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Anticancer mechanisms of temporin-1CEa, an amphipathic α-helical antimicrobial peptide, in Bcap-37 human breast cancer cells

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ABSTRACT

Aims: Temporin-1CEa, a 17-residue antimicrobial peptide, is known to exert broad-spectrum anticancer activity that acts preferentially on cancer cells instead of normal cells. However, the mechanism of cancer cell death induced by temporin-1CEa is weakly understood.
Main methods: Here, we investigated the cytotoxic and membrane-disrupting effects of temporin-1CEa on human breast cancer cell line Bcap-37, using MTT assay, electronic microscope observation, fluorescence imaging and flow cytometry analysis.
Key findings: The MTT assay indicated that one-hour temporin-1CEa treatment led to rapid cell death in either caspase-dependent or -independent manner. The electronic microscope observation suggested that temporin-1CEa exposure resulted in profound morphological changes in Bcap-37 cells. The fluorescence imaging and flow cytometry analysis demonstrated that temporin-1CEa exhibited membrane-disrupting property characterized by induction of cell-surface phosphatidylserine exposure, elevation of plasma membrane permeability, and rapid transmembrane potential depolarization. Moreover, temporin-1CEa might also induce rapid cell death through mitochondria-involved mechanisms, including rapid intracellular Ca^{2+} leakage, collapse of mitochondrial membrane potential ($\Delta\varphi m$) and over-generation of reactive oxygen species (ROS).
Significance: In summary, the present study indicates that temporin-1CEa triggers a rapid cytotoxicity in Bcap-37 cells through membrane-destruction and intracellular mechanisms involving mitochondria. These intracellular mechanisms and direct membrane-destruction effect were evaluated helping to understand the detail action of antimicrobial peptides in mammalian cancer cells.

Introduction

A major limitation inherent to most conventional anticancer chemotherapeutic agents is their lack of tumor selectivity and therefore the deleterious side effects. Moreover, cancer cells can develop resistance to conventional chemotherapy agents by cellular changes through multimechanisms (Gatti and Zunino, 2005). These limitations associated with conventional chemotherapy have stimulated the search for new classes of anticancer drugs with new modes of action.

Antimicrobial peptides (AMPs) recently have received attention as alternative chemotherapeutic agents that overcome the limitations of current drugs. AMPs have several advantages over currently used oncolytic therapeutics, such as selective cytotoxicity for cancer cells, ability to bypass the multidrug-resistance mechanism, and additive effects in combination therapy (Papo and Shai, 2005). AMPs are expressed in many diverse species and have an important function in the host innate immunity to microbial pathogens (McPhee and Hancock, 2005; Zasloff, 2002). In addition to antimicrobial activity, some certain kinds of synthetic AMPs or natural AMPs, including cecropin B, magainins, melittin, tachyplesin, BMAP-28 and lactoferrin, have recently been shown to exert exciting potentials as a new class of anticancer agents. These AMPs exert rapid and selective cytotoxicity against malignant cells but show relatively lower cytotoxicity against untransformed proliferating cells (Eliassen et al., 2003; Furlong et al., 2006; Mader et al., 2005; Risso et al., 1998; Simmaco et al., 1990; Wang et al., 2012), thereby suggesting that these peptides may be administered in vivo with minimal nonspecific toxicity. The cytotoxic effect of these AMPs on microorganisms and neoplastic cells is commonly believed to be a function of the cationic nature and secondary structure of these peptides. Due to the net positive charge and amphipathic structure of AMPs, the direct interaction of these peptides with plasma membrane is a distinct mechanism from those conventional chemotherapeutic agents that are currently used in the treatment of human malignancies, thus preventing the development of side-effects and multiple drug resistance (Leuschner and Hansel, 2004; Mader and Hoskin, 2006).

Temporin-like peptides were initially identified in methanol extracts of the skins of the Asian frog *Rana erythraea* and the European hybrid frog *Rana esculenta* (Simmaco et al., 1990). In 1996, 10 structurally

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related peptides, endowed with antimicrobial properties, were discovered (Simmaco et al., 1996). These peptides were isolated from skin secretions of mild electrically stimulated specimens of the European red frog *Rana temporaria* and were designated as temporins from A to L. New members were identified in skin secretions of other ranid frogs of both North American and Eurasian origin, thereby enlarging the temporin family to more than 100 different isoforms (Mangoni, 2006; Mangoni and Shai, 2009). Previous research have reported that two temporin-like peptides, temporin-1DRa and temporins-ALa, exert potent anticancer activities (Conlon et al., 2007; Lu et al., 2006). Recently, temporin-1CEa, one novel 17-residue (FVDLKKIANIINSIFGK) AMP isolated from the skin secretions of the Chinese brown frog (*Rana chensinensis*), has been shown to exhibit a rapid cytotoxicity against human breast cancer cell lines (Shang et al., 2009; Wang et al., 2012). Moreover, temporin-1CEa has a lower hemolytic effect on human erythrocytes and has no cytotoxicity against normal human umbilical vein smooth muscle cells at concentrations that induce cancer cell death (Wang et al., 2012).

The anticancer mechanisms of temporin-1CEa against two human breast cancer cell lines, MCF-7 and MDA-MB-231, have been explored in our previous study, in which temporin-1CEa induces significant membrane disruptions, Ca^{2+}-releasing and ROS over production (Wang et al., 2013). In the present study, to clarify the cell line specificity of anticancer activity of temproin-1CEa, the anticancer activity of temporin-1CEa was further evaluated using Bcap-37, an ERα negative human breast cancer cell line that first established in China. The influences of temporin-1CEa on cell membrane and possible intracellular mechanisms of temporin-1CEa-induced cancer cell death were also investigated and discussed.

Materials and methods

Cell culture

The human breast cancer cell line, Bcap-37, was obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100-U/mL penicillin and 100-U/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO_2 (Wang et al., 2009).

Effects of caspase inhibitors on temporin-1CEa-induced Bcap-37 cell death

To clarify the possible involvement of capspase-related pathways in temporin-1CEa induced cancer cell death, caspase-3 inhibitor (Ac-DEVD-fmk), caspase-8 inhibitor (Ac-IETD-fmk), or caspase-9 inhibitor (Ac-LEHD-fmk) of 100 μM concentration were co-incubated with Bcap-37 cells for 60 min. Then cells were exposed to various concentrations of temporin-1CEa (20–40 μM) for 1 h. The cells' death and sensitivities' cell death to caspase inhibitors were evaluated using a 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

Morphological analysis using electronic microscope

Some AMPs can target nonpolar lipid cell membranes resulting in the target cells death. Therefore, in the present study, morphological changes of Bcap-37 cells after 1 h peptide treatment were examined by scanning electronic microscopy (SEM, KYKY-1000B) and transmission electron microscope (TEM, JEM-200EX) using standard protocols.

Assessment of cell-surface phosphatidylserine exposure and plasma membrane integrity using FITC-annexin V and propidium iodide (PI) staining

To evaluate the effects of temporin-1CEa on cell-surface phosphatidylserine (PS) exposure and plasma membrane integrity, Bcap-37 cells were seeded in a 96-well plate and incubated with various concentrations of temporin-1CEa (20–40 μM) or were left untreated (control) for 60 min. After treatment with peptides, the cells were stained with FITC-annexin V and PI according to manufacturer's instructions (FITC-Annexin V Apoptosis Detection Kit, BD Biosciences, USA). The cell-surface phosphatidylserine (PS) exposure and plasma membrane integrity were analyzed using FACSCanto flow cytometer (BD Biosciences). Cells that are considered viable are FITC-annexin V and PI negative (lower-left quadrant, Q3); cells with membrane lipid asymmetry and PS exposure are FITC-annexin V positive (lower-right quadrant, Q4); and cells with interrupted membrane integrity are both FITC-annexin V and PI positive (upper-right quadrant, Q2).

