The Mechanism Study and Target Modifications on the AMP Temporin-PEa Triggering TNF-α Necroptosis Pathway in Lung Cancer Cell Death

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Abstract: NSCLC is the most typical lung cancer with one of the highest lethal rates in the world. However, the drug resistance and poor prognosis make the conventional therapies less effective. A previous study has reported that a novel AMP temporin-PEa shows anticancer activity on NSCLC, which provides more potential for lung cancer treatment. This study investigates the antiproliferation mechanism of temporin-PEa, as well as the therapeutic effect of this AMP, and two other modified versions in both in vitro and in vivo conditions. The experiments will utilize NSCLC cell lines A549 and NCI-H157, and HMEC-1 cell line, and Xenograft mice models. Antiproliferation activity is measured by MTT assay. Cytotoxicity is detected by LDH assay, and haemolytic activity is detected using horse blood. The TNF-α necroptosis will be measured by Annexin V-FITC/PI assay and western blot. ROS over-generation will be measured by ROS assay kit with H2DCFDA ROS probe. The results of this study will provide important information for the exploration of the anticancer mechanism of AMPs and target modifications improving drug therapeutic values. Further studies should mainly focus on exploiting the specific target of temporin-PEa for further anticancer drug development, and in vivo drug delivery system design, which could help the drug overcome various biological barriers.

1 INTRODUCTION

Non-small-cell lung cancer (NSCLC) accounts for 85-90% of all lung cancers, and is one of the leading causes of cancer death in the world (R.L. Siegel, K.D. Miller, A. Jemal, Cancer Statistics, 2017). However, the poor prognosis and resistance to conventional chemotherapy and radiotherapy have greatly motivated the development of novel efficacious therapeutics in treating this lethal disease (Liu, Pei, Yang, Li, Amin, Liu, Buchan, Cho 2017).

Antimicrobial peptides (AMPs), which are commonly believed to show strong antimicrobial activity against a broad spectrum of microorganisms, have been reported to represent cell-line-dependent anticancer activity (Tornesello, Borrelli, Buonaguro, Buonaguro, Tornesello 2020). Compared to normal cells, cancer cell membranes exhibit more anionic, according to the changes of the tumor microenvironment, which might lead to dysregulation of phospholipid transporters (Ran, Downes, Thorpe 2002). Therefore, the electrostatic attraction between the anