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ORIGINAL RESEARCH PAPER

Anti-melanoma activity of hybrid peptide P18 and its mechanism of action

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Abstract The anticancer properties and mechanism of action of a hybrid peptide -P18 were investigated. It had significant cytotoxic activity against human melanoma cells and low toxicity to normal NIH-3T3 cells. It also induced cell death via necrosis rather than classical apoptosis. The peptide targets the cells membrane, causing a sustained depolarization of transmembrane potential resulting in the cells swelling and bursting, thereby triggering cytolysis. P18 peptide initially binds to the melanoma cell membrane via electrostatic interaction, causing the cell membrane to rupture. The effect may be mediated by the amphiphilic α-helical structure of P18 peptide, coupled with changes in ion channels and an increase in plasma membrane permeability that eventually leads to melanoma cell death.

Keywords Anticancer · Antimelanoma · Melanoma cells · Membrane permeability · Peptide P18 · Plasma membrane

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Introduction

Malignant melanoma can be effectively treated by surgical resection at an early stage. However, once metastasis occurs, it is extremely difficult to treat with traditional surgery, chemotherapy or radiotherapy (Kalkman and Baxter 2004). Accordingly, there is a need to develop new therapeutic strategies to overcome the invasiveness and metastasis of melanoma cells in order to improve prognosis and survival rates. Several crude drugs and artificial products have been tested for their anti-melanoma activity both in vitro and in vivo (Bae et al. 2005; Malafa et al. 2002). These antitumor agents are effective in inhibiting the growth and metastasis of melanoma, but their antitumor mechanisms of action remain unclear. Moreover, the development of drug resistance towards current anticancer agents is a major obstacle, which must be taken into account when designing novel anticancer therapeutics.

A new class of anticancer agents without the side effects of conventional chemotherapeutic agents, and to which cancer cells do not drug resistance, is urgently required. In previous studies, cationic antimicrobial peptides (AMPs) showed potent cytotoxicity against a broad spectrum of bacterial cells and tumors with no hemolysis of normal erythrocytes cells (Giuliani et al. 2007). Also, currently used anticancer drugs need to enter the cancer cells and interact with a specific target protein to be effective. This makes it possible for cells to develop resistance

to these drugs by simply pumping them out again. However, most AMPs typically induce cell lysis and death of bacterial and tumor cells by targeting the cell membrane. They are, therefore, unaffected by common mechanisms of chemoresistance. However, AMPs from natural sources, such as insects, amphibians and mammals, have shown either limited activity against bacterial and fungal cells, or cytotoxicity to host mammalian cells (Hancock and Chapple 1999).

Consequently, numerous studies have attempted to design novel synthetic peptides that are complementary to these native AMPs. P18 (KWKLFKKIPKFLHLAKKF) is an α-helix hybrid antibiotic peptide designed by combining the positively charged *N*-terminal region of cecropin A with the COOH-terminal amphipathic region of magainin 2 (Shin et al. 2001). P18 shows significant cytotoxicity against bacterial cells without any hemolytic activity (Shin et al. 2001). Nevertheless, no information has been reported regarding the mechanism underlying the antitumor effects of peptide P18. In this study, we assessed the cytotoxicity of P18 against both human melanoma cells and normal cells. In addition, we investigated the anti-melanoma mechanism of P18, with the aim of facilitating the further development and utilization of AMPs-derived novel hybrid peptides for cancer therapy.

Materials and methods

Peptide synthesis

P18 peptides (Bo Tai) were synthesized by the solid-phase method using standard Fmoc-chemistry. The molecular mass of the synthetic peptides was confirmed by MALDI-MS. The radiochemical purity of the purified peptides, as measured by analytical reversed-phase HPLC, was ≥95%. All peptide solutions used in the study were prepared in sterile-deionized water.

Cell lines and culture conditions

Human A375 malignant melanoma cells, M14 melanoma cells, and NIH-3T3 cells were purchased from the replanted immune laboratory of Sichuan University in China. All cells were maintained as adherent monolayers in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), 100 μg streptomycin/ml and 100 U penicillin/ml under a 5% CO_2 atmosphere at 37°C. The cells were detached from the culture flasks with trypsin–EDTA (Gibco) twice weekly.

MTT cytotoxicity assay

A375, M14 and NIH-3T3 were seeded into 96-well plates in 100 μl DMEM medium containing 10% (v/v) FBS at 2500, 5000, 5000 cells/well for respectively. After incubation for 24 h, the cells were treated with various concentrations of P18. Control cells were treated with the same volume sterile-deionized water alone. After 48 h, 20 μl MTT (5 mg/ml in PBS) was added per well for an additional 4 h. The purple formazan crystals were dissolved in 10% (v/v) SDS with 0.01 M HCl at room temperature overnight. The absorbance at 570 nm was then measured using an ELISA assay reader. Cell viability was determined relative to the controls. Three independent experiments were performed in triplicate. The percentage of cell viability was calculated as: $\text{viability}\ (\%) = \text{absorbance of sample}/\ \text{absorbance of control} \times 100$

Flow cytometry

To investigate the death pathways induced human melanoma cells by P18, the Vybrant Apoptosis Kit, including YO-PRO-1 and propidium iodide (PI), was used (Gawlitta et al. 2004). A375 cells were cultured in 100 mm Petri dishes at 10^6 overnight. After treatment with either 20 μM P18, or sterile-deionized water, the cells were resuspended in 1 ml cold PBS and stained with 1 μl YO-PRO-1 and 1 μl PI for 30 min on ice in the dark. Flow cytometry was used to quantify cell death induced by the P18 (FACStar, Beckton Dickinson). Samples stained with YO-PRO-1 alone or PI alone and one sample containing unstained cells was used to set the gating and compensation for the experiment. All experiments were performed in triplicate.

Confocal fluorescence microscopy

P18 was labeled with FITC without affecting its anticancer activity. A375 cells were cultured overnight in 30 mm Petri dishes with glass cover slips in

the bottom (MatTek) at 10^4 cells/dish. After treatment with 40 μM FITC-labeled P18 peptide, the cells were washed twice and covered with PBS, then observed using LTCS-SP confocal system (Leica) equipped with an inverted DMIRBE microscope immediately. Three independent experiments were performed.

Fluorescence spectrophotometry

Alterations in the plasma membrane potential were measured using a fluorescent potential-sensitive anionic dye, $DiBAC_4$ (3), which only exhibits green fluorescence when bound cytoplasmic proteins (Tsukimoto et al. 2005). A375 cells were washed twice and resuspended in serum-free medium at 10^6 cells/ml. After incubation with 2 μM $DiBAC_4$ for 10 min at 37°C, the cells were subjected to time scanning by a fluorescence spectrophotometer (F-7000, Hitachi) with laser excitation at 488 nm and emission at 518 nm. When the fluorescence intensity was stable, the cells were treated with either 40 μM P18, or sterile-deionized water. Membrane depolarization was monitored by observing the changes in the intensity of fluorescence emission of the membrane potential $DiBAC_4$ dye consecutively. The assay was repeated three times.

Fluorescence microscopy

The assay was carried out using the LIVE/DEAD Viability/Cytotoxicity Assay Kit (Molecular Probes Inc.) with calcein AM and ethidium homodimer (EthD-1) colour fluorescent dyes. A375 and M14 melanoma cells were seeded into 96-well plates at 2,500 or 5,000 cells/well respectively overnight. After treatment with either 20 μM P18 or sterile-deionized water for 24 h, the medium was removed and 20 μl dye containing 2 μM calcein AM and 4 μM EthD-1 were added for 30 min in dark. Live and dead cells were distinguished under a fluorescence microscope. Three independent experiments were performed in triplicate.

Statistical analysis

All data were expressed as mean ± standard error (SEM). Significant differences between the treated groups and the control were assessed using Student's *t* test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Results

Anticancer activity of P18

We tested the ability of P18 to inhibit the growth of both cancer and non-cancer cell lines. As shown in Table 1, P18 is remarkable cytotoxic against melanoma cells and low toxicity to normal NIH-3T3 cells. The survival rate of both melanoma cell lines was less than 50% after treatment with 40 μM P18, while up to 80 μM P18 was need to achieve similar results in NIH-3T3 cells. There was a significant difference in survival rates of between the melanoma cell lines A375 ($P < 0.01$), M14 cells ($P < 0.05$) and NIH-3T3 cells. Also, P18 inhibited the growth of melanoma cells in a dose-dependent manner.