Cell membrane permeability assay using calcein AM and ethidium homodimer (EthD-1) staining

The cell membrane integrity and permeability were determined using the LIVE/DEAD® Viability/Cytotoxicity Assay Kit (Molecular Probes, Inc., USA), which is a two-color fluorescence assay with two probes that measure recognized parameters of cell viability, including intracellular esterase activity and plasma membrane integrity. An increased fluorescence intensity of EthD-1 or a decreased fluorescence intensity of calcein means enhanced membrane permeability and interrupted membrane integrity. Bcap-37 cells were seeded into 96-well plates at 5×10^4 cells/mL. After treatment with temporin-1CEa (20–40 μM) or were left untreated (control) for 60 min, the medium was removed, and 20 μL of dye containing 2 μM calcein AM and 4 μM EthD-1 was then added and incubated for 30 min in the dark. The fluorescence intensity was distinguished by FACS analysis with Ex485nm/Em530nm for calcein and Ex530nm/Em645nm for EthD-1.

FITC-labeled peptides uptake

To detect the possible dynamic processing of temporin1CEa through cell membranes, Bcap-37 cells were incubated with FITC-labeled temporin-1CEa (20–40 μM) for 5, 10 or 60 min. Myelin and other lipophilic areas on cell membrane were stained with the red-orange fluorescent tracker DiI (Molecular Probes, Inc., USA). The FITC-labeled peptides were traced and recorded at each time point using laser scanning confocal microscopy.

Transmembrane potential measurements

Since membrane depolarization has been implicated in the mode of action of AMPs, cell transmembrane potential depolarization was measured using the membrane potential sensitive dye, bis-(1,3-dibutylbarbituric acid) trimethin eoxonol [$DiBAC_{4(3)}$]. As previously described (Franco et al., 2006), after incubation with 2 μM $DiBAC_{4(3)}$ for 10 min at 37 °C, the cells were subjected to time scanning using a fluorescence spectrophotometer (Varioskan Flash, Thermo Scientific) with Ex488 nm/Em518 nm. When the fluorescence intensity was stable, the cells were treated with either temporin-1CEa or sterile-deionized water. Membrane depolarization was monitored by observing the changes in the intensity of fluorescence emission of the membrane potential dye $DiBAC_{4(3)}$.

Cytosolic calcium (Ca^{2+}) concentration determination

To detect cytosolic Ca^{2+} levels, the Ca^{2+}-specific fluorescent dye, Fluo3-AM, was loaded into Bcap-37 cells using a modified procedure adapted from the manufacturer (Beyotime, China). Briefly, cells were incubated for 30 min at 37 °C with 4 μM Fluo3-AM in Hank's buffered salt solution (HBSS) with or without 1.3 mM Ca^{2+}. The cells were then washed two times with fresh HBSS and incubated in HBSS at room temperature prior to detection. The Fluo3-AM green fluorescence (Ex488 nm/Em526 nm) was proportional to cytosolic Ca^{2+} levels. The changes of Fluo3-AM intensity in response to the peptides in cell populations were monitored using flow cytometry.

Measurement of mitochondrial membrane potential

To investigate the possible involvement of mitochondria in temporin-1CEa-induced cytotoxicity, the mitochondrial membrane potential was measured using rhodamine 123 (rh123). Briefly, the peptide-treated cells were incubated with rh123 (0.1 mg/mL) for 30 min at 37 °C, washed with FACS buffer, and resuspended in FACS buffer. The rh123 staining was determined by analysis on a FACSCanto flow cytometer.

Determination of reactive oxygen species (ROS) production

The intracellular accumulation of ROS was determined using a sensitive free-radical indicator, 2',7'-dichlorofluorescin-diacetate (DCFH-DA). The nonfluorescent 2',7'-dichlorofluorescin (DCFH) can be oxidized by ROS to form green fluorescent molecule, 2',7'-dichlorofluorescein (DCF). Temporin-1CEa-treated Bcap-37 cells were incubated with 25 μM DCFH-DA in darkness for 30 min. After incubation, cells were collected, washed with PBS, resuspended in PBS and then subjected to flow cytometry analysis. The group without temporin-1CEa addition was a negative control. Rosup-induced intracellular peroxide production was used as a positive control.

Results

Temporin-1CEa induces Bcap-37 cell death in either caspase-dependent or -independent manner

As shown in Fig. 1, one-hour treatment of Bcap-37 cells with temporin-1CEa (20–40 μM) induced rapid cell death in a concentration-dependent manner. Moreover, the cell death induced by 20 or 40 μM temporin-1CEa was failed to be ameliorated by three typical caspase inhibitors, Ac-DEVD-fmk (caspase-3 specific), Ac-IETD-fmk (caspase-8 specific) and Ac-LEHD-fmk (caspase-9 specific), whereas the caspase-3 specific inhibitor Ac-DEVD-fmk partially blocked the cytotoxic effect of 30 μM temporin-1CEa. These findings indicated that temporin-1CEa might exert an interesting dose-independent caspase-mediating mechanism.

Temporin-1CEa induces morphological changes of Bcap-37 cells

Morphological examination via SEM (Fig. 2A) or TEM (Fig. 2B) revealed that Bcap-37 cells cultured in the presence of various concentrations (20–40 μM) of temporin-1CEa for 60 min showed dramatic morphological changes. While untreated Bcap-37 cells showed an intact membrane and smooth surface, the cells exposed to 20 μM temporin-1CEa showed a minor disruption in the cell membrane. Additionally, temporin-1CEa at higher concentrations (30–40 μM) induced striking morphological changes in membranes of Bcap-37 cells. The cell membranes were shriveled and disrupted, which may in turn resulted in formation of ion-permeable channels or membrane pore, which lead to depolarization, irreversible cytolysis and final death of the target cells.

Temporin-1CEa induces phosphatidylserine exposure, plasma membrane integrity disruption in Bcap-37 cells

In the present study, the FACS analysis indicated that while the viable cells captured in Q3 quadrant were reduced from 99.2% (control) to 58.7% (20 μM), 35.6% (30 μM) and 16.4% (40 μM), the cells captured in Q2 or Q4 quadrant were increased in a dose-dependent manner (Fig. 3). These results indicated that temporin-1CEa could affect Bcap-37 cells by disrupting their membrane integrity (as shown by the PS exposure) and increasing membrane permeability (as indicated by uptake of PI into cells).

Temporin-1CEa induces enhancement of membrane permeability in Bcap-37 cells

Two-color fluorescence dyes, calcein AM and ethidium homodimer (EthD-1), were used to confirm the effect of temporin-1CEa on membrane permeability of Bcap-37 cells. Live cells have intracellular esterases that convert nonfluorescent, cell-permeable calcein AM to the intensely fluorescent green calcein, which is retained within the cells with intact membrane. EthD-1 is excluded by the intact plasma membrane of live cells. However, in membrane-disrupted or dead cells, EthD-1 enters into intracellular space of cells and produces bright-red fluorescence when bound to nucleic acids. As shown in Fig. 4, after one-hour exposure of temporin-1CEa, the fluorescence intensity of calcein was reduced (Fig. 4A); meanwhile, the fluorescence intensity of EthD-1 was increased in Bcap-37 cells (Fig. 4B). These results suggested that temporin-1CEa disrupted the cell membrane of BCAP-37 cells and led to an increase in membrane permeability.

