Melanoma cell surface-expressed phosphatidylinerse as a therapeutic target for cationic anticancer peptide, temporin-1CEa

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Abstract
We have previously reported that temporin-1CEa, a cationic antimicrobial peptide, exerts preferential cytotoxicity toward cancer cells. However, the exact molecular mechanism for this cancer-selectivity is still largely unknown. Here, we found that the negatively charged phosphatidylinerse (PS) expressed on cancer cell surface serves as a target for temporin-1CEa. Our results indicate that human A375 melanoma cells express 50-fold more PS than non-cancerous HaCaT cells. The expression of cell surface PS in various cancer cell lines closely correlated with their ability to be recognized, bound and killed by temporin-1CEa. Additionally, the cytotoxicity of temporin-1CEa against A375 cells can be ameliorated by annexin V, which binds to cell surface PS with high affinity. Moreover, the data of isothermal titration calorimetry assay further confirmed a direct binding of temporin-1CEa to PS, at a ratio of 1:5 (temporin-1CEa:PS). Interestingly, the circular dichroism spectra analysis using artificial biomembrane revealed that PS not only provides electrostatic attractive sites for temporin-1CEa but also confers the membrane-bound temporin-1CEa to form α-helical structure, therefore, enhances the affinity and membrane disrupting ability of temporin-1CEa. In summary, these findings suggested that the melanoma cells expressed PS may serve as a promising target for temporin-1CEa or other cationic anticancer peptides.

Keywords
Cancer specificity, cationic antimicrobial peptide, melanoma, phosphatidylserine, temporin-1CEa

Introduction
Malignant melanoma, the deadliest form of skin cancer, is a malignant neoplasm that arises from melanocytes (or cells that derive from melanocytes). Although melanoma was once considered uncommon, the annual incidence has increased dramatically over the past few decades and now is the fifth most common cancer for males and seventh most common for females in the United States [1]. Malignant melanoma at its early stage can be effectively treated by surgical resection, whereas advanced melanoma usually do not benefit from conventional surgery, chemotherapy or radiotherapy treatment due to its highly metastatic and chemoresistant nature [2]. Moreover, short-term angiogenesis inhibitor treatment even accelerates melanoma metastasis [3]. Therefore, the development of more effective therapeutic strategies with high melanoma specificity is currently needed.

One major difference between cancerous and non-cancerous cells is the enhanced exposure of negatively charged phosphatidylinerse (PS) on the outer leaflet of cancer cell membrane. As for melanoma, the previous research results have indicated that human melanoma cells express a higher level of PS [4] and are more susceptible than other cancer cell lines to the cytotoxicity induced by activated monocytes [5]. Consistently, one recent study has also revealed that mouse B16F10, a highly metastatic melanoma cell line, produces large quantities of PS-containing microvesicles, which favors the establishment of melanoma metastasis [6]. In addition, another recent study has revealed that human host defense peptides induce melanoma cells death through interactions with the negatively charged membrane PS [7]. Therefore, the high levels of PS content on melanoma cells surface may serve as a potential target for melanoma therapy.

Many cationic antimicrobial peptides (AMPs) and their derivatives have recently been shown to have a preferential cytotoxicity toward cancer cells [8–10], including melanoma cells [4,7,11,12]. Our previous studies have found that temporin-1CEa, a 17-residue cationic AMP (FVDLKKIANIINSIFGK) isolated from the skin secretions of Chinese brown frog (Rana chensinensis), exerts potent cytotoxicity against a wide variety of human cancer cell lines via cellular membrane disruption, but display weak cytotoxicity toward normal non-cancerous cells [13–15]. However, the preferential cytotoxic effects of temporin-1CEa toward melanoma cells and the exact mechanisms of cell surface PS on this conferred susceptibility still remain largely unknown.

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Therefore, the aim of this study was to determine the preferential cytotoxicity of temporin-1CEa against human melanoma A375 cells in vitro. The contributions of cell-surface PS to temporin-1CEa-induced cytotoxicity on A375 melanoma cells were further evaluated.

Materials and methods

Cell culture

Seven different cancer cell lines, including A375 human melanoma, B16 mouse melanoma, MCF-7 human breast carcinoma, BEL-7402 human hepatocellular carcinoma, A-549 human lung adenocarcinoma, HK-2 human squamous cell carcinoma, HT-29 human colon carcinoma, were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were routinely cultured in RPMI 1640 (Gibco BRL, Gaithersburg, MD) or Dulbecco’s modified Eagle’s medium (DMEM, Gibco BRL) containing 10% fetal bovine serum (FBS), 1% l-glutamine, 1% sodium pyruvate, 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco BRL). Normal human HaCaT keratinocytes (obtained from KeyGen Biotech, Nanjing, China) were grown in DMEM containing 10% FBS and antibiotics. Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO2.

Cytotoxicity detection by MTT assay and LDH leakage assay

The cells were seeded in 96-well plates at a density of approximately 5 × 10^3 cells/well, and were incubated for 24 h before treatment of various concentrations of temporin-1CEa. After peptides treatment, the cytotoxicity of temporin-1CEa on either cancer cells or HaCaT cells was determined using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay or lactate dehydrogenase (LDH) leakage assay according to the manufacturers’ protocol. Experiments were run in triplicate. On the basis of the MTT assay, IC_{50} values were calculated using GraphPad Prism version 5.02 (GraphPad Software Inc., San Diego, CA).

Membrane PS content determination using Annexin V-FITC staining

The cells were seeded in 24-well tissue culture plates at a density of approximately 2.5 × 10^5 cells/well. The cells were harvested at indicated time points and put on ice before fluorescence-activated cell sorting (FACS) analysis. The basal levels of membrane PS of various cell lines (either cancer cells or HaCaT cells) were monitored by annexin V-fluorescein isothiocyanate (FITC) staining (excitation 494/ emission 518) using FACS analysis.

Secondary structure analysis by circular dichroism spectroscopy

The circular dichroism (CD) spectra of peptides were recorded using CD spectrophotometer (J-810, Jasco, Victoria, BC, Canada) under nitrogen flush in 1 mm path length cell at 25 °C. The analysis was performed under the indicated conditions [50% trifluoroethanol (v/v), water and 0.8 mm liposomes]. The liposomes were composed of phosphatidylcholine (PC), PS and cholesterol (CHO) with PC/PS/CHO ratio at 7:3:1 or 5:5:1 (mol/mol). The spectra were recorded between 190 and 250 nm. The percentage of the α-helical structure was calculated by Jasco secondary structure estimation software.

Fluorescence dyes leakage from liposome vehicles

In order to detect potential roles of PS on the membrane-damaging potential of temporin-1CEa, phospholipid liposomes consist of PC, PS and CHO and entrapping fluorescent molecule calcine were prepared using the reverse-phase evaporation (film dispersion) method. As artificial biomembranes, phospholipid liposomes containing 7:3:1 or 5:5:1 molar ratios of PC/PS/CHO were constructed. Calcine, one fluorescence dye (molecular weight, 623 Da; diameter, ~1 nm) was encapsulated into liposome vehicles. Liposome vesicles were seeded onto 96-well microplates and co-incubated with temporin-1CEa for 1 h. The fluorescence intensity was detected under excitation and emission wavelengths of 485 nm and 520 nm, respectively.

Direct binding of PS with temporin-1CEa as assessed by isothermal titration calorimetry (ITC)

The isothermal titration calorimetry (ITC) analysis was performed at 30 °C on a Microcal high-sensitivity ITC calorimeter (ITC-200, Microcal, Northampton, MA). The lipid vesicles of 1.5 mM PS were injected into the chamber containing 1.0 mM temporin-1CEa. Each injection of 2 μl was done in a 5-s period with the 120-s interval between individual injections. The heats of dilution were determined in control experiments by injecting vesicles into buffer solutions and subtracting the heat produced from the corresponding peptide-PS binding experiment. Data acquisition and analysis were performed using Microcal Origin software (Microcal Software Inc., Northampton, MA).

Measurement of the interactions of FITC-labeled temporin-1CEa with cells using FACS

A375 and HaCaT cells were seeded at 2.5 × 10^5 cells/well in 24-well tissue culture plates and were treated with various concentrations of FITC-labeled temporin-1CEa for 1 h. The interactions of temporin-1CEa with cells were determined by FACS analysis and were expressed as deviations between FITC fluorescence density of peptides (Fp) and fluorescence intensity of control (F0).

Transmembrane potential measurements using fluorescence spectrophotometer

After incubation with 2 μM bis-(1,3-dibutylbarbituric acid)-trimethine oxonol (DiBAC4(3)) for 10 min at 37 °C, cells were subjected to time scanning using a fluorescence spectrophotometer (Varioskan Flash, Thermo Scientific, Waltham, MA) with Ex488nm/Em518nm. When the fluorescence intensity was stable, A375 cells were treated with various concentrations of 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 DiBAC4(3).
Results

Preferential cytotoxicity of temporin-1CEa against A375 melanoma cells

The results of MTT assay indicated that human melanoma A375 cells and mouse melanoma B16 cells were much more sensitive to temporin-1CEa when compared their IC50 values with other cancer cell lines (Table 1). In addition, while temporin-1CEa exhibited significant cytotoxicity against human A375 melanoma cells in a dose-dependent manner (Figure 1A) with an IC50 value of approximate 20µM (Table 1), temporin-1CEa at 5–40µM showed no cytotoxicity to HaCaT cells (Figure 1B). This preferential cytotoxicity of temporin-1CEa against A375 cells was further confirmed by the LDH leakage assay (Figure 1C and D).

In addition, when A375 cells were double stained with annexin V-FITC and propidium iodide (PI) after 1-h temporin-1CEa treatment, the percentage of cells distribution in Q3 quadrant was declined, suggesting a decrease of cell viability. Moreover, the percentages of cells in both Q2 and Q4 quadrants were increased, indicating an elevated membrane PS exposure and a loss of cell integrity and viability (Figure S1), which was confirmed by the cell membrane permeability assay using Calcein acetoxyethyl ester (Calcein AM)/Ethidium homodimer (EthD-1) staining (Figure S2). All these data suggested that the cytotoxicity of temporin-1CEa against melanoma A375 cells might be due to membrane disrupting.

Cytotoxicity of temporin-1CEa is closely associated with cell surface PS

The previous study has revealed that A375 cells surface contains a higher level of PS and thus is more sensitive to activated monocytes-induced cytotoxicity. In order to confirm whether the above mentioned higher sensitivity of A375 and B16 melanoma cells to temporin-1CEa was due to the higher levels of PS on these cells surface, the basal levels of cell surface PS of different cancer cell lines were determined using FITC-annexin V staining. As expected, the data indicated that, under the non-apoptotic condition, A375 and B16 melanoma cells possessed higher basal levels of cell surface PS, as evidenced by higher annexin V-FITC fluorescence intensities than other five tested cell lines, especially 50-folds higher than non-cancerous HaCaT cells (Table 2). Interestingly, the correlation analysis indicated that the PS levels of different cell lines were closely associated with their IC50 values following 1-h temporin-1CEa exposure (Table 2, \( r = -0.815, \ p = 0.014 \)). All these data suggested that cell viability in Q3 quadrant was declined, suggesting a decrease of cell viability. Moreover, the percentages of cells in both Q2 and Q4 quadrants were increased, indicating an elevated membrane PS exposure and a loss of cell integrity and viability (Figure S1), which was confirmed by the cell membrane permeability assay using Calcein acetoxyethyl ester (Calcein AM)/Ethidium homodimer (EthD-1) staining (Figure S2). All these data suggested that the cytotoxicity of temporin-1CEa against melanoma A375 cells might be due to membrane disrupting.

Table 1. Cytotoxicity effects (IC50 value) of temporin-1CEa on seven tested cancer cell lines and permanent epithelial cells HaCaT.

| Carcinoma type      | Cell line | 1 h | 6 h | 24 h | 48 h |
|---------------------|-----------|-----|-----|------|------|
| Human melanoma      | A-375     | 19.68 | 20.77 | 22.38 | 22.92 |
| Mouse melanoma      | B16       | 18.78 | 19.69 | 23.56 | 24.17 |
| Breast cancer       | MCF-7     | 31.78 | 27.84 | 31.91 | 30.28 |
| Epithelial carcinoma| Hela      | 43.87 | 40.6 | 36.31 | 35.17 |
| Hepatoma            | BEL-7402  | 36.90 | 37.10 | 38.98 | 39.22 |
| Hepatoma            | SMMC-7721 | 44.92 | 46.73 | 50.32 | 49.76 |
| Lung cancer         | A-549     | 52.82 | 53.12 | 53.31 | 57.41 |
| Epithelial cell     | HaCaT     | 70.22 | 68.66 | 70.81 | 68.27 |

