Anticancer mechanism of peptide P18 in human leukemia K562 cells

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Studies on the anticancer mechanism of peptide P18 in human leukemia K562 cells revealed that P18 causes the death of most K562 cells by depolarizing plasma membrane potential and enhancing membrane permeability, rather than activating the classical apoptosis pathway. The mechanistic studies indicate that disrupting plasma membrane is an effective approach to kill cancer cells and help design more effective peptide analogues in future cancer therapies.

Over the past 50 years, tremendous efforts have been made to treat cancers, but unfortunately cancer is still one of the major causes of mortality in the world. Cancer is a highly complex disease caused by different genetic mutations and multiple molecular alterations,[1,2] and traditional anticancer drugs generally do not differentiate between cancerous and normal cells, leading the inefficiency and severe side effects.[3] Therefore, the characteristics of promising anticancer drugs should have excellent curative properties and favorable toxicity profiles.[4] Recently, antibiotic peptides, which play an important role in the host defense and innate immunity of insects, amphibians and mammals,[5–10] have been developed as new anticancer drugs. Some of these antibiotic peptides have shown attractive anticancer activity at concentrations that are nontoxic toward normal mammalian cells.[11,12] Hence, antibiotic peptides may be a class of effective agents for cancer treatment.[8,13–16]

P18 (KWKLFKKIPKFLHLAKKF) (Fig. 1), an antibiotic peptide, is designed from the hybrid of cecropin A[17] and magainin 2,[18] which were isolated from *Hyalaphora cecropia pupae* and the skin of the African clawed frog *Xenopus Iaevis*, respectively. Previous studies have shown that P18 is highly active against bacteria and fungi,[19,20] the selective inhibitions of P18 against LPS-stimulated inflammatory responses make P18 become a promising anti-inflammatory agent.[21] Most interestingly, P18 has significant anticancer activity with low side effects,[20,22] and these properties make P18 a promising agent for cancer treatment. However, the mechanism by which P18 exerts its anticancer activity and causes the death of cancer cells is not well understood. Here, the primary goal is to investigate the anticancer mechanism of P18 in human leukemia K562 cells.

In this study, peptide P18 was synthesized by Shanghai Bootech Bioscience & Technology Co., Ltd. with high purity (≥95%). Crude peptide was purified by reverse-phase high-pressure liquid chromatography (RP-HPLC). The identity of P18 was confirmed by electrospray ionization mass spectrometry in an ion trap mass spectrometer (Finnigan LCQ, San Jose, CA, USA). The working solutions of P18 were prepared at different concentrations with sterile water (18 MΩ; Millipore Milli-Q system).

We first examined the cytotoxicity of P18 by using the MTT assay.[23] MTT assay showed that P18 molecules have high anticancer activities, causing remarkable dose-dependent death of human cancer K562 cells (IC_{50} = 8.28 μM) (Fig. 2). Meanwhile, P18 showed relatively low cytotoxicity against normal fibroblast cell line NIH 3T3 (Fig. 2). These results indicated that P18 had significant anticancer activity with low side effect.

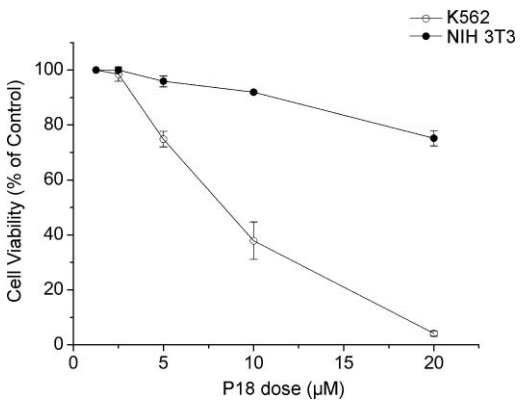

Fig. 2 The cytotoxicity of P18 against human leukemia cell K562 and fibroblast cell NIH3T3, bars, SD.

P18

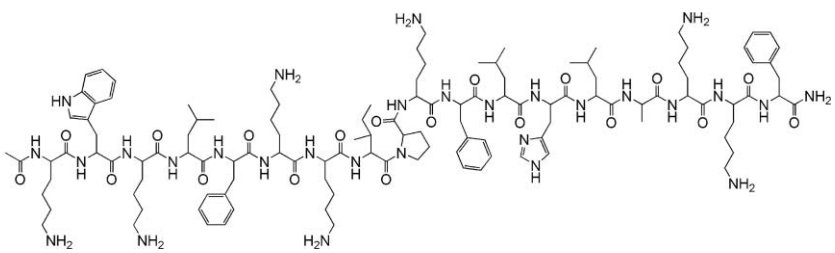

Fig. 1 The chemical structure of peptide P18 (KWKLFKKIPKFLHLAKKF).