Distributions of temporin-1CEa in Bcap-37 cells

To determine whether temporin-1CEa has the potential to penetrate or influx into Bcap-37 cells to trigger intracellular events,the peptide was labeled with FITC and co-cultured with Bcap-37 cells for 1 h. The dynamic changes of intracellular distributions of the FITC-labeled-temporin-1CEa were evaluated using laser scanning confocal microscopy. The

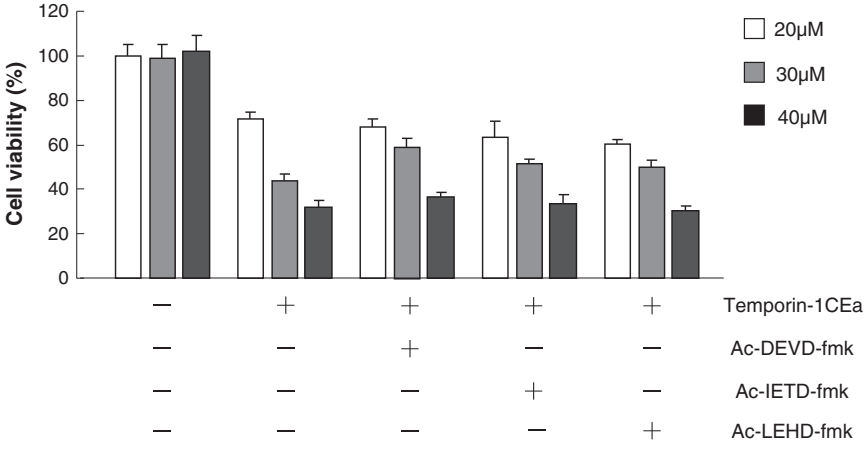


Fig. 1. Temporin-1CEa-induced Bcap-37 cell death and effects of caspase inhibitors. The cells treated with 20–40 μM temporin-1CEa were co-incubated with or without inhibitors of caspase 3, caspase 8 or caspase 9 (Ac-DEVD-fmk, Ac-IETD-fmk or Ac-LEHD-fmk, respectively) for 1 h. Cell viability was determined using the MTT method.

results indicated that the FITC-labeled peptides bound to cell membrane, and transferred through the cell membrane into the intracellular space, characterized by an increase of intracellular green fluorescence (Figs. 5A–C). The fluorescence imaging also indicated that at the lower concentration (20 μM), most of the peptides were located on cell membrane as shown by the green fluorescence. At 30–40 μM concentrations, temporin-1CEa disrupted the membrane integrity (as shown by intermittent red fluorescence from DiI on membrane) and caused uptake into cells (as shown by increased intracellular green fluorescence from FITC). The plasma membrane disruption during temporin-1CEa exposure is a progressive dose-response process with gradually increased permeability. After being exposed to peptides of lower concentration, the plasma membrane became permeable to only small molecules, including PI (668 Da) and EthD-1 (857 Da). However, after being exposed to peptides of higher concentrations, the membrane was disrupted to be permeable for FITC-labeled temporin-1CEa (about 2133 Da).

Transmembrane potential depolarization induced by temporin-1CEa in Bcap-37 cells

After one-hour temporin-1CEa treatment, Bcap-37 cells were incubated with the anionic and membrane-potential-sensitive dye $DiBAC_{4(3)}$. Depolarization of cell membranes leads to an uptake of $DiBAC_{4(3)}$ inside the cells, resulting in an increased fluorescent signal. As seen in Fig. 6, the exposure of temporin-1CEa to Bcap-37 cells led to an immediate and dramatic increase in the fluorescence intensity of $DiBAC_{4(3)}$, which was not observed in control cells.

Temporin-1CEa increases cytosolic calcium level in Bcap-37 cells

To clarify the possible involvement of Ca^{2+} in the peptide-induced membrane potential depolarization, the intracellular Ca^{2+} concentration was evaluated in either a Ca^{2+}-containing or Ca^{2+}-free situation. In the Ca^{2+}-containing situation, FACS analysis indicated that one-hour treatment of Bcap-37 cells with temporin-1CEa resulted in an increase of intracellular Ca^{2+} concentration (Figs. 7A–D). This upregulation of the Ca^{2+} content might be due to the influx of extracellular Ca^{2+}, and/or an endogenous Ca^{2+} release from the intracellular Ca^{2+} stores.

To further clarify whether temporin-1CEa treatment would cause an endogenous Ca^{2+} release from the intracellular stores, the cytosolic Ca^{2+} concentration was determined in a calcium-free situation. FACS analysis demonstrated that one-hour treatment of Bcap-37 cells with 20–30 μM temporin-1CEa caused a leakage of intracellular Ca^{2+} (Figs. 7E–G). However, the up-regulation of intracellular Ca^{2+} level was declined in cells treated with a higher dose of temporin-1CEa (40 μM) (Fig. 7H), which might be due to Ca^{2+} efflux induced by transmembrane