Figure 1. Temporin-1CEa-induced A375 melanoma cells death. The A375 cells or HaCaT cells were treated with various concentrations of temporin-1CEa for a different time. Cell viability or cytotoxicity was determined using the (A, B) MTT assay or (C, D) LDH leakage test. Each bar represents the mean value from three determinations with the standard deviation.
Phosphatidylserine as a therapeutic target for temporin-1CEa

Table 2. PS content (mean fluorescence intensity of FITC-annexin V) of seven tested tumor cell lines and permanent epithelial cells HaCaT.

| Carcinoma type       | Cell line | Mean fluorescence intensity | Correlation between PS level and IC50 |
|----------------------|-----------|----------------------------|--------------------------------------|
| Human melanoma       | A-375     | 548.62                     |                                      |
| Mouse melanoma       | B16       | 593.19                     |                                      |
| Breast cancer        | MCF-7     | 126.22                     |                                      |
| Hepatoma             | BEL-7402  | 75.41                      |                                      |
| Hepatoma             | SMMC-7721 | 72.33                      |                                      |
| Lung cancer          | A-549     | 61.25                      |                                      |
| Epithelial carcinoma | Hela      | 45.74                      |                                      |
| Epithelial cell      | HaCaT     | 12.03                      |                                      |

Table 3. Membrane-bound ability (mean FITC fluorescence intensity) of temporin-1CEa (40 μM) on seven-tested cancer cell lines and permanent epithelial cells HaCaT.

| Carcinoma type       | Cell line | Mean fluorescence intensity | Correlation between PS level and peptide binding |
|----------------------|-----------|----------------------------|-----------------------------------------------|
| Human melanoma       | A-375     | 8003.26                    |                                               |
| Mouse melanoma       | B16       | 6792.39                    |                                               |
| Breast cancer        | MCF-7     | 3595.82                    |                                               |
| Hepatoma             | BEL-7402  | 3217.01                    |                                               |
| Hepatoma             | SMMC-7721 | 4556.25                    |                                               |
| Lung cancer          | A-549     | 3176.34                    |                                               |
| Epithelial carcinoma | Hela      | 2633.41                    |                                               |
| Epithelial cell      | HaCaT     | 2224.28                    |                                               |

Surface PS confers higher vulnerability of melanoma cells to temporin-1CEa-induced cytotoxicity.

Membrane PS favors temporin-1CEa binding to cells surface

Due to the negatively charged nature of cell surface PS and a positive net charge of temporin-1CEa, we speculated that the cell surface PS might attract extracellular temporin-1CEa to bind on the cell surface and subsequently induce cancer cell death. To confirm this hypothesis, in this study, FITC-labeled temporin-1CEa was co-incubated with various cancer cell lines, and then the binding ability of temporin-1CEa to the cell surface of different cell lines was evaluated by detecting fluorescence intensity of FITC. As summarized in Table 3, among those tested cancer cell lines, human melanoma A375 cells exerted the highest affinity to temporin-1CEa. In addition, the binding ability of temporin-1CEa to various cancer cells was negatively correlated with the IC_{50} values ($r = -0.792$, $p = 0.025$), suggesting that a higher binding affinity of temporin-1CEa with cancer cells causes a stronger cytotoxicity. Additionally, temporin-1CEa bound to A375 cells in a dose-dependent manner (Figure 2A), but with lower affinity to normal HaCaT cells (Figure 2B).

Direct bindings of temporin-1CEa with PS as assayed by ITC

To further confirm the direct binding of negatively charged PS with cationic temporin-1CEa, the ITC binding assay was performed. As shown in Figure 3(A), temporin-1CEa showed a clear interaction with PS. The binding process is exothermic with stoichiometry ($N$) value of 0.202, which suggests a binding ratio of PS/temporin-1CEa around 1/5. Moreover, the MTT assay also indicated that the cytotoxicity of temporin-1CEa against A375 melanoma cells can be partially blocked by masking PS residues using annexin V (Figure 3B), confirming that cancer cell surface PS is involved in temporin-1CEa-induced melanoma cell death.

