. To do this we used two approaches. The biologic approach included fluorescent staining and a flow cytometric assay. The chemical methods included measurements of the CMC (critical micelle concentration) and GC (gas chromatography) analysis of fatty acid composition of the cellular membrane. Furthermore, the effects of lipopeptides on other tumor, as well as normal, cells were also investigated. The results of this study help to further understand the potential functions of lipopeptides in inducing apoptosis in tumor cells and shed light on the potential applications of lipopeptides in the chemotherapy of breast cancer.

RESULTS

Effect of lipopeptide chain length on cytotoxic activity

To select the most effective lipopeptides to do the experiments, cytotoxic activities of lipopeptides with different lipid chain lengths were investigated. It showed that the IC_{50} values of C_{13}-lipopeptide, C_{14}-lipopeptide and C_{15}-lipopeptide on Bcap-37 cells were 60.81 ± 0.94 μg/mL, 41.26 ± 9.60 μg/mL and 29.7 ± 2.44 μg/mL, respectively (Fig. 1A).

At the concentration of 75 μg/mL, C_{15}-lipopeptide significantly reduced the cell viability to 5.0%, while C_{13}-lipopeptide and C_{14}-lipopeptide reduced the cell viability to 36.9% and 19.3%, respectively (Fig. 1B).

Effect of lipopeptide concentrations and treatment time on cytotoxic activity

C_{15}-lipopeptide was selected for further investigations. The surface tension of a PBS solution of C_{15}-lipopeptide was reduced from 70.1 mN/m to a constant value of 26.5 mN/m with the increase of lipopeptide concentration. The CMC of C_{15}-lipopeptide was determined to be 10.2 μg/mL (Fig. 2A). It showed that the inhibitory activity of the lipopeptide on tumor cells was correlated with its interfacial behavior, and the inhibitory concentrations were above the CMC (Fig. 2B).

The cytotoxic effects of lipopeptides on Bcap-37 cells (Fig. 2C) decreased with increasing treatment time when the concentration of the lipopeptide was less than 25 μg/mL. This phenomenon was especially obvious for the treatment time of 12 h and 48 h (Fig. 2D). At concentrations of 12.5 μg/mL and 25 μg/mL, the cytotoxicity of lipopeptides significantly decreased when the treatment time increased from 12 h to 48 h. However, at a lipopeptide concentration of 75 μg/mL, the cytotoxicity activity significantly increased with the treatment time (12 h to 48 h).

Observations of morphological changes in Bcap-37 cells

To study the effects of lipopeptides on the morphology of tumor cells, untreated and treated cells were analyzed by microscope. In the control group, the cells treated with vehicle (0.1% DMSO) aggregated in patches. The surface of each cell was convex and the boundaries of cells were well defined. Compared with the control cells, most cells treated with

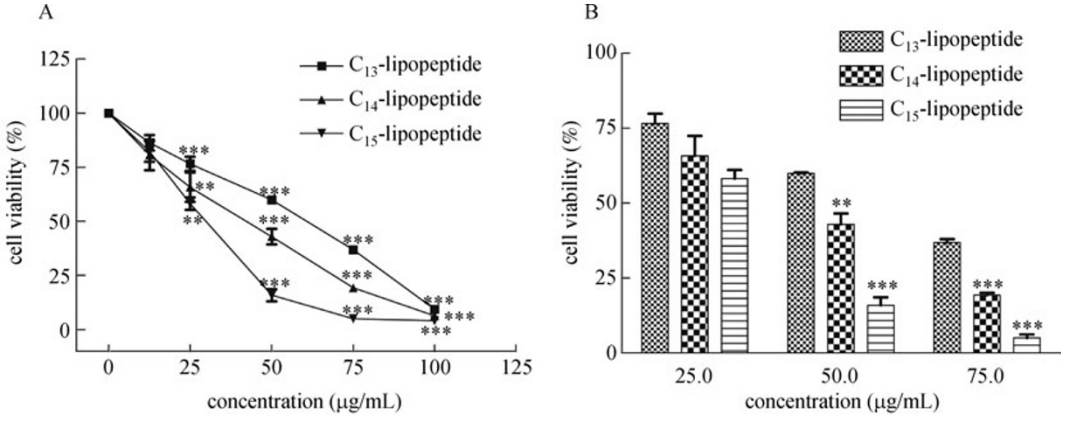

Figure 1. The dose-dependent inhibition of cell viability with various concentrations of the three peptides. (A) Cells treated lipopeptides for 24 h. (Significant differences from untreated control were indicated by ** $P < 0.01$; *** $P < 0.001$.) (B) Comparison of effects of lipopeptides with different chain lengths. (Significant differences from effects of C_{13}-lipopeptide were indicated by ** $P < 0.01$; *** $P < 0.001$.)