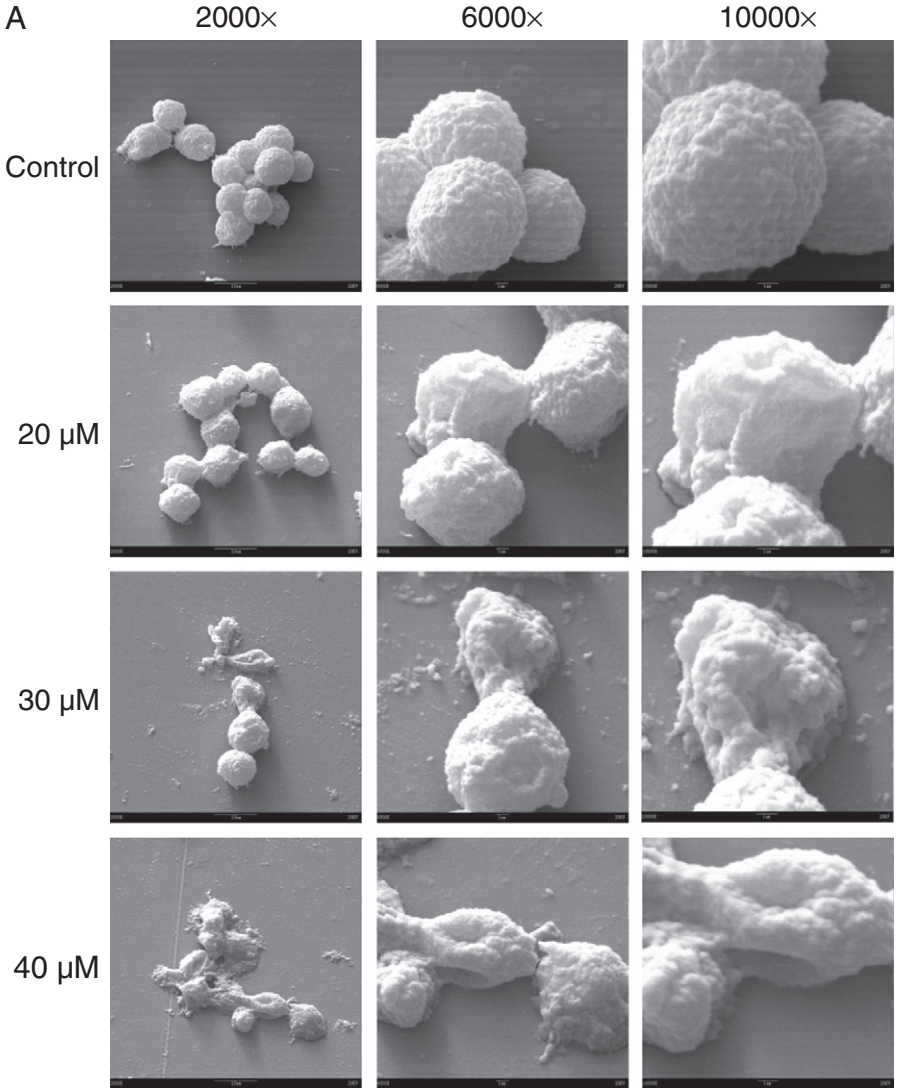


Fig. 2. Morphological changes of Bcap-37 cells upon exposure to temporin-1CEa for 60 min. (A) SEM evaluation of Bcap-37 cells treated with temporin-1CEa. (B) TEM evaluation of Bcap-37 cells treated with temporin-1CEa.

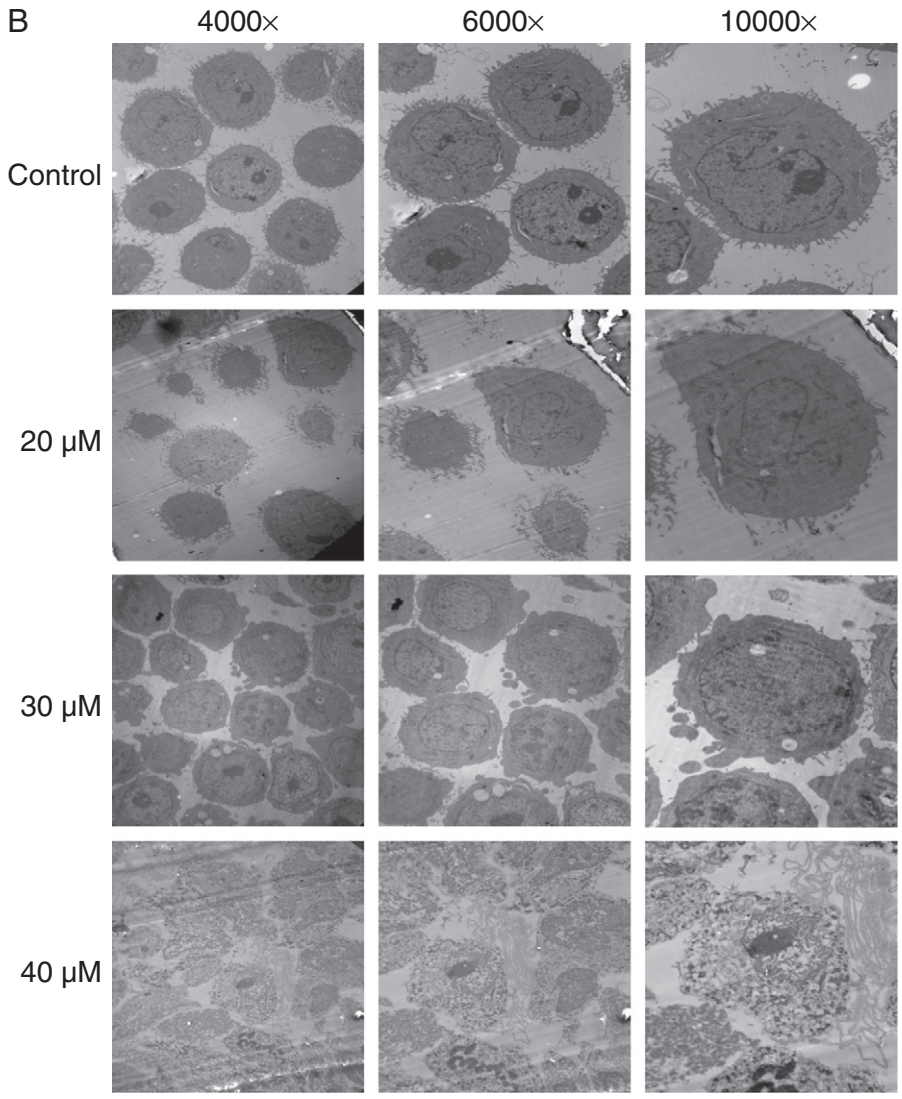


Fig. 2 (*continued*).

Ca^{2+} gradient or due to the seriously disrupted membrane structure during the late phage of peptides exposure. These results suggested that temporin-1CEa could induce an intracellular release of Ca^{2+} and that this effect was independent of extracellular Ca^{2+} concentration.

Temporin-1CEa disrupts the mitochondrial membrane potential (Δφm) in Bcap-37 cells