P18-induced death pathways in melanoma cells

We used the YO-PRO-1/PI dual staining method to investigate the P18-induced death pathway in A375 malignant melanoma cells. The staining pattern, resulting from the simultaneous use of these two dyes, made it possible to distinguish viable, apoptotic, and necrotic cell populations. Figure 1 shows the percentage of necrotic cells within the P18-treated population (92.6 ± 0.8%) compared with control cells (2.6 ± 0.6%). The viability of P18-treated cells and control cells was 4.8 ± 0.4 and 93.5 ± 0.9% respectively. Only a small proportion of P 18-treated cells (0.57 ± 0.2%) and control cells (3.1 ± 0.1%)

Table 1 Effect of various concentration of P18 on cell viability

Peptide concentration (μM)	NIH 3T3 cell viability (% of control)	A375 cell viability (% of control)	M14 cell viability (% of control)
10	99 ± 1.6	86 ± 0.57	92.2 ± 1.02
20	98 ± 1.6	63.8 ± 0.8	71.1 ± 2.84
40	75.2 ± 5.6	47.5 ± 1.66	50 ± 3.09
60	70.8 ± 3.6	26.9 ± 0.5	31.5 ± 1.49
80	47.7 ± 3.1	6.5 ± 1.07	13.8 ± 1.44

Each value represents the mean ± standard error (SEM)

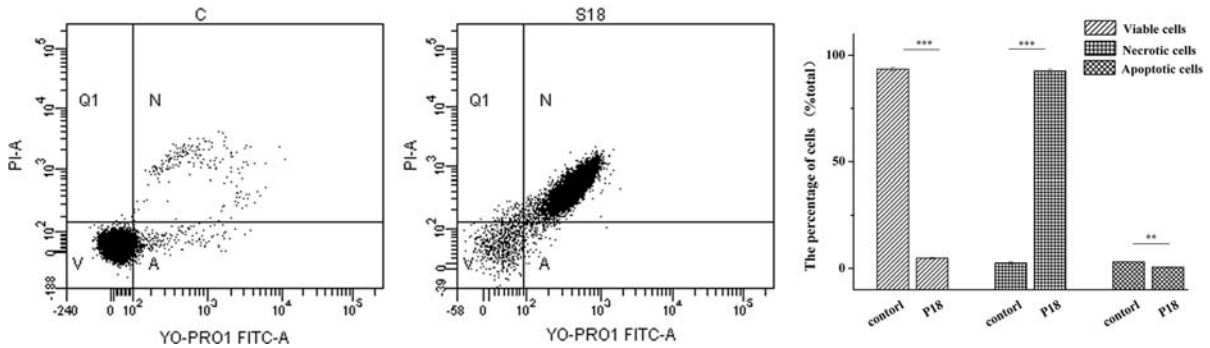

Fig. 1 Flow cytometry. YO-PRO-1 enters the early apoptotic and dead cells, while PI enters the dead cells. "N", "V", and "A" represent necrotic cells, viable cells and apoptotic cells respectively. Clear differences in the proportion of live and necrotic cells are seen between the treatment groups and the controls (*** $P < 0.001$). Also, the proportion of apoptotic cells was significantly greater in the treatment groups compared with the controls (** $P < 0.05$)

were apoptotic, suggesting that P18 induces cell death via necrosis rather than apoptosis.

P18 peptide binding affinity and cell lysis

To identify the P18 binding target, and to test its lytic effect on melanoma cells, we used confocal microscopy to analyze the cellular distribution of peptide P18 along with changes in cell morphology. The results showed that, after treatment for 2 min, the FITC-labeled P18 peptide was mainly bound to the melanoma cell membrane (77 ± 0.05%) (Fig. 2a). Further experiments revealed the effect of P18 on the integrity of the melanoma cells (Fig. 2b). After 5 min of treatment, FITC-conjugated P18 remained at the cell membrane, with no fluorescence detectable inside the nucleus (82 ± 0.05%). Disruption of cell plasma membrane, along with cell swelling was clearly visible, and was followed by the release of the intracellular contents. Interestingly, P18 treatment led to the loss of all cytoplasmic contents only leaving the nucleus intact. Nuclear morphology appeared to be relatively well preserved.

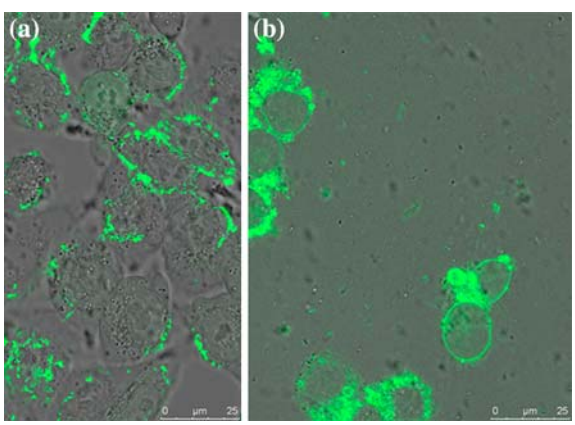

Fig. 2 Confocal laser scanning microscopy images of A375 cells. **a** 2 min treatment and **b** 5 min treatment with 40 μM FITC-labeled peptide P18 (*green*). P18 mainly binds to the plasma membrane. The integrity of the cell membrane was disrupted in response to P18. The confocal images were taken using 40 × objectives with living cells at room temperature. Bar = 25 μm

