Research on the Anti-tumor Mechanism of Natural Antimicrobial Peptide TA

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Abstract: Natural antimicrobial peptides exhibit broad-spectrum antibacterial properties and the capacity to selectively eradicate tumour cells, presenting a promising avenue for the development of innovative anti-cancer medications[1,2]. Among these peptides, TA is a significant natural antimicrobial peptide currently undergoing investigation for its anti-tumour efficacy. This investigation focuses on human non-small cell lung cancer cells (A549 cells) as the experimental model. Cell morphology will be examined using scanning electron microscopy, while the mitochondrial membrane potential changes in A549 cells following various drug treatments will be monitored in real-time using the mitochondrial membrane potential probe DiSC3(5). The overall cell count and apoptotic cell distribution will be assessed alongside DAPI/PI double staining to elucidate the mechanism and effectiveness of TA in inducing cell death in A549 cells. Intriguingly, our experiments have revealed that TA can trigger the apoptotic pathway by disrupting the mitochondrial membrane potential of A549 cells, demonstrating significantly greater cytotoxicity compared to 5-FU and PT compounds. These findings provide empirical support for considering TA as a potential therapeutic option for lung cancer and propose a novel strategy for targeted therapy of non-small cell lung cancer.

***Keywords*:** natural antimicrobial peptide TA; anti-tumor mechanism of action; cell apoptosis

1. Introduction

Cancer, the most fatal disease globally, poses challenges in treatment despite numerous studies[3]. Traditional chemotherapy drugs face limitations in clinical use due to their lack of specificity and propensity to trigger drug resistance. Natural antimicrobial peptides, known for their wide antibacterial spectrum and ability to selectively eliminate tumour cells, offer a promising approach to combat chemotherapy resistance[4,5]. Among these peptides, TA stands out for its capacity to induce tumour cell apoptosis by disrupting membranes and mitochondrial pathways[6,7]. Nonetheless, its efficacy and mechanism against human non-small cell lung cancer cells require further elucidation. This research aims to investigate and compare the apoptosis-inducing effects of TA antimicrobial peptides with the chemotherapy drugs 5-fluorouracil and PT compounds on A549 cells, shedding light on TA's anti-tumour mechanism.

2. Experimental instruments and reagents

Fmoc-Pro-(2-CTC)-resin (1% cross-linked, 100-200 mesh, 0.476 mmol/g) was procured from GL Biochemical (Shanghai) Co., LTD. HAtu, HBtu, N, N-dimethylformamide (DMF), dichloromethane (DCM), and methanol (MeOH) were utilised. Liquid-phase analysis was conducted using Waters e2659 and an Eclipse XDB-C18 chromatographic column (4.6 x 250 mm, 5μm). Agilent Technologies employed FLB-1025-5-10 10A (10mm×250mm, 5μm) for purification. Human non-small cell lung cancer cells (A549 cells) were observed under an OLYMPUS fluorescence microscope. Fetal bovine serum was also included.

3. Experimental section

3.1 Compound Experiment

The Fmoc-Pro resin should be weighed and transferred into the T-type synthetic tube. Subsequently, DMF is added for swelling and washing, followed by the introduction of a deprotective solution (20% piperidine /DMF) to eliminate the protecting groups of the resin. The mixture is then shaken for 10 minutes and dried. Next, 4-5mL of DMF, methanol, and DCM are added for two washes each, followed by drying. The amino acids are then added to the peptide synthesis tube, shaken on a shaker for 1 hour, the resin washed, and the deprotective solution used to remove the protective groups on the amino acids. After shaking for 10 minutes and washing the resin again, the second amino acid is added, and the aforementioned steps are repeated to synthesise the peptide according to the sequence. Upon completion of the condensation of the last amino acid in

the peptide chain, the resin is washed, and 4-5 ml of the pre-prepared cutting solution (5%DCM/95%TFA) is added to cut the resin. The mixture is then shaken for 30 minutes, cut twice, and the cutting solution is collected in a 50mL centrifuge tube. Nitrogen is blown over the solution until approximately 1mL of solvent remains. Subsequently, 40mL of methyl tert-butyl ether is added, stirred evenly with a plastic dropper, and placed in a -20℃ refrigerator to freeze and precipitate for 15 minutes. The solution is then centrifuged, the supernatant aspirated and discarded, the precipitate collected, and a small amount of crude peptide taken for confirmation via ESI mass spectrometry[8].

3.2 DAPI-PI Fluorescence Staining Experiment

The A549 cells in the logarithmic growth phase were trypsinized to generate a cell suspension, which was subsequently seeded into 96-well microplates at a density of 1×10^5 cells per well. The cells were allowed to adhere and reach a confluence of 70%-80%. Subsequently, different drugs (5-FU, PT, TA) and blank control groups were administered based on the experimental design. The microplates were then placed in a 37 °C, 5% CO_2 incubator for 24 hours. Following incubation, the culture medium in the plates was aspirated and discarded, and the cells were gently rinsed twice with pre-cooled PBS. Add 4% paraformaldehyde fixative and fix at room temperature for 15 minutes. Remove the fixative, then rinse the cells twice with PBS for 5 minutes each. Following fixation, introduce DAPI staining solution into the 96-well microplate, and let it incubate at room temperature in the dark for 10 minutes. Afterward, remove the DAPI staining solution, wash the cells twice with PBS, add PI staining solution, and incubate in the dark at room temperature for 8 minutes. Discard the PI staining solution, rinse the cells twice with PBS to eliminate excess dye, transfer the sample onto a slide, and examine the DAPI (blue fluorescence) and PI (red fluorescence) channels separately using an OLYMPUS fluorescence microscope (10× objective lens). Capture images to document cell distribution and quantify both the total cell count and the number of apoptotic cells.

3.3 Plasma membrane depolarization

The fluorescence of the cationic dye DiSC(3)5 undergoes self-quenching upon accumulation at the cytoplasmic membrane. However, following membrane rupture, DiSC(3)5 is released and resuspended in the buffer, leading to an increase in fluorescence. The specific procedure is as follows: Suspend mid-logarithmic A549 cells in a prepared 5 mM HEPES buffer and incubate at 37 °C in the dark for 20 minutes. Subsequently, centrifuge at 1000 rpm for 5 minutes, aspirate, and discard the supernatant. Add growth medium at 37 °C to resuspend the cells, then centrifuge and wash twice. Culture the adherent cells on sterile glass coverslips, remove the coverslips, aspirate, and discard the excess culture medium, placing them in a humidity chamber. Add 100 μL of the dye working solution to cover the cells and incubate at 37 °C for 20 minutes. After incubation, discard the working solution and wash the cells twice with preheated growth medium. In each instance, cover the cells with the medium and incubate for 7 minutes before discarding it. The extent of membrane depolarisation was assessed by monitoring changes in fluorescence intensity of the DiSC(3)5 dye at an excitation wavelength of 622 nm and an emission wavelength of 670 nm.

3.4 Scanning Electron Microscopy Experiment

Following the resuscitation of A549 cells, they were inoculated into RPMI-1640 medium supplemented with 10% fetal bovine serum and cultured in an incubator at 37°C with 5% CO_2. Upon reaching 80% confluence, A549 cells were transferred into 96-well plates at a density of 1×10^5 cells per well. After 24 hours of culture, various treatment solutions were introduced, and the culture was continued for an additional 10 hours. Subsequent to drug treatment, the culture medium was aspirated and discarded. The cells were gently washed twice with PBS, followed by the addition of 2.5% glutaraldehyde fixative. They were then fixed at 4 °C for 2 hours. The cells were washed twice with PBS, centrifuged at 4000 RPM for 10 minutes, and subsequently dehydrated using 30%, 50%, 70%, 80%, 90%, and 100% ethanol for 15 minutes each. The samples were incubated in a refrigerator at 4 °C for 15 minutes before being centrifuged again at 4000 RPM for 15 minutes. The dehydrated samples were dispersed in anhydrous ethanol, and 10 μl was placed onto a slide. After drying the slide with an alcohol lamp, the surface was coated with gold for 30 seconds and finally examined under a scanning electron microscope[9].

4. Results and Discussion

4.1 Compound Experiment

Tyrocidine A(TA): >95% purity, 8 mg, 11.5% overall yield from spp.ms (ESI+): m/z calcd for C66H87N13O13+:1270.5 [M+H]+; Found: 1269.65.

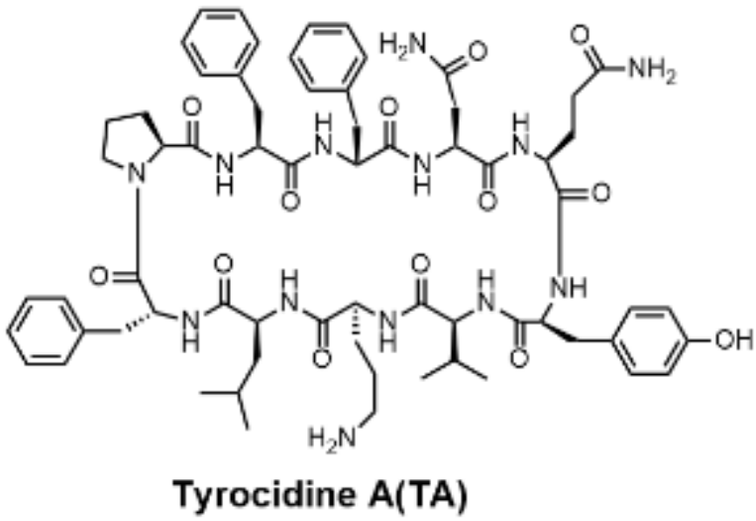

Figure 1. Structural formula of natural antimicrobial peptide TA

4.2 DAPI-PI Fluorescence Staining Experiment

In the TA group, the red fluorescence signal was the most intense, indicating a higher number of dead cells. Conversely, the blank group exhibited minimal red fluorescence, resembling the characteristics of healthy cells. The 5-FU and PT groups displayed only a slight presence of red fluorescence, with a relatively low proportion of dead cells. The diminished nuclei stained with DAPI and elevated dead cells stained with PI in the TA group serve as direct evidence of TA's efficacy in inducing apoptosis in A549 cells .

4.3 Plasma membrane depolarization

The results of the aforementioned experiments indicate a substantial increase in the fluorescence intensities of TA-1 and TA-2, whereas the fluorescence levels in the 5-FU, PT, 0.9% NaCl, and Control groups have remained approximately at zero. This observation suggests that the membrane potential regulatory activity of TA-1 and TA-2 on A549 cells is markedly more potent than that of the other test substances. The effect value exhibited a rapid increase following TA treatment, demonstrating that TA can significantly elicit a specific response in A549 cells under the given experimental conditions. Furthermore, the effect of TA-1 surpasses that of TA-2, which may be attributed to factors such as differences in formulation and the binding efficiency at the target site.