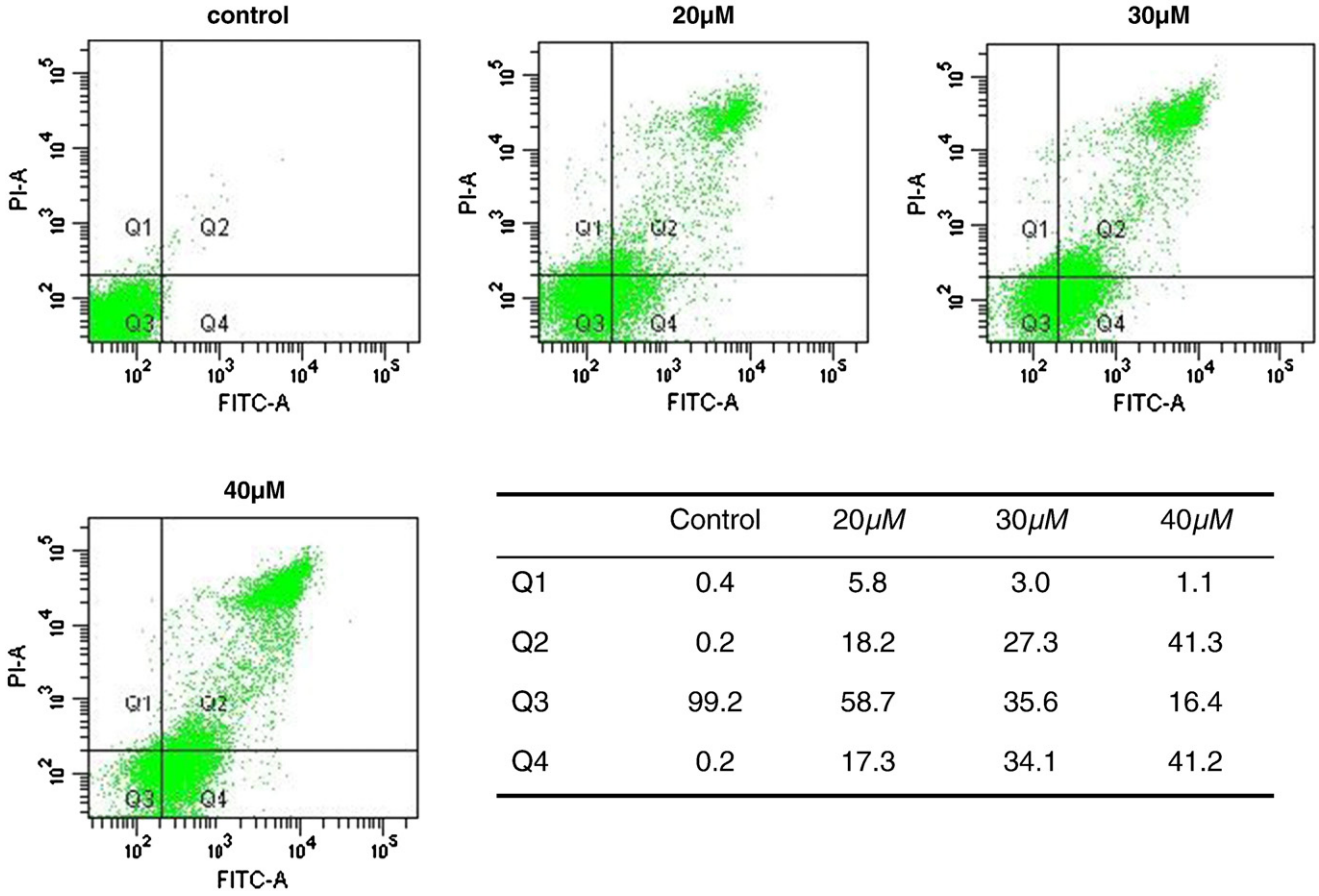
Temporin-1CEa of 30–40 μM disrupted the membrane integrity and uptake into cells. Given the negative charge of mitochondrial membranes, mitochondria are possibly the preferential intracellular structural targets for internalized temporin-1CEa. In the present study, temporin-1CEa-treated Bcap-37 cells experienced significant decreases of Δφm (Fig. 8). For temporin-1CEa of higher concentrations, we hypothesized that the internalized peptides and the peptides-induced intracellular Ca^{2+} overload would trigger an increase in the mitochondrial membrane permeability and cause impairment of mitochondrial structure and function. However, temporin-1CEa at 20 μM was excluded from Bcap-37 cells. Whether the collapse of mitochondrial membrane potential induced by lower concentrations of temporin-1CEa is a result of increased intracellular Ca^{2+} production or a secondary result from an attack on metabolic pathways, is not possible to determine based on these present experiments.

ROS generation in temporin-1CEa-treated BCAP-37 cells

Temporin-1CEa-induced intracellular ROS generation was evaluated using intracellular peroxide-dependent oxidation of DCFH-DA to form fluorescent DCF. DCF fluorescence was detected after cells were treated with 20–40 μM temporin-1CEa for 60 min. ROS production was significantly increased upon treatment with 20–40 μM temporin-1CEa compared with negative control (Fig. 9).

Discussion

Cancer treatment with conventional chemotherapy is hindered by toxic side effects and the frequent development of multidrug resistance by cancer cells. Recently, a growing number of studies have shown that some of the cationic AMPs, which are toxic to bacteria but not normal mammalian cells, exhibit a broad spectrum of cytotoxicity against cancer cells. These peptides might be a promising new class of natural-source anticancer candidates, which may avoid the shortcomings of



	Control	20μM	30μM	40μM
Q1	0.4	5.8	3.0	1.1
Q2	0.2	18.2	27.3	41.3
Q3	99.2	58.7	35.6	16.4
Q4	0.2	17.3	34.1	41.2

Fig. 3. Membrane integrity and phosphatidylserine exposure of temporin-1CEa-treated Bcap-37 cells. The cells were incubated with different concentrations of temporin-1CEa (0, 20, 30 and 40 μM) for 1 h and then were stained with Annexin-V-FITC/PI. Fluorescence intensity was determined using FACS analysis.

conventional chemotherapy due to their selective cytotoxicity against human cancer cells and an avoidance of multiple drug resistance (Hoskin and Ramamoorthy, 2008).

AMPs have been commonly considered to cause cancer cells to undergo rapid cell death through a direct cell membrane-damaging effect, but some AMPs can trigger cancer cell death through non-membranolytic intracellular actions (Ausbacher et al., 2012; Paredes-Gamero et al., 2012). For example, LfcinB, a cationic antimicrobial peptide isolated from cow's milk, disrupts cancer cell membranes, causing the loss of membrane integrity due to the formation of transmembrane pores that allow an uptake of the peptide into the cytoplasmic compartment of the cancer cell. The internalized LfcinB further colocalizes with the negatively charged mitochondria (Eliassen et al., 2006; Mader et al., 2007). Although LfcinB triggers mouse fibrosarcoma cells and human neuroblastoma cells to die primarily via necrosis through a cell membrane-lytic effect (Eliassen et al., 2006), LfcinB treatment of human leukemia and breast carcinoma cells results in cell death via an apoptotic process that involves the sequential generation of reactive oxygen species, the loss of mitochondrial

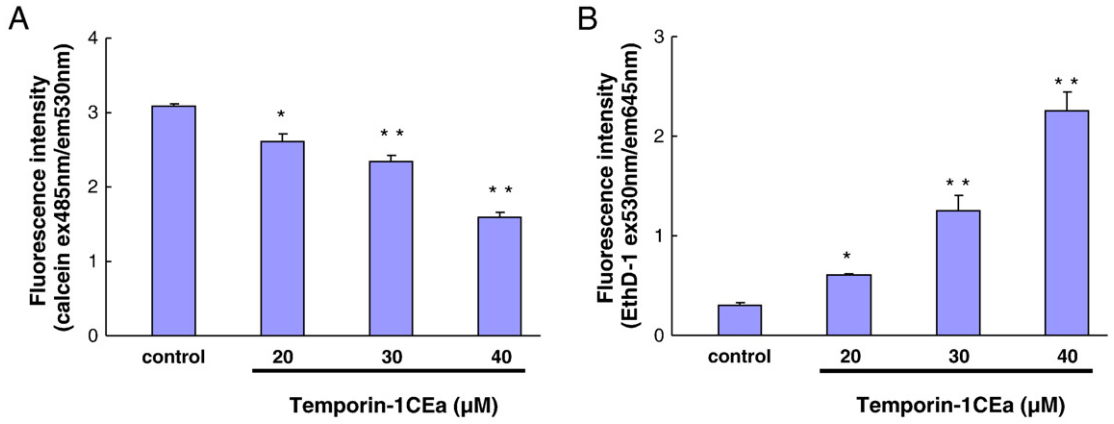


Fig. 4. Temporin-1CEa induces enhancement of membrane permeability in Bcap-37 cells using calcein AM/EthD-1 staining. Bcap-37 cells were seeded into 96-well plates at 5×10^4 cells/mL. After treatment with temporin-1CEa (20–40 μM) or were left untreated (control) for 60 min, the medium was removed, and 20 μl of dye containing 2 μM calcein AM and 4 μM EthD-1 was then added for 30 min in the dark. The fluorescence intensity was distinguished by FACS analysis.