Temporin-1CEa disrupts cell membrane integrity, elevates membrane permeability and induces transmembrane potential depolarization

The negatively charged PS attracts cationic temporin-1CEa to interact with cancer cell surface, and subsequently leads to the disrupted membrane integrity and elevated membrane permeability. Since disrupting the plasma membrane causes rapid depolarization and a loss of membrane potential, in the present
A375 cells were incubated with DiBAC$_{4}(3)$, one anionic and membrane-potential-sensitive dye. Depolarization of cell membranes leads to an uptake of DiBAC$_{4}(3)$ inside the cells, resulting in an increased fluorescent signal. As expected, our data revealed that 1 h exposure of various concentrations of temporin-1CEa (5–40 µM) to A375 melanoma cells led to an immediate and dramatic increase in the fluorescence intensity of DiBAC$_{4}(3)$, which was not observed in those untreated controls.

Figure 2. FITC-labeled temporin-1CEa binding to cells. The A375 cells or HaCaT cells were treated with various concentrations of temporin-1CEa for 1 h. The binding affinity of temporin-1CEa to cells was determined using FACS analysis and was presented as FITC fluorescence intensity. (A) A375 cells and (B) HaCaT cells. *p<0.05, compared with control; **p<0.01, compared with control.
control cells (Figure 3C). This rapid and dose-dependent depolarization of cellular transmembrane potential suggested a disruption of the cell membrane and an enhancement of membrane permeability after temporin-1CEa exposure. This membrane-disrupting effect was also confirmed by annexin V-FITC-PI assay (Figure S1), EthD-1/calcein AM double-staining assay (Figure S2) and morphological observations using electronic microscopy (Figure S3).

**PS confers temporin-1CEa to form α-helical structure and induce calcein leakage from liposomes**

In consistent with those data derived from assays of the cell-based system, the *in vitro* test using biomembrane mimic liposomes also confirmed the pivotal role of PS in temporin-1CEa-mediated membrane disruption. As shown in Figure 4(A–C), temporin-1CEa incubation dose-dependently caused fluorescence molecule calcein leakage out of the liposome vesicles. Interestingly, this leakage potency also showed a PS-dependent manner, as shown by a stronger extent of calcein leakage from liposome vesicles containing a higher level of PS (PC/PS/CHO ratio at 5:5:1) than from those at 7:3:1. All these data strongly suggested that membrane PS is involved in peptide-cell interactions and the peptides induced membrane disruption.

Interestingly, the data from CD analysis suggested that temporin-1CEa showed a higher percentage of the α-helical structure content when incubated with liposome vehicles containing high levels of PS (PC/PS/CHO ratio at 5:5:1) (Figure 4D). This finding indicated that PS can not only provide negatively charged attracting sites for cationic temporin-1CEa but also confers the membrane-bound peptides to form α-helical structure, which has been proved to be a key factor in the activity of AMPs [16,17].

**Morphological changes induced by temporin-1CEa in A375 cells**

The morphological observation using both scanning electron microscope (SEM) (Figure S3A) and transmission electron microscope (TEM) (Figure S3B) revealed that 1 h incubation of A375 cells with various concentrations of temporin-1CEa induced dramatic morphological changes. While untreated control cells showed an intact membrane and smooth surface, the temporin-1CEa-treated A375 cells membrane were shriveled, invaginated and disrupted, which may in turn resulted in irreversible cytolysis and finally death of the target cells.