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It is well known that aspartate-specific cysteine proteases (caspases) play critical roles in the initiation and execution of apoptotic pathways.[24] Caspase-8 is one of the initiator caspases for the death receptor pathway of apoptosis,[25] and caspase-9 is associated with the mitochondria-dependent pathway of apoptosis,[26] whereas caspase-3 plays critical roles in execution of apoptosis as one of the executioner caspases.[27] Therefore, we have chosen caspase-3, 8 and 9 as molecular markers and examined whether P18-mediated cell death is through the classical caspase-dependent apoptosis. After incubation with 20 μM P18 solution or control solution (sterile water), K562 cells were harvested and lysed, and the changes of caspases were detected by a Western blot. We found that P18 treatment did not cause the cleavage of procaspase-8 (Fig. 3a), 9 (Fig. 3b), 3 (Fig. 3c), and did not generate active subunits (*i.e.* caspase-8, 9, 3). Western blot analysis showed that P18 kills K562 cells without activating the caspase-dependent apoptosis pathway.

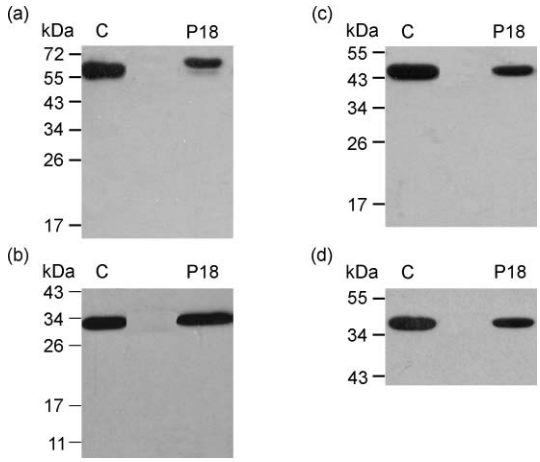

Fig. 3 P18-induced K562 cell death is unrelated to the classical apoptosis triggered by caspases. P18 treatment did not cause the cleavage of procaspase-8 (a), 9 (b) and 3 (c) and no active subunits were detected. (d) The expression of β-actin was used as a control.

The results of the Western blot made it necessary to distinguish the cell death type of K562 cells treated by P18, and a flow cytometry-based assay including the Vybrant Apoptosis Assay kit # 7 (Molecular Probes) was used according to the manufacturer's instructions. In this kit, green-fluorescent dye YO-PRO-1 can stain early apoptotic cells as well as necrotic cells, whereas red-fluorescent dye PI can enter necrotic cells and produce a red fluorescence. After stained with fluorescent dye YO-PRO-1 and PI for 30 min, live (YO-PRO-1$^-$ PI$^-$), apoptotic (YO-PRO-1$^+$ PI$^-$) and necrotic (YO-PRO-1$^+$ PI$^+$) cells were analyzed by flow cytometry (FACSAria, BD). We observed most K562 cells treated by control solution (sterile water) were alive, because only 6.3% and 5.6% cells were stained positive for YO-PRO-1 and PI, respectively (Fig. 4). However, P18 treatment significantly increased the percentage of necrotic cells (80.8% cells stained positive for YO-PRO-1 and 81.0% cells stained positive for PI) compared with the basal level of necrosis seen in the control cells (Fig. 4). The results of flow cytometry analysis were consistent with those of western blot and indicated that peptide P18 caused K562 cells necrosis rather than apoptosis.

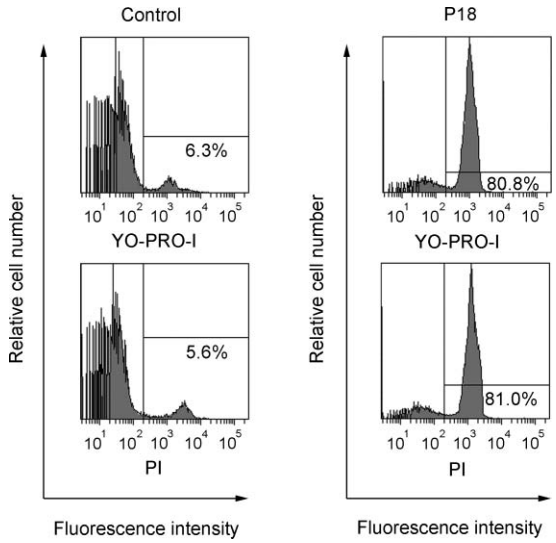

Fig. 4 Peptide P18 caused necrosis of K562 cells. Most K562 cells treated by control solution were alive, while P18 treatment significantly increased the percentage of necrotic cells.