Transmembrane potential depolarization

To test whether cell death was related to cell membrane damage, cell transmembrane potential depolarization experiments were performed using the anionic dye $DiBAC_4$. Depolarization of the transmembrane potential occurred when the dye entered cells, causing the increase of intracellular fluorescence intensity. On the contrary, fluorescence intensity was reduced when the transmembrane potential hyperpolarized. As shown in Fig. 3, the addition of P18 led to immediate and dramatic increases in the fluorescence intensity of $DiBAC_4$. This was not seen in the controls. P18 caused a sustained depolarization of the transmembrane potential, indicating that the peptide has a powerful and lasting impact on the permeability of A375 melanoma cells membranes.

Membrane permeability assay

Two-color fluorescence dyes, calcein AM and ethidium homodimer (EthD-1), were used to confirm the cytotoxicity of P18, and to detect changes in cell

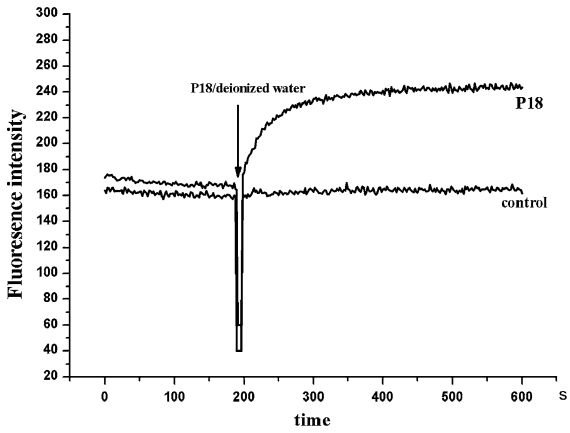

Fig. 3 The depolarization of A375 cells plasma membrane potential. Fluorescence intensity was monitored consecutively at excitation/emission wavelengths of 488 nm/518 nm using a fluorescence spectrophotometer. The fluorescence intensity maintains a relatively constant level before treatment. About 200 s later, A375 cells were treated with 40 μM P18, or sterile-deionized water (control). The fluorescence intensity of the control still maintains a relatively constant level, whereas the addition of P18 peptide leads to an increase in the fluorescence intensity

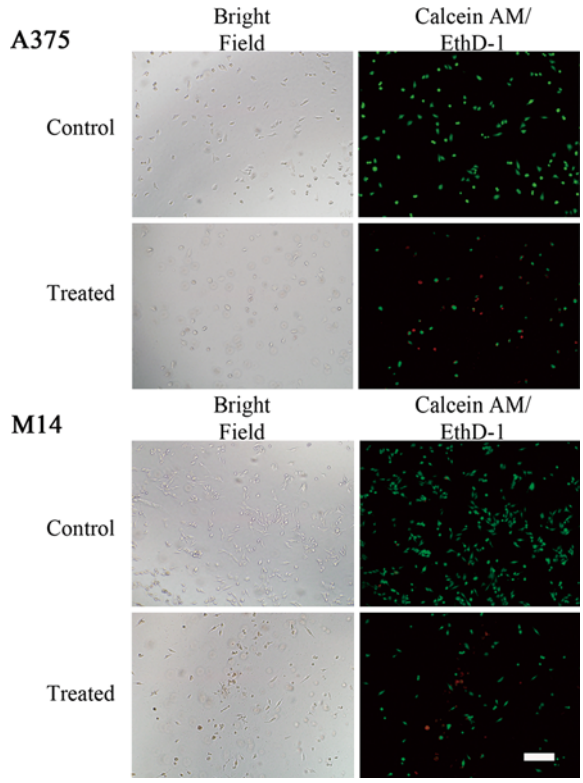

Fig. 4 Fluorescence microscopy images of A375 and M14 melanoma cells. Having been treated with 20 μM P18 or sterile-deionized water (control) for 24 h, the cells were stained by calcein AM and ethidium homodimer (EthD-1) fluorescent dyes. Calcein AM enters the live cells producing green fluorescence. EthD-1 only enters cells with damaged membranes, producing a bright red fluorescence. The proportion of cells with damaged membranes was significantly different between the treated cells, A375 ($P < 0.01$) and M14 ($P < 0.05$) and controls. Bar = 50 μm, in all images