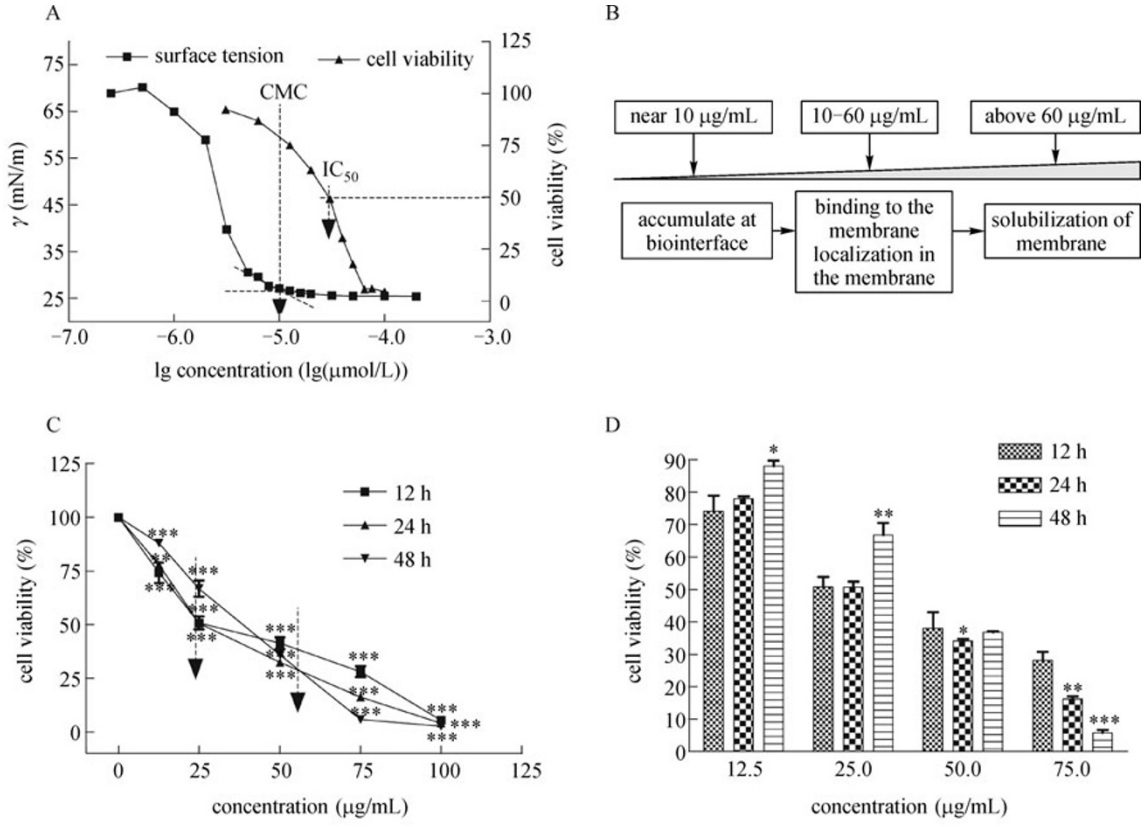

Figure 2. The relationship of surface tension and time on cell viability. (A) The relation between lipopeptide concentrations, surface tension and cell viability. (B) Deduced action modes of the lipopeptide on tumor cell membranes. The concentrations 10 (CMC), 10–60 and > 60 μg/mL represented less-inhibitory, inhibitory and killing concentrations, respectively. (C) Effects of treatment time on the cytotoxicity of lipopeptides (Significant differences from control: ** $P < 0.01$, *** $P < 0.001$.) (D) Effects of treatment time on the cell viability at different concentrations. (Significant differences from treatment time of 12 h: ** $P < 0.01$, *** $P < 0.001$.)

75 μg/mL lipopeptides were of a shrunken size and cell membranes were blebbing.

Staining with Hoechst 33258 showed fragmentation and condensation of chromatin in the cells treated for 24 h with 75 μg/mL lipopeptides (Fig. 3B). Control cells (Fig. 3A) which exhibited a normal nuclear morphology were characterized by a diffuse chromatin structure and therefore light staining.

Effects of lipopeptide on the population of Bcap-37 hypodiploid

To verify whether the lipopeptides induced apoptosis in Bcap-37 cells, flow cytometric analysis was performed to detect hypodiploid cells, which were identified as giving DNA fluorescence in the sub-diploid regions. Ten thousand cells in each sample were analyzed and the percentage of apoptotic cell accumulation in the sub-G1 peak was calculated. As shown in Fig. 4B–E, the sub-G0/1 population in lipopeptide-treated groups increased with the concentration of the lipopeptide. After a 24 h incubation with 20, 30, 40 and 60 μg/mL lipopeptides, the percentages of hypodiploid cells were 1.49 ± 0.93%, 2.05 ± 0.27%, 4.19 ± 0.53% and 18.31 ± 0.72%, respectively (Fig. 4F).

Lipopeptide induced changes in lipid composition of cell membranes

GC/MS analysis of the membrane fatty acids of Bcap-37 cells was performed to investigate the effects of lipopeptides on tumor cellular membranes. It showed that the total amount of fatty acids was significantly decreased in a dose-dependent manner after the tumor cells were treated with lipopeptides (Fig. 5). Compared to the amounts of fatty acids in the negative control group, the amounts of those in the positive control groups treated with 30 μg/mL and 60 μg/mL lipopeptides were decreased by 51.2% and 67.1%, respectively.

To explore the effects of lipopeptides on membrane disturbance, the lipid composition of cancer cell membranes

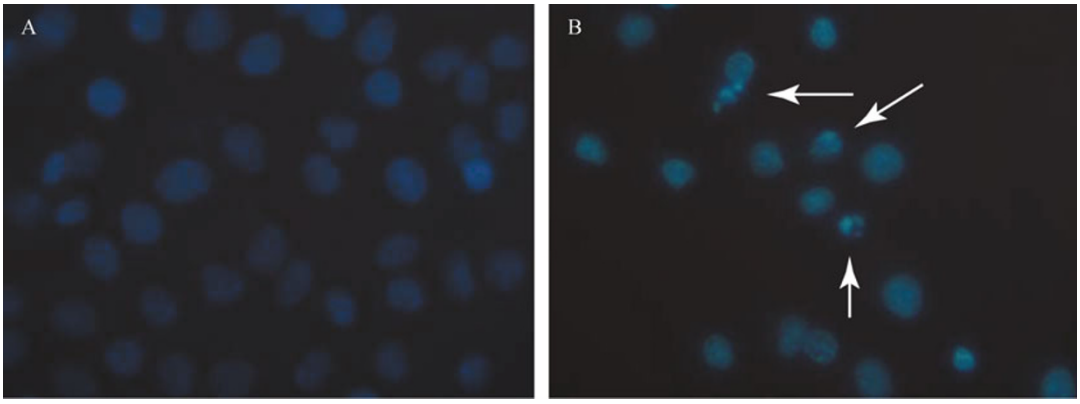

Figure 3. The fluorescent staining of nuclei in Bcap-37 cells by Hoechst 33258. Cells were treated with or without 75 μg/mL C_{15}-lipopeptide for 24 h. Cells with condensed and fragmented nuclei and apoptotic bodies (arrows) were observed in the lipopeptide-treated cells (B), but not in the cells with control treatment (A). Magnification: 400 ×.

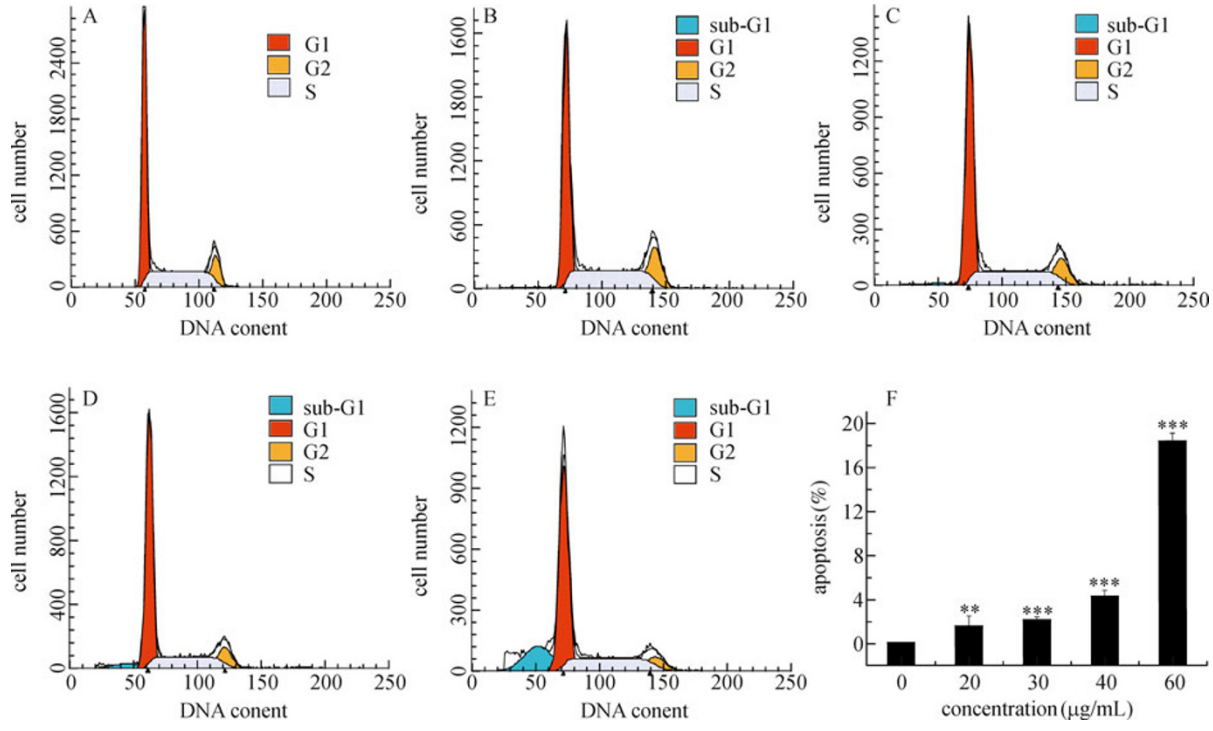

Figure 4. Analysis of the population of hypodiploid cells in Bcap-37 cells by flow cytometry. (A) Representative of the profiles of cell cycle distribution in three independent experiments per concentration was shown. Bcap-37 cells treated with 0 (A), 20 μg/mL (B), 30 μg/mL (C), 40 μg/mL (D) and 60 μg/mL (E) C_{15}-lipopeptide for 24 h. (F) The percentage of hypodiploid cells after staining with propidium iodide. (Significant differences from control: *** $P < 0.001$.)

was examined (Table 1). The fatty acids $C_{16:1}$, $C_{18:1}$ and $C_{20:4}$ showed a decrease of 56.6%, 13.6% and 45.2%, respectively, in cancer cell lines after being cultured for 24 h in the medium containing 60 μg/mL lipopeptides. The changes in lipid composition induced by the lipopeptide were probably consequences of membrane permeabilization due to the perturbing action of lipopeptides. In addition, the unsaturated degree (Geiser et al., 1994) of cellular fatty acids treated with the 30 μg/mL and 60 μg/mL lipopeptides was decreased from 29.3% to 28.3% and 23.4%, respectively, which indicated that the physiologic properties of membranes, such as fluidity and cellular functions, may be changed (Hagve, 1988).

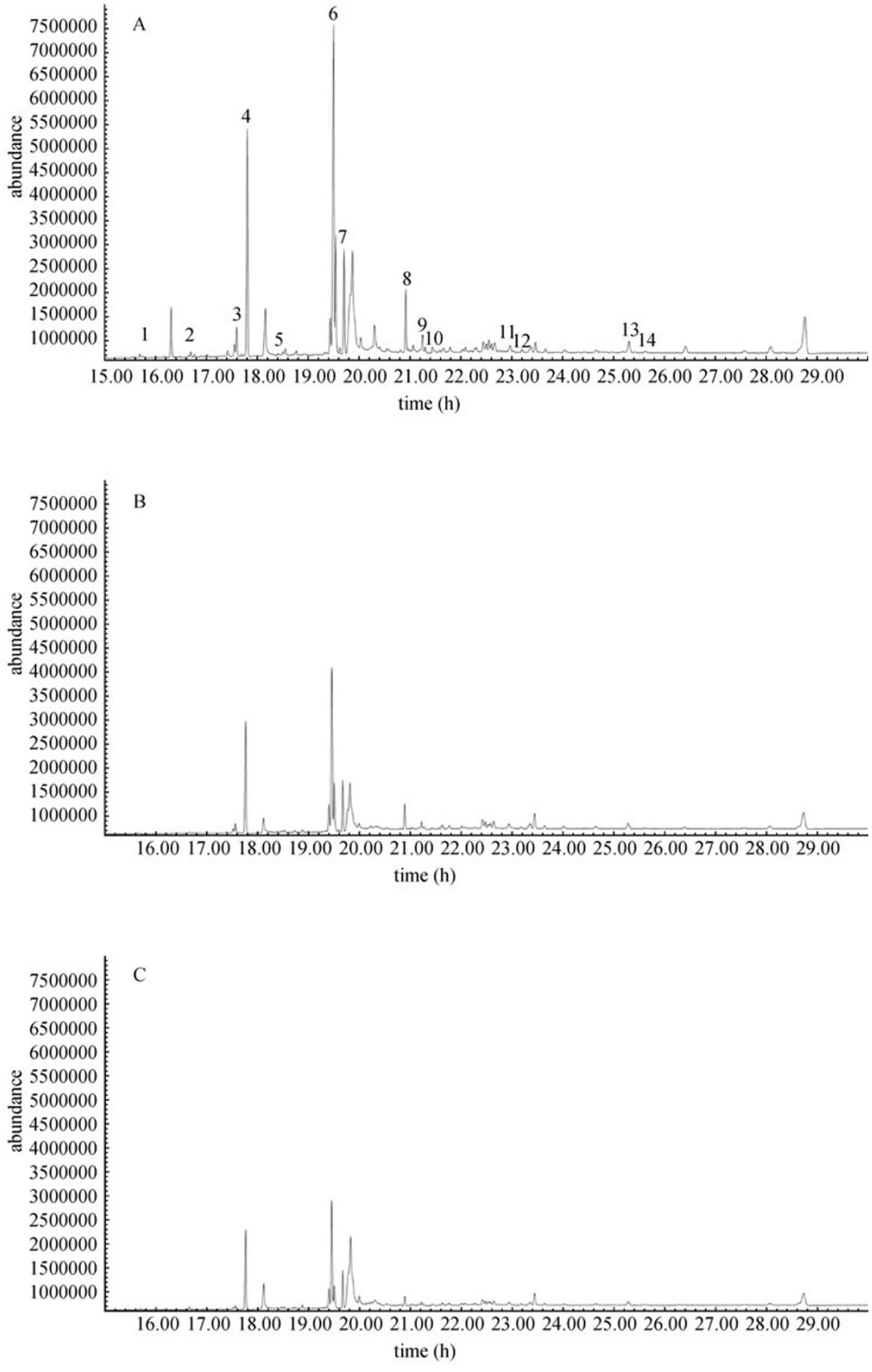

Figure 5. GC/MS analysis of fatty acid composition of membrane lipids of Bcap-37 cells. (A) Control cells; (B) and (C) Cells were treated with 30 μg/mL (B) and 60 μg/mL (C) lipopeptides for 24 h. Each group showed same peaks (peak 1–14 were shown in Table 1).

Table 1 Effects of C_{15}lipopeptide on fatty acid composition of membrane lipids

peak	Rt	fatty acid	percentage (%)		
			0	30 (μg/mL)	60 (μg/mL)
1	15.68	$C_{14:0}$	0.82 ± 0.029	0.58 ± 0.058[a]	0.45 ± 0.047[b]
2	16.76	$C_{15:0}$	0.58 ± 0.029	0.52 ± 0.033	0.38 ± 0.017[b]
3	17.58	$C_{16:1}$	2.21 ± 0.005	1.71 ± 0.052[b]	0.96 ± 0.015[b]
4	17.79	$C_{16:0}$	49.32 ± 0.623	48.96 ± 0.426	50.29 ± 0.305
5	18.40	$C_{17:0}$	0.36 ± 0.019	0.51 ± 0.024[b]	0.68 ± 0.010[b]
6	19.49	$C_{18:1}$	21.63 ± 0.443	21.27 ± 0.538	18.68 ± 0.386[b]
7	19.69	$C_{18:0}$	20.38 ± 0.062	21.52 ± 0.208[b]	23.68 ± 0.282[b]
8	20.91	$C_{20:4}$	0.84 ± 0.078	0.76 ± 0.070	0.46 ± 0.094[a]
9	21.24	$C_{20:1}$	0.86 ± 0.050	0.87 ± 0.178	0.57 ± 0.145
10	21.44	$C_{20:0}$	0.55 ± 0.035	0.65 ± 0.030	0.94 ± 0.096[a]
11	22.97	$C_{22:1}$	0.31 ± 0.007	0.42 ± 0.036[a]	0.38 ± 0.018[a]
12	23.19	$C_{22:0}$	0.72 ± 0.021	0.76 ± 0.102	1.00 ± 0.096[a]
13	25.30	$C_{24:1}$	0.89 ± 0.060	1.02 ± 0.105	0.91 ± 0.030
14	25.62	$C_{24:0}$	0.48 ± 0.046	0.47 ± 0.073	0.63 ± 0.092

[a] $P < 0.01$ versus control.
[b] $P < 0.001$ versus control.

Cytotoxic effects of the lipopeptide on cancer/normal cells

To explore the therapeutic potential of C_{15}-lipopeptide, it was important to investigate the differential toxicity between tumor cells and normal cells. As shown in Fig. 6, the IC_{50} values for cell lines B16, Bel-7402, HeLa, Bcap-37, KB-3-1 and Sw-1990 cells were 20.8 ± 1.6 μg/mL, 36.7 ± 12.4 μg/mL, 38.8 ± 4.7 μg/mL, 29.7 ± 2.4 μg/mL, 59.0 ± 2.7 μg/mL and 59.9 ± 1.7 μg/mL, respectively. These results showed that the cytotoxic activity of C_{15}-lipopeptide was cell line specific. Both Bcap-37 cells and B16 cells were very sensitive to the C_{15}-lipopetide. However, the IC_{50} value for Hacat cells was 100 μg/mL.

DISCUSSION

The interactions of surfactin-like lipopeptides with various cell lines have been studied by many researchers. The 50% cytotoxic concentrations of surfactin for the adhesive and nonadhesive cell lines were as follows: ML, 40 μmol/L; 293, 30 μmol/L; Hep2, 42 μmol/L; CV1, 50 μmol/L; Molt 4/8, 35 μmol/L; MT-4, 30 μmol/L; and H9, 43 μmol/ L (Vollenbroich et al., 1997). The IC_{50} of surfactin on LoVo cells was about 60 μmol/L after treated 24 h (Kim et al., 2007), and in addition, a new cyclic lipopeptide inhibited proliferation in K562 cells with an IC_{50} value approximately to 30 μg/mL (Wang et al., 2007). Combined with our present work, it can be concluded that surfactin-like lipopeptides at a concentration of 20–

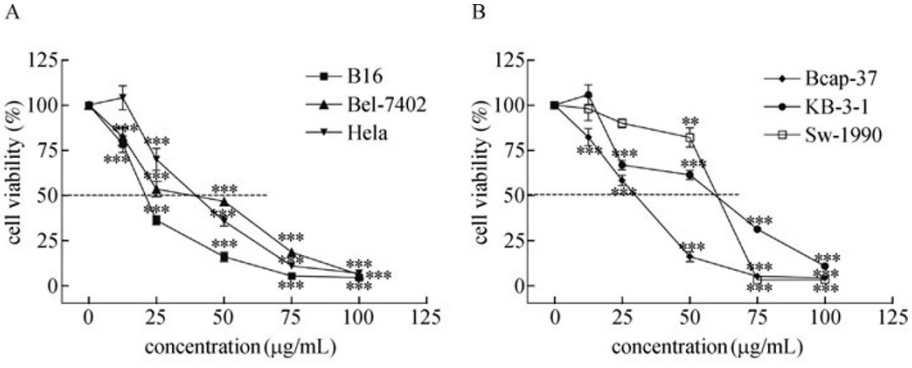

Figure 6. Cytotoxic effects of the lipopeptide. (A) B16 (■), Bel-7402 (▲) and HeLa (▼) cells, or (B) Bcap-37 (♦), KB-3-1 (●) and Sw-1990 (□) cells were treated for 24 h in the presence of the lipopeptide in medium. Cytotoxicity was then determined by MTT assay and was expressed as mean ± S.D. of three separate experiments ($n = 3$ each in the three experiments). (Significant differences from untreated control were indicated by ** $P < 0.01$; *** $P < 0.001$.)

60 μmol/L have potential cytotoxic effects on different tumor cells. For normal cells, the 50% cytotoxic concentration (CC_{50}) value of antimicrobial lipopeptides (surfactin and fengycin) for the Porcine Kidney (PK-15) cells was 32.87 μmol/L (Huang et al., 2006). The present study showed that the IC_{50} value for Hacat cells was 100 μg/mL, which was larger than those for other tested tumor cells. This confirmed a good toxic selectivity of the lipopeptide on tumor cells such as Bcap-37 cells.

Recently, surfactin has been reported to induce the apoptosis of LoVo (human colon carcinoma cell line) and MCF-7 (human breast cancer cell line) cancer cells, and the mechanisms of surfactin induced apoptosis on cancer cell lines have been investigated on the molecular level (Kim et al., 2007; Cao et al., 2009a, b, c, 2010). For example, the apoptosis of LoVo cells induced by surfactin was mediated by inhibiting extracellular-related protein kinase and phosphoinositide 3-kinase/akt activation, as assessed by phosphorylation levels (Kim et al., 2007). It was reported that the apoptosis effects of surfactin on MCF-7 cell lines were associated with caspase-3 (Cao et al., 2009b). It also revealed that surfactin induced accumulation of the tumor suppressor p53 and cyclin kinase inhibitor p21waf1/cip1, and inhibited the activity of the G2-specific kinase, cyclin B1/p34cdc2, and the elevation of $[Ca^{2+}]i$ may play an important role in the apoptosis of MCF-7 (Cao et al., 2009a). Surfactin also induces apoptosis of MCF-7 cell lines through a ROS/JNK-mediated mitochondrial/caspase pathway (Cao et al., 2010). Our results that lipopeptides inhibited the growth of Bcap-37 cells by inducing apoptosis were consistent with the findings of Kim (2007) and other authors (Wang et al., 2007). It is worthy to note that surfactin inhibits many enzymes or lipopolysaccharides that are located in the cellular membrane. At the same time, surfactin is a powerful biosurfactant that may accumulate at the interface and interact with cell membranes. In the present study, we therefore focused on the effects of surfactin on the cellular fatty acid composition of membranes to elucidate its apoptosis mechanisms. We hypothesized that lipopeptides exert their inhibitory effects by interaction with membrane or cellular fatty acids as lipopeptides can interact with membrane phospholipids and penetrate into the membrane in a concentration dependent relationship (Carrillo et al., 2003; Heerklotz and Seelig, 2007). Our experiments showed that the composition of cellular fatty acids was influenced by lipopeptides. This may be due to the disturbing effects of lipopeptides on the cell membranes, resulting in the observed change in lipid composition of tumor cells. Moreover, the unsaturated degree of fatty acids of Bcap-37 cell membrane lipids also decreased with increasing concentration of lipopeptides. It is well known that the unsaturation degree is associated with the phase transition and fluidity of membrane lipids (Howlett and Avery, 1997). It has also been reported that an increase in the relative proportion of saturated fatty acids in the membrane can make cancer cells more susceptible to cryodamage (Rakheja et al., 2005). These results lead to the conclusion that lipopeptides exert an inhibitory effect on tumor cells by disturbing the lipid composition, which sensitizes the tumor cells.

It has been shown that fatty acid composition of specific lipids may be important in the tumor cells that are resistant to killing agents (Schlager et al., 1978). Different tumor cells have different saturated cellular fatty acids, which may account for their differential sensitivities. For example, the unsaturated index of B16 (Ando et al., 2006), Bel-7402 (Qi et al., 2007), Hela (Geyer et al., 1962) and KB (Pelz et al., 2006) cells (Table 2) are 74.1%, 73.5%, 90.4% and 63.0%, respectively. Despite that fact that other lipids, such as glycolipds, are also included in the membranes of tumor cells, the different fatty acid composition of different tumor cells becomes a reasonable explanation of their different IC_{50} values.

Table 2 The fatty acid compositions of different tumor cells cultured in RPMI1640 medium

fatty acid	composition percentage (%)				
	B16	Bcap-37	Bel-7402	Hela	KB
$C_{14:0}$	4.2		3.4	1	2.8
$C_{15:0}$		0.58			
$C_{16:0}$	28.8	49.3	24	18	16.5
$C_{16:1}$	8.6	2.2		5	16.6
$C_{17:0}$		0.36			
$C_{18:0}$	10.1	20.4	28.9	12	
$C_{18:1}$	29.6	21.6	16.1	30	16.3
$C_{18:2}$	1.5		25.5	25	
$C_{20:0}$		0.55			
$C_{20:1}$		0.86			
$C_{20:4}$	6.2	0.84	1.5		
$C_{21:0}$				3	
$C_{22:0}$		0.72			
$C_{22:1}$		0.31			
$C_{24:0}$		0.48			
$C_{24:1}$		0.89			
UI (%)	74.1	26.7	73.5	90.4	63.0

In this paper, the cytotoxic activity of C_{15}-lipopeptide on Bcap-37 cells exerted mainly above (Fig. 2A). The CMC value of C_{15}-lipopeptide was similar to that of other homologs, such as C_{13}-lipopeptides and C_{14}-lipopeptides, so it could be possible that the cytotoxicity of these lipopeptides was related to a particular concentration. This correlation was in accordance with effects of lipopeptides on blood cells in our previous work (Liu et al., 2008). In addition, lipopeptides with different concentrations exerted different effects on tumor cell viability (Fig. 2C). At low concentration, the lipopeptides

exerted some cytotoxicity but this activity did not persist; the inhibited tumor cells were not killed and were able to proliferate, resulting in increasing cell viability with increased treatment time (Fig. 2C). However, at a high concentration (75 μg/mL), most tumor cells were killed by lipopeptides and did not proliferate with increased treatment time (Fig. 2C and 2D). This indicates there was a threshold concentration for maximum cytotoxicity activity. Furthermore, based on the CMC value, the lipopeptide was a good surface active compound, and we predicted that the surface activity of lipopeptide may be important in its interaction with tumor cell membranes or membrane binding proteins (Fig. 2B).

Our present results (Fig. 2) showed an important role of hydrophobic nature of lipopeptides in their antitumor activities. When the lipopeptide molecule penetrates into the outer sheet of the lipid bilayer, its fatty acid chain interacts with the acyl chains of the phospholipids while its headgroup has affinity to the polar heads of the lipids. It has been shown that surfactin penetration is promoted by longer lipopeptide chain length (Eeman et al., 2006). The data suggested that particularly high hydrophobic lipopeptides have an increased cytotoxic activity on cells (Deleu et al., 2003; Dufour et al., 2005). Furthermore, the significant difference of cytotoxicity in Fig. 2C revealed that the effects of hydrophobic nature at high concentrations were more important than those at low concentrations, which in turn suggested that micelles formed by lipopeptides were key factors in their antitumor activity.

Our conclusion was that lipopeptides exerted their cytotoxicity on tumor cells in a dose-dependent manner until a threshold was reached and the tumor cells no longer maintained the structure of their membrane, which resulted in cell apoptosis. It is worthy to note that in addition to the surface active properties of lipopeptides, the free carboxyl acid residues in structures of lipopeptides play an important role in its effects on the alkaline phosphatase (Bortolato et al., 1997). Moreover, the lipopeptide can change the conformation of plasminogen (Kikuchi and Hasumi, 2002). These activities may also contribute to the further interaction of lipopeptide with enzymes of tumor cell membranes. Whether the lipopeptides influence on fatty acid composition is correlated to their effects on the fatty acid synthase will be an interesting topic.

MATERIALS AND METHODS

Chemicals

Cyclic lipopeptides (MW1007, 1021, 1035, which were C_{13}-lipopeptide, C_{14}-lipopeptide and C_{15}-lipopeptide, respectively) were isolated from *Bacillus subtilis* HSO121 according to previous isolation and purification procedures (Liu et al., 2007, 2008; Haddad et al., 2008). The structures of the C_{13}-lipopeptide, C_{14}-lipopeptide and C_{15}-lipopeptide were shown in Fig. 7. The lipopeptides were first dissolved in dimethyl sulfoxide (DMSO) with a final DMSO concentration of 0.1% (*v*/*v*) for each sample. Previous experiments have shown that DMSO at this concentration did not influence cellular activities (Ye et al., 2004). The control cells were treated with vehicle alone, 0.1% (*v*/*v*). MTT was purchased from Bioseen Technology, Inc; Rosewell Park Memorial Institute (RPMI) 1640 medium was purchased from GIBCO BRL, Grand Island, NY, USA; Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Co., Ltd; Phosphate buffered saline (PBS, pH 7.4) was composed of: NaCl 8.0 g, KCl 0.2 g, $Na_2HPO_4 \cdot 12H_2O$ 3.49 g, KH_2PO_4 0.2 g, per liter of double distilled water.

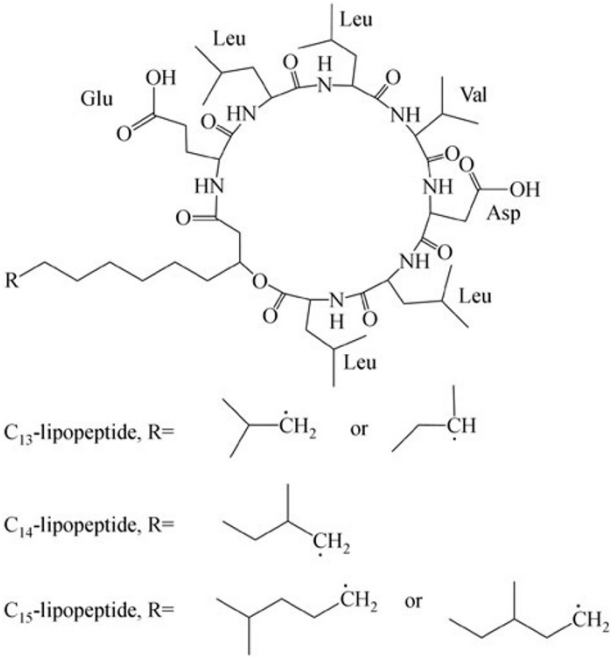

Figure 7. The chemical structures of lipopeptides.