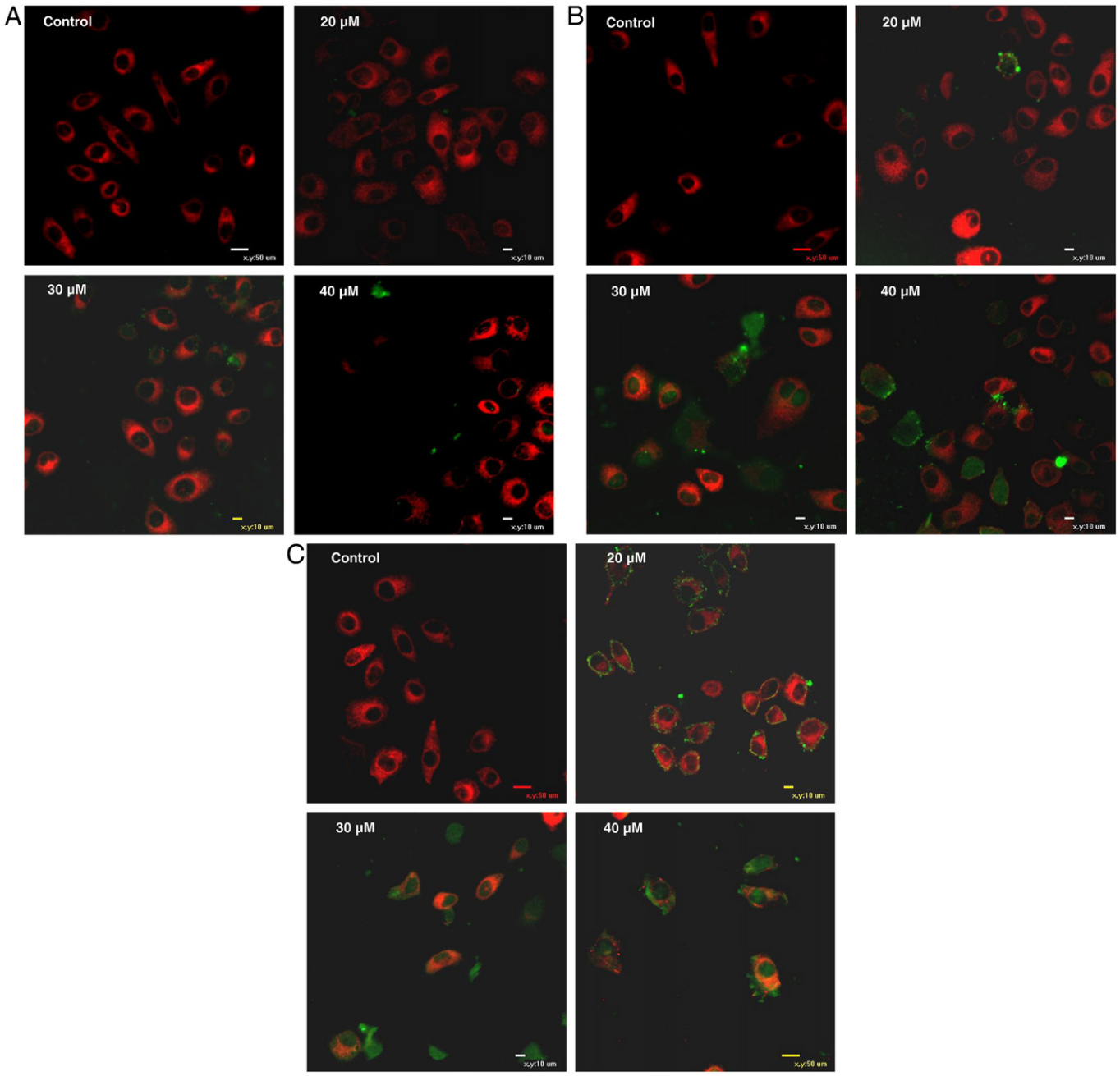


Fig. 5. Laser scanning confocal microscopy images of Bcap-37 cells treated with FITC-labeled temporin-1CEa for (A) 5 min, (B) 10 min, and (C) 60 min.

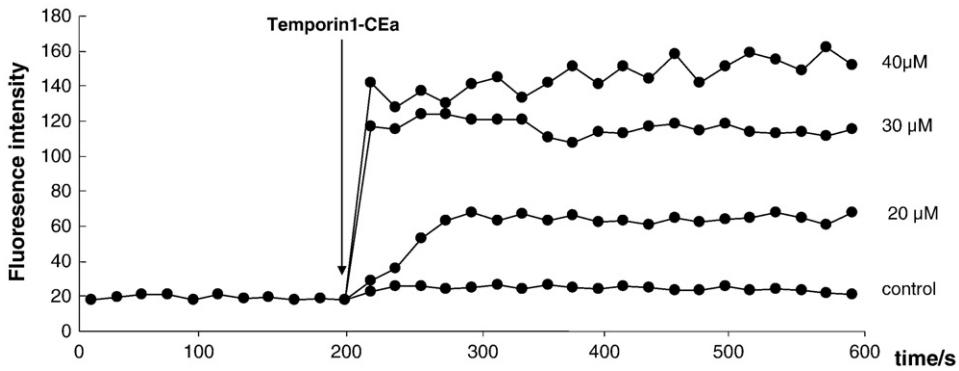


Fig. 6. Transmembrane potential of Bcap-37 cells after peptide treatment. The cell transmembrane potential depolarization experiments were performed using the anionic dye, $DiBAC_{4(3)}$.