**Discussion**

Metastatic melanoma is the most malignant skin cancer, and its incidence has steadily increased in the past few decades [18,19]. Although melanoma represents only 10% of all skin cancer diagnoses, it accounts for at least 65% of all skin cancer-related deaths [18]. Due to the highly metastatic, relatively resistant and lower specificity of malignant melanoma to currently available chemotherapeutic agents, more
potent and cancer-selective treating strategies have been required urgently. PS, as an important phospholipid constituent of cytoplasmic membranes, plays critical roles in the maintenance of cell membrane structure. PS exposure on the external leaflet of the plasma membrane is widely observed during apoptosis and forms the basis for the annexin V binding assay to detect apoptotic cell death. Much more importantly, previous studies [4] and our present data (Table 2) also revealed an enhanced exposure of negatively charged PS on the outer leaflet of non-apoptotic cancer cells compared with non-cancerous cells, even though no apoptotic cell death has been observed. Previous studies have also found that cell surface PS may interact with extracellular molecules to modulating cell functions, or even cause cell death. Heat shock protein HSP70 interact with PS on the surface of PC12 cells resulting in a decrease of viability [20]. Surface exposed PS also serves as a surface marker for recognition and clearance by monocytes and macrophages [21]. As for cancer cells, one previous research report has indicated that activated human monocytes bind to and lyse three tumorigenic cell lines, A375 melanoma and A431 and colo-16 carcinomas, while the normal human epidermal keratinocyte line (NHEK) were neither bound nor killed [5]. Semi-quantitative analysis of PS in the outer leaflet of these cells revealed that the tumorigenic cells expressed 3–7-fold more PS than did the non-tumorigenic NHEK cells. More interestingly, A375 cell line exerts a two-fold higher PS content, much more bindings to activated monocytes and more vulnerable to activated monocytes-mediated cytotoxicity than A431 and colo-16 cells [5]. Schröder-Born et al. analyzed the interaction of NK-2 with normal human lymphocytes and seven different human cancer cell lines and demonstrate that some of these cells surface expose negatively charged PS, which presumably facilitates killing of the cells by NK-2 [22]. All these research data may predict a hypothesis that PS could serve as a potential and preferential target for cancer therapy. The positively charged small peptides are able to discriminate between cancerous and non-cancerous cell membranes and specifically interact with negatively charged lipids on cancer cell membrane, which would minimize side effects upon treatment and could reach all cancer types exposing PS, even those resistant to conventional chemotherapy. Consistent with this consumption, in our previous studies, we found that temporin-1CEa shows specific anticancer activity against a broad spectrum of cancer cell lines, with a relatively low toxicity to non-cancerous normal cells. Temporin-1CEa triggers a rapid cytotoxicity in cancer cells through membrane destruction and intracellular mechanisms involving mitochondria [14,15]. Here, we further found temporin-1CEa exerts a preferential cytotoxicity against human A375 melanoma cells (Figure 1) with an IC₅₀ value of around 20 μM, through a membrane-disrupting effect (Figures 3C and 4A–C). Much more importantly, this cytotoxicity is partially blocked by masking PS residues with annexin V (Figure 3B), a molecule binds to cell membrane PS with high affinity. In addition, in the cell-based system, the FITC-labeled temporin-1CEa preferentially binds to PS-enriched melanoma cells (Table 1, Figure 2A), compared with the relative lower binding affinity of temporin-1CEa to other cancer
cell lines and HaCaT cells (Table 1, Figure 2B). Moreover, the PS level in cancer cells is closely correlated with their susceptibility to temporin-1CEa-induced cytotoxicity (Table 2), suggesting the negatively charged PS may provide binding sites on the cell surface for temporin-1CEa by an electrostatic attraction. This PS-involved membrane binding and disrupting properties of temporin-1CEa is further confirmed by ITC assay (Figure 3A) and by artificial membrane system using liposome vehicles (Figure 4A–C).

More importantly, using CD spectrum analysis, our present study discovered that negatively charged PS can not only provide binding sites for temporin-1CEa but could also favor the membrane-bound temporin-1CEa to form z-helical structures (Figure 4D). It is well known that an increased helicity correlates well with an enhanced hemolytic and antibacterial activity of AMPs [23–26]. Inversely, a decreased helical conformation results in a loss of both hemolytic and antimicrobial activity [27–29]. In previous research reports, cationic AMPs exerted their cytolytic activity by folding into an amphipathic helix upon binding and then inserting into the target membrane, leading to the breakdown of the membrane structure, leakage of cell contents and cell death [30]. The data of 3D structure simulation in our present study (Figure S4) and CD spectroscopy analysis in our previous study [14] have shown that temporin-1CEa exerts z-helical structure. This structure promotes peptide to disrupt the integrity of the cytoplasmic membranes of cancer cells. Consistent with those previous findings, the CD spectrum data in our present study revealed that PS elevates z-helical contents of temporin-1CEa (Figure 4D), and this pivotal role of PS may contribute to the stronger membrane disrupting effect of temporin-1CEa on A375 melanoma cells.

**Conclusion**

In summary, our results indicated that the preferential antimelanoma action of temporin-1CEa was PS-related. The higher levels of membrane PS content in melanoma cells favors binding ability, helical structure formation and cytotoxicity of temporin-1CEa, and may serve as a novel target for malignant melanoma treatment using cationic anticancer peptides.

**Declaration of interest**

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Supplementary material available online
Supplementary Figures S1 - S4