Necrosis might be initiated by physical or chemical insults, such as osmotic imbalance and energy deprivation, thus, we wondered whether P18 molecules directly affect on the plasma membrane of K562 cells, change the cellular physiological balance and trigger the necrosis of K562 cells. The plasma membrane integrity and permeability of P18-treated K562 cells were then examined by using fluorescent probe ethidium homodimers (EthD-1),[28] which enter cells with damaged membranes and are excluded by the intact plasma membrane of live cells. We found that most cells treated by control solution (sterile water) were alive and had intact plasma membrane because they stained negative for EthD-1, while most cells treated by P18 had damaged membrane and stained positive for EthD-1 (Fig. 5).

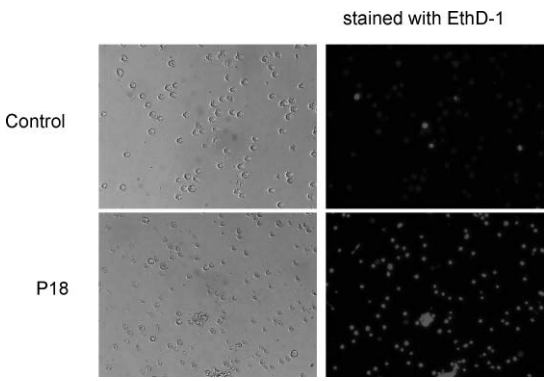

Fig. 5 Peptide P18 enhanced the plasma membrane permeability of K562 cells. Most K562 cells treated by control solution (sterile water) stained negative for EthD-1, while most cells treated by P18 had damaged membrane and stained positive for EthD-1.

Previous studies have shown that plasma membrane potential plays an important role in cell functions by controlling ion fluxes across the cell membrane. Osmotic balance and signal transduction are both influenced by the potential across the plasma membrane.[29] The movement of ions and the resulting alterations in the electrical field across the membrane lead to

plasma membrane depolarization, which could disturb membrane potential, cause ionic unbalance, destroy osmotic balance and initiate cell death.[29–33] In this study, we analyzed P18's depolarization effect on the plasma membrane of K562 cells by using a fluorescent potential sensitive anionic dye, $DiBAC_4$ (3).[34–36] $DiBAC_4$ (3) molecules can enter cells through depolarized plasma membrane and enhance fluorescence intensity while binding to intracellular membranes or proteins. Thus, the increase of fluorescence intensity implies more influx of the anionic dye, indicating the increased permeability and depolarization of plasma membrane. $DiBAC_4$ (3) therefore becomes a standard anionic probe for measuring plasma membrane depolarization.

The fluorescence emission of $DiBAC_4$ (3) was firstly recorded with excitation at 488 nm in four media, including serum-free RPMI 1640 medium, P18 solution, NIH 3T3 and K562 cells suspension. The fluorescence emission spectra showed that the positions of maximum emission were at the same wavelength (518 nm) in the four media above (Fig. 6). The results indicated that the membrane potential analysis is directly related to the intensity of the fluorescence emission at excitation/emission wavelengths of 488/518 nm, avoiding any influence of spectral shift caused by the different media.

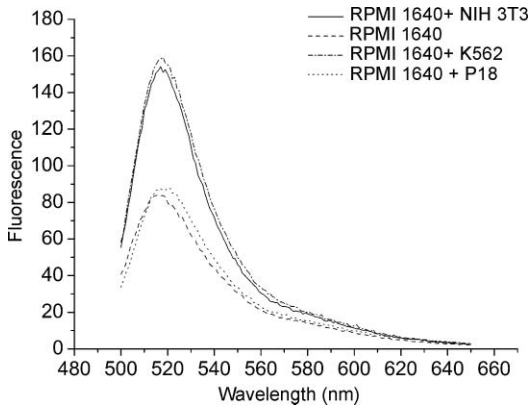

Fig. 6 Fluorescence emission spectra of DiBAC4 (3) with excitation at 488 nm in serum-free RPMI 1640 medium, P18 solution (P18 was diluted in serum-free RPMI 1640 medium), NIH 3T3 and K562 cell suspension (NIH 3T3 and K562 cells were suspended in serum-free RPMI 1640 medium, respectively).

The membrane depolarization effect of P18 on NIH 3T3 and K562 cells was then investigated by using fluorescence spectrophotometer with $DiBAC_4$ (3) at excitation/emission wavelengths of 488/518 nm. The stimulus-induced fluorescence changes were corrected for the background fluorescence, relative fluorescence intensity changes were expressed as F/F_0, where F is the fluorescence measured at any given time, and F_0 represents the baseline fluorescence before the addition of stimulus. The relative fluorescence intensity changes were used as an indication of the changes in membrane potential.[37,38] After P18 solution was added into the RPMI 1640 medium, there was little P18-dependent enhancement in the fluorescence intensity (Fig. 7a), suggesting that P18 solution itself does not enhance the fluorescence intensity of $DiBAC_4$ (3). Similarly, control solution (sterile water) did not cause the change of fluorescence intensity (Fig. 7b). However, an immediate and significant enhancement of relative fluorescence intensity was observed in K562 cell suspension treated by P18,

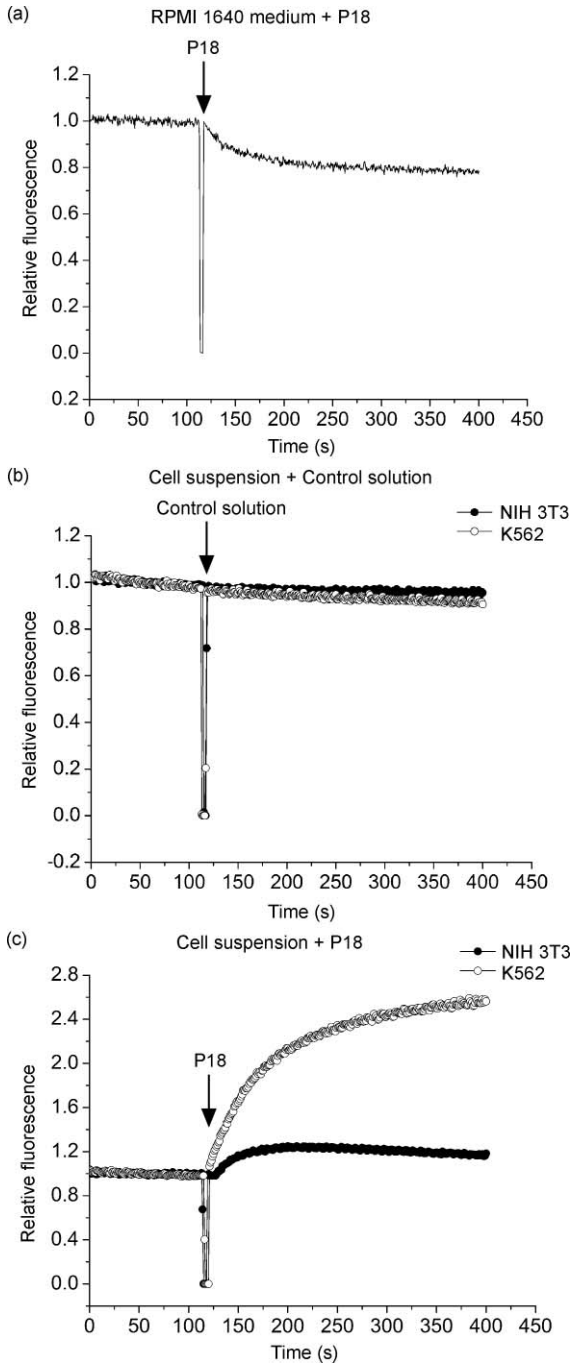

Fig. 7 The membrane depolarization effect of P18 on NIH 3T3 and K562 cells. (a) P18 solution was added into the RPMI 1640 medium. (b) Control solution did not cause the change of fluorescence intensity in NIH 3T3 and K562 cell suspension. (c) P18-treatment induced the significant enhancement of fluorescence intensity in K562 cells as compared with NIH 3T3 cells, indicating the membrane depolarization induced by P18 in K562 cells is significantly more enhanced than that in NIH 3T3 cells.

whereas the enhancement was obviously weak in NIH 3T3 cell suspension (Fig. 7c), indicating that the membrane depolarization induced by P18 in K562 cells is significantly more enhanced than that in NIH 3T3 cells, and the selectivity in depolarization may be one of the key factors that make P18 have significant anticancer

activity against K562 cells with low cytotoxicity against NIH 3T3 cells (Fig. 2). Taken together, these results indicated that P18 could rapidly affect on the plasma membrane of K562 cells, enhance membrane permeability and induce the intracellular contents to leak, evoke significant membrane depolarization and disturb the electrolyte balance, resulting in the death of cancer cells.

We have examined the anticancer mechanism of P18 in human leukemia K562 cells. We found that P18 is able to cause most of K562 cells necrosis without activating the classical caspase-dependent apoptosis pathway. We further observed that P18-treated K562 cells have quickly lost their membrane potential and profoundly enhanced their plasma membrane permeability. Most interestingly, the membrane depolarization induced by P18 in K562 cells is significantly more enhanced than that in NIH 3T3 cells, and the selectivity in depolarization is consistent with the results of MTT assay, in which P18 had significant anticancer activity against K562 cells with low cytotoxicity against NIH 3T3 cells. Our data indicated that membrane disruption changes the cellular physiological balance and triggers the necrosis of cancer cells. This mechanism may be an effective approach to kill cancer cells and help produce more peptide analogues for the future cancer therapy.

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