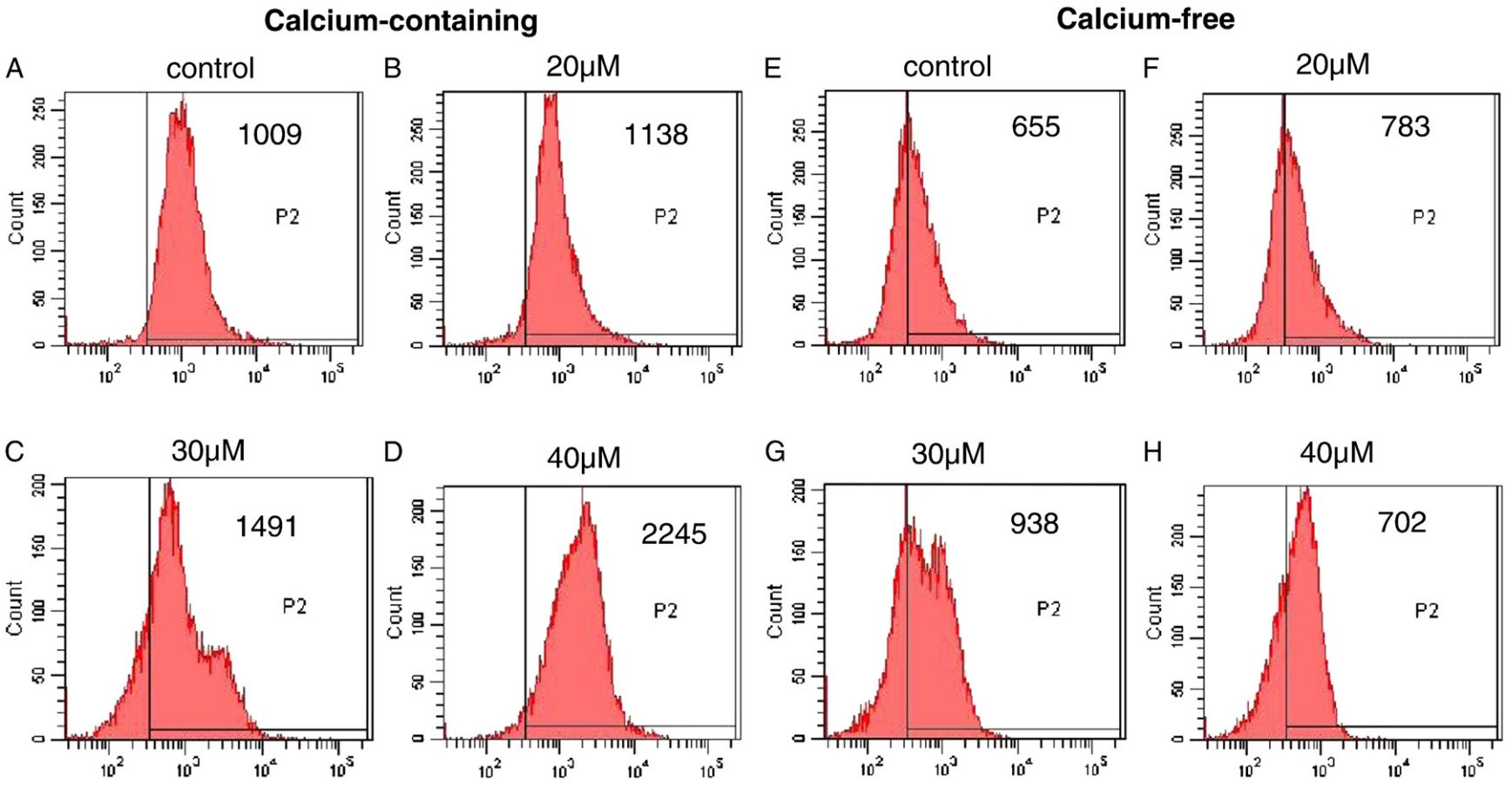


Fig. 7. Increase in the Cytosolic calcium concentration following temporin-1CEa treatment of Bcap-37 cells in either a calcium-containing solution (A–D) or calcium-free solution (E–H). Fluo3-AM green fluorescence was used to determine the cytosolic calcium levels.

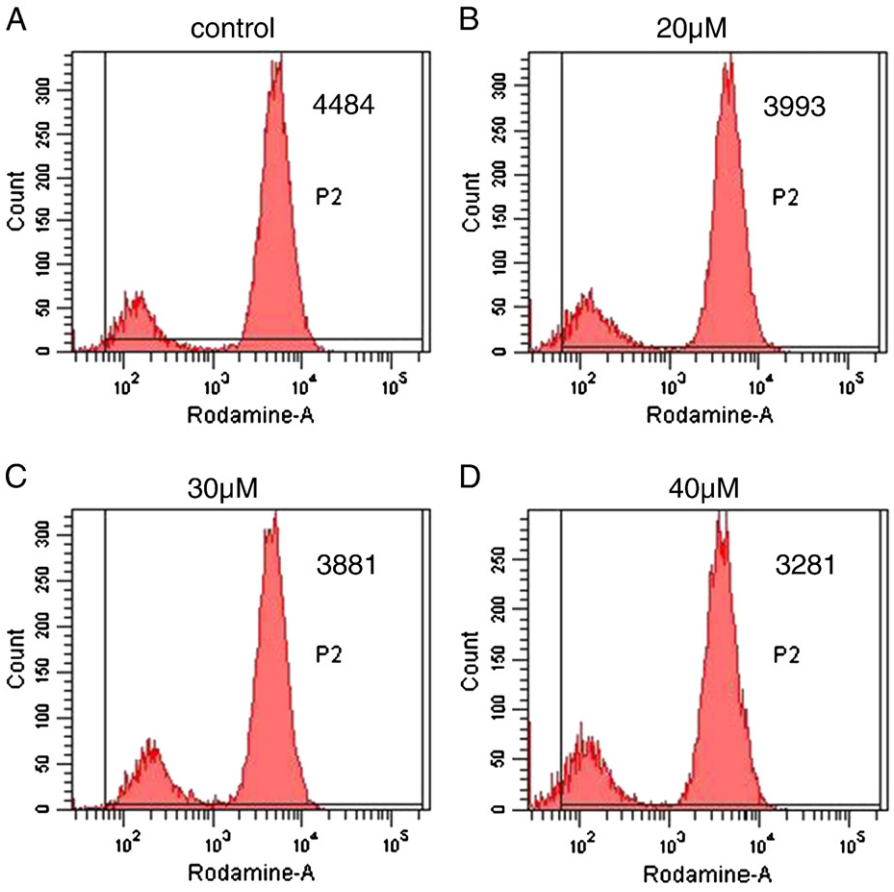


Fig. 8. Disruption of mitochondrial membrane potential in Bcap-37 cells after temporin-1CEa treatment. Mitochondrial membrane potential was measured using the cell-permeable fluorescent cationic dye, Rhodamine 123.

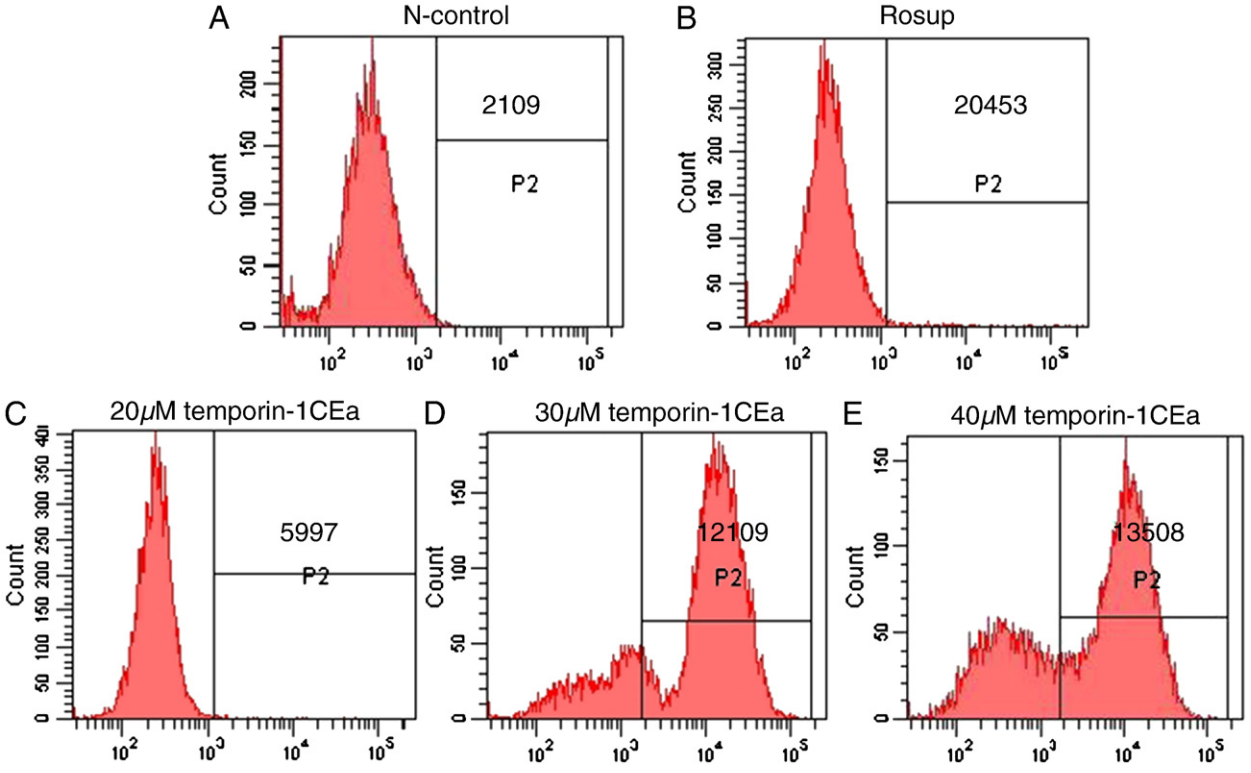


Fig. 9. Release of intracellular ROS in Bcap-37 cells after temporin-1CEa treatment. ROS production was measured by FACS analysis using a sensitive free-radical indicator, 2',7'-dichlorofluorescin-diacetate (DCFH-DA).