membrane permeability. As shown in Fig. 4, P18-treated cells showed both green and red fluorescence, whereas almost all the control cells showed green fluorescence only, indicating viability. The proportion of A375 cells with damaged membranes in the treated and control groups were 21.3 ± 0.01 and $0.27 \pm 0.001\%$ respectively ($P < 0.01$). The proportion of M14 cells with damaged membranes in the treated and control groups were 28 ± 0.07 and $0.9 \pm 0.001\%$ respectively ($P < 0.05$). This suggests that P18 disrupts the melanoma cell membrane and increases membrane permeability, further leading to the cells death. Some cells within the treated groups began to become rounded, even detached from the culture plates. The microscope is unable to focus simultaneously on both floating and adherent cells, and so the treated groups appeared little in comparison with the control in the Fig. 4.

Discussion

Our results demonstrate that the hybrid cationic peptide, P18, has significant cytotoxic activity against both human A375 malignant melanoma cells and M14 melanoma cells, and little toxicity to NIH-3T3 cells. It suggests that P18 has potent and selective activity against human melanoma cells. Differences in membrane characteristics between malignant cancer cells and normal cells contribute to the highly selective cytotoxic activity of antimicrobial peptides (Cruciani et al. 1999). Cancer cell membranes mainly carry a net negative charge due to the over-expression of anionic molecules such as phosphatidyl serine (Dobrzynska et al. 2005) and *O*-glycosylated mucins (Yoon et al. 1996). In contrast, normal cell membranes are composed of zwitterionic molecules such as sphingomyelin and phosphatidyl choline, which have an overall neutral charge (Zachowski 1993). Such characteristics are a major factor in the preferential binding of cationic antimicrobial peptides to

the negatively charged cancer cell membrane by electrostatic interactions that trigger membrane rupture. Therefore, the confocal laser scanning microscope experiments in our study were done to verify that P18 typically targets the membrane of melanoma cells. Rapid cell swelling, membrane rupture and release of cytoplasmic content were observed within a few minutes of P18 binding, suggesting necrosis. It was surprising that P18 treatment resulted in the loss of all the cytoplasmic contents of the melanoma cells, but did not enter and destroy the nucleus, which maintained its integrity after cell death. As expected, flow cytometry confirmed that P18 induced cell death via necrosis rather than classical apoptosis.

In general, necrosis is modulated by factors such as cytokines, ion channels, and redox reactions. However, our results show that P18 induces a dramatic and sustained depolarization of the transmembrane potential. Melanoma cell death was preceded by severe disturbance of inorganic ion homeostasis, characterized by clear increases in cytosolic [Ca^{2+}], [Na^{+}] and [Mg^{2+}]. The concentrations of these ions are crucial for signal transduction, and changes can lead to destabilization of the cell membrane (Barros et al. 2002). Consequently, one of the mechanisms of P18-induced melanoma cell necrosis works via ion channels. Furthermore, as illustrated by the fluorescence microscope assay, the permeability of the melanoma cell membrane increased remarkably.

Most naturally occurring antibacterial peptides induce bacterial or cancer cell death via destruction of the plasma membrane, followed by leakage of the intracellular contents and subsequent cytolysis (Dekker et al. 2001). *Furthermore, studies of* cecropin and magainin have shown that their amphiphilic α-helical structure may play an important role in killing bacteria or cancer cells (Oh et al. 1999). Electrostatic interaction between these positively charged antimicrobial peptides and negatively charged lipids enables these cationic peptides to bind to plasma membranes. This interaction is non-specific, but the conformation of these cationic antimicrobial peptides allows their hydrophobic *C*-terminal α-helix to insert into and span the cell membrane, then forming transmembrane ionic channels that result in membrane destabilization, mediating a series of follow-on effects. In other words, although the initial interaction and selective recognition are driven by electrostatic forces, hydrophobic interactions also play a very important role after peptide binding, due to the partial amphiphilic α-helical structure.

In this study, P18 initially bound to the melanoma cell membrane through electrostatic interactions, and caused the cell membranes rupture. This may have been mediated by the α-helical structure of P18 inducing the formation of ion channels, thus raising membrane permeability, and eventually leading to melanoma cell death. These results give an insight into the mechanism of P18 (or other antimicrobial peptides) induced cancer cell death, and may aid the development of more potent and highly selective AMPs for cancer treatment. However, it is unclear whether the molecular mechanism underlying the anticancer activity of all antimicrobial peptides is the same. Further study at the molecular level is needed to deduce the exact mechanism of interaction of AMPs with cancer cells. In the near future, more potent antimicrobial peptides, with notable advantages over the current drugs, may be successfully used for cancer therapy.

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