Cell lines and culture conditions

The cancer cell lines included human oral epidermoid carcinoma cell line (KB-3-1), human breast cancer cell line (Bcap-37), human liver cancer cell line (Bel-7402), human pancreas cancer cell line (Sw-1990), human cervix uteri cell line (HeLa), and rat melanoma cell line (B16). The normal cell line was human keratinocytes cell line (HaCaT). All cells were purchased from the Chinese Type Culture Collection (Shanghai Institute of Cell Biology, Chinese Academy of Science, Shanghai, China). They were cultured in RPMI 1640 medium with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL). All cells were incubated at 37°C with 5% CO_2 in an air atmosphere.

Cytotoxicity assay

The MTT colorimetric assay was performed as described by Mosman (Mosmann, 1983). KB-3-1, Bcap-37, Bel-7402, SW-1990, HeLa and B16 cells were placed in 96-well culture plates (10^4 cells/well) and allowed to attach for 24 h before treatment. The cells were treated with lipopeptide ranging from 12.5 to 100 μg/mL or without the

lipopeptide (vehicle control, 0.1% DMSO). Each experiment was repeated in triplicate. The lipopeptide cytotoxicity activities on the cell lines were measured after 24 h of culture using the MTT assay. Absorbance in control and lipopeptide-treated wells was measured at 570 nm, with a reference wavelength of 655 nm, using an Automated Microplate Reader (Microplate Reader, 550 Bio-Rad, USA). The cytotoxicity of the lipopeptide was expressed as IC_{50} (inhibitory concentration of 50% cytotoxicity), which was extrapolated from linear regression analysis of the experimental data.

Surface tension measurement

The lipopeptides were dissolved in DMSO and then diluted using PBS. The surface tension was measured at 25.0°C with a DCA 315 series system (Thermo-Cahn Instruments, Inc. USA).

Fluorescent staining of the nuclei of Bcap-37 cells

Bcap-37 cells from exponentially growing cultures were seeded in 24-well culture plates and were allowed to attach for 24 h before treatment. The cells were treated for 24 h with 12.5, 25, 50, 75 and 100 μg/mL lipopeptides or with a vehicle control. After treatment, cells were washed two times with PBS, and were fixed with MeOH-HAc (3:1, *v*/*v*) for 10 min at 4°C. Cells were stained with Hoechst 33258 (5 μg/mL in PBS) for 10 min at room temperature and then examined in a Leica DMIRB fluorescent microscope at 356 nm.

Flow cytometric assay for Bcap-37 cells

Flow cytometric analysis of the Bcap-37 cell cycle was performed according to the method described previously (Walker et al., 1993). The cells were collected by centrifugation at 1000 rpm on a TGL-80-2B centrifuge (ShangHai Anting Scientific Instrument Factory) for 5 min and thoroughly rinsed with PBS. The pellets were re-suspended in ice-cold 70% ethanol and fixed at −20°C overnight (12 h). The fixed solution was washed three times with PBS and centrifuged (1000 rpm for 5 min). The cell pellets were re-suspended in 500 μL PBS containing 50 μL of 5 mg/mL RNaseA and stained with 10 μL of 5 mg/mL propidium iodide (PI), then incubated in a black box at room temperature for 30 min. Cells were analyzed using a FACScan (Becton Dickinson, USA) with Cell Fit software. Cells that have lost DNA due to the apoptosis will take up less stain and will appear to the left of the G1 peak (sub-G1) (Gorczyca et al., 1993; Gong et al., 1994; Elstein et al., 1995).

Fatty acid analysis by GC/MS

The fatty acid composition of Bcap-37 cell membranes was determined after the cells were treated for 24 h with 30 μg/mL or 60 μg/mL lipopeptide, or were a control. The digested and undigested cells were collected by centrifugation for 5 min at 2000 rpm on a TGL-80-2B centrifuge (ShangHai Anting Scientific Instrument Factory), and then washed three times in PBS. The total lipids were extracted from the cells using the procedure of Bligh and Dyer (Bligh and Dyer, 1959). The preparation of fatty acid methyl esters was divided into four steps: soaping with 3 mol/L NaOH and methanol (1:1, *v*/*v*) at 65°C for 30 min; esterification with H_2SO_4/MeOH (1:10, *v*/*v*) at 55°C for 6 h; extraction with ether; and evaporation under a stream of nitrogen at ambient temperature. The extracted fatty acid methyl ester was subjected to gas chromatography/mass spectrometry (instrument model 6890N-5975; Hewlett-Packard Co., Avondale, USA) with HP 3392A integrator using a HP-5MS capillary column (30.0 m × 0.25 mm × 0.25 μm nominal) packed with 5% phenyl methyl siloxane. One microliter of the extract (dissolved in 0.5 mL methanol) was injected for analysis by the 7683B series injector. The column oven temperature started at 80°C for 3 min, then reached 260°C at a rate of 10°C/min and was maintained at 260°C for 10 min. Fatty acid methyl esters were identified by searching the standard MS library.

Statistical analysis

The scientific statistic software *GraphPad Instat* version 3.06 was used to evaluate the significance of differences between groups. Each experimental value was expressed as the mean ± standard deviation (S.D.). Comparisons between groups were done using a one-way ANOVA followed by a Student-Newman-Keul's test, and the criterion of statistical significance was taken as $^{**}P < 0.01$ or $^{***}P < 0.001$.

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ABBREVIATIONS

CMC, critical micelle concentration; FBS, fetal bovine serum; GC, gas chromatography; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl

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