transmembrane potential, and the activation of the caspase cascade (Mader et al., 2005; Yoo et al., 1997). Our previous research has indicated that temporin1CEa, one novel antimicrobial peptide from the skin secretions of the Chinese brown frog (*R. chensinensis*), triggered a rapid cell death in MCF-7 and MDA-MB-231 cells through both plasma membrane-destruction and some specific intracellular mechanisms, including calcium-leakage, ROS overproduction and collapse of mitochondrial membrane potential (Wang et al., 2013).

In our present study, using Bcap-37 cell line, temporin1CEa showed anticancer properties similar to those in MCF-7 and MDA-MB-231 cells, which indicated non-specific anticancer mechanisms of temporin1CEa against various human breast cancer cell lines. The SEM and TEM observations indicated that treatment with temporin-1CEa resulted in striking morphological changes in Bcap-37 cells membrane. The enhanced green fluorescence on membrane of Bcap-37 cells treated with 20 μM FITC-labeled temporin-1CEa, as detected by fluorescence imaging, suggested that temporin-1CEa at lower concentrations mainly bound to the cell membrane. When the peptides cumulated on the membrane to reach the threshold concentration, the membrane-bound peptides would induce membrane lipid asymmetry, disrupt the membrane integrity and increase membrane permeability (as indicated by increased membrane PS exposure and PI/EthD-1 uptake). Moreover, although temporin-1CEa at 20 μM concentration was excluded from Bcap-37 cells, the peptides are still able to trigger intracellular events, including a slight increase of intracellular calcium concentration (mediated mainly by intracellular calcium leakage from stores), induction of mitochondrial membrane potential collapse and intracellular ROS accumulation.

The calcium-related mechanisms have been identified to be involved in cell death induced by some certain antimicrobial peptides. For example, the cationic peptide tritrpticin exerts ion channel-like activity in various types of planar lipid bilayers (Salay et al., 2004). Cecropins have the ability to form specific amphipathic α-helices, which allow them to target nonpolar lipid cell membranes. Upon membrane targeting, cecropins form ion-permeable channels subsequently resulting in cell depolarization, irreversible cytolysis and final cell death (Bechinger, 1997; Boman, 2003). Treatment with pexiganan causes a rapid rise in the amount of intracellular Ca^{2+} (Kulkarni et al., 2009), which is also consistent with our present study. Moreover, in the present study, the increased cytosolic Ca^{2+} concentration induced by temporin-1CEa exposure might be due to the endogenous Ca^{2+} released from intracellular stores and have pivotal roles in temporin-1CEa-induced Bcap-37 cell death, although the detailed molecular mechanisms involved await further investigation.

At higher concentrations (30–40 μM), temporin-1CEa might directly disrupt cell membranes to lysis. This membrane-disrupting effect can also result in PS exposure and even the release of cytoplasmic contents out of the cell, which ultimately leads to cell death. The extracellular or membrane-bound temperin-1CEa might also cause an influx of extracellular Ca^{2+} into the intracellular compartment or activation of the membrane Ca^{2+} channels, which led to a rapid increase of intracellular Ca^{2+} concentration and a significant transmembrane potential depolarization.

The disrupted cell membrane induced by higher concentrations of temporin-1CEa may also permit extracellular peptides uptake into the intracellular space (as shown by increased intracellular green fluorescence from FITC-labeled temporin-1CEa) to initiate cell death through intracellular mechanisms. Given the negative charge of mitochondrial membranes and their structural similarity with bacteria membrane, mitochondria are possibly the preferential intracellular structural targets for internalized temporin-1CEa, supported by previous studies indicated that AMPs disrupt mitochondrial potential and other mitochondrial functions (Helmerhorst et al., 1999; Paredes-Gamero et al., 2012; Segura et al., 2007). The internalized peptides may also trigger endogenous calcium leakage from the intracellular calcium stores, such as the mitochondria or endoplasmic reticulum. The cytosolic calcium released from the intracellular stores, together with Ca^{2+} influxed from extracellular space, resulted in calcium overload and triggered either necrotic or apoptotic cell death. The increased cytosolic calcium concentration may induce an opening of the mitochondrial permeability transition pore (PTP), thus triggers mitochondrial membrane permeabilization, the loss of $\Delta\varphi M$ and ROS overproduction, and final activation of cells death (Ly et al., 2003; Orrenius et al., 2003). Meanwhile, the increased membrane permeability or even the disruption of cell membranes induced by temporin-1CEa may also lead to a release of intracellular materials out of the cell, which can induce a rapid cell death and cause toxic inflammation to other cells (Kroemer et al., 1998; Ono et al., 2003; Vande Velde et al., 2000).

Moreover, previous research reports have indicated that lower concentrations of AMPs may promote apoptosis of K562 cancer cells through intracellular calcium mechanisms, free radicals and caspase-3 signaling pathway, while treatment with higher concentrations of AMPs primarily resulted in cancer cell death through membrane disruption (Paredes-Gamero et al., 2012). The results of our present study also showed a concentration-related variation of anticancer manner. Lower concentration of temporin-1CEa (20 μM) mainly bound to the cell membrane (as detected by fluorescence imaging) and showed a relative minor morphological change in membrane (as shown in SEM and TEM). However, higher concentrations of temporin-1CEa can influx or penetrated in to intracellular spaces (as indicated by the increased green fluorescence) and remarkable morphological changes (as indicated by SEM and TEM observation). Moreover, the caspase-dependence of target cell death induced by temporin-1CEa was variable under different concentration conditions. The cytotoxic effect of temporin-1CEa at 20 and 40 μM was caspase-independent, whereas the peptide at 30 μM induced cell death in a caspase-3-dependent manner. These clearly distinguished mechanisms of various dose conditions may help to understand the real action of AMPs in mammalian cells and provide potential theoretical supports for future anticancer drugs development.

Conclusion

In summary, temporin-1CEa induced Bcap-37 cell death through both directive membrane-destructive effect and intracellular mechanisms, including rapid intracellular Ca^{2+} leakage, collapse of mitochondrial membrane potential ($\Delta\varphi m$) and over-generation of reactive oxygen species (ROS). Further work is needed to elucidate the detailed molecular signaling pathways involved in Bcap-37 cell death induced by temporin-1CEa, such as the release of cytochrome C, the formations of mitochondrial PTP and the dynamic changes in the intracellular calcium concentrations after peptide treatment. Moreover, additional pharmacological studies are needed to examine the possible inhibitory effects of calcium-channel blockers on temporin-1CEa induced cell death.

Conflict of interest

The authors declare that they have no conflict of interest.

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