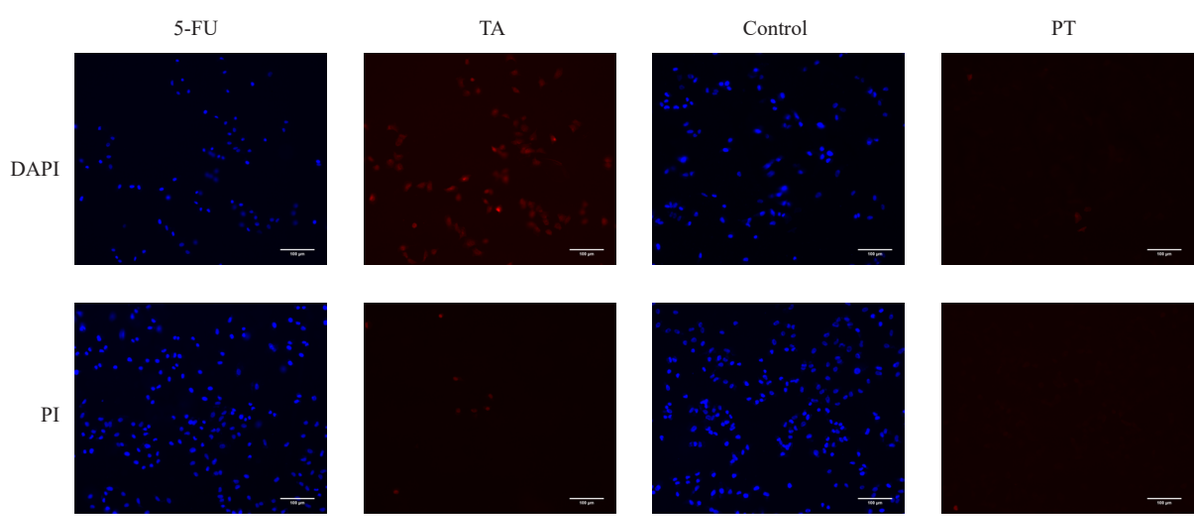

Figure 2. Fluorescence microscopy images of TA, 5-FU, and PT

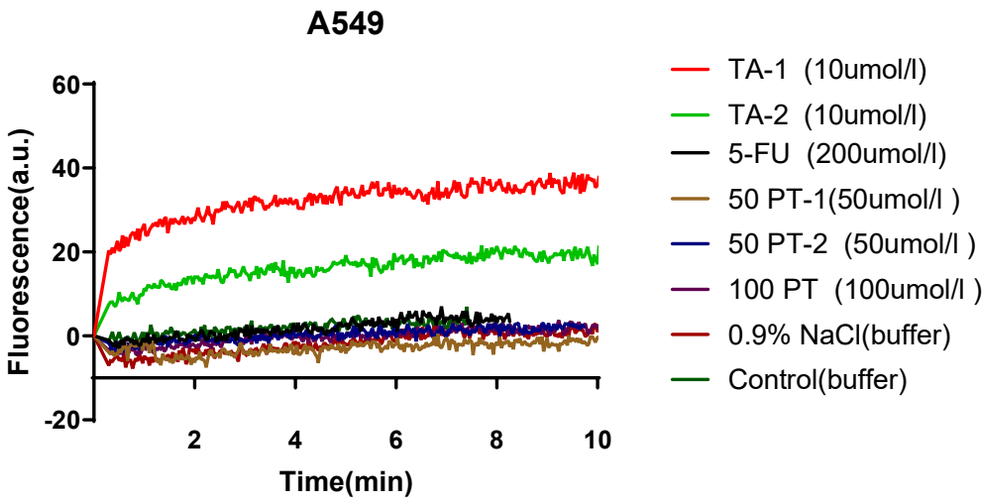

Figure 3. Cytoplasmic membrane depolarization results for TA, 5-FU, and PT

4.4 Scanning Electron Microscopy Experiment

The experimental findings revealed that the control group exhibited typical morphological features of standard in vitro cell culture. In the PT group, wrinkles on the cell membrane surface were slightly dense, yet the membrane remained continuous and intact. Similarly, the distribution of surface folds and membrane continuity in the 5-FU group mirrored those of the control group, with no evident morphological harm observed. Conversely, cells in the TA group displayed compromised membrane integrity, starkly differing from the control, PT, and 5-FU groups, suggesting that TA exerts a more pronounced detrimental impact on cellular structure. It is postulated that TA may induce cell morphology disintegration by damaging the cell membrane, cytoskeleton, and other cellular components.

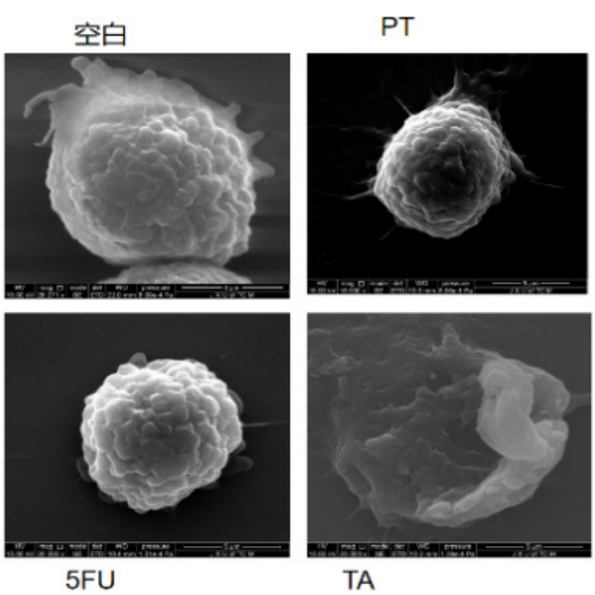

Figure 4. TA, 5-FU, PT scanning electron microscopy results

5. Conclusion

In conclusion, our experiments demonstrate that the cytotoxic effect of TA on tumour cells is more pronounced than that of 5-FU and PT, thereby further substantiating that TA induces apoptosis as a mechanism of its anti-tumour activity. Additionally, its selective toxicity towards tumour cells coupled with low cytotoxicity towards normal cells positions TA as a promising alternative to conventional chemotherapy agents. Future research will aim to further elucidate the role of TA in oncology.

Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (No. 22577044, 22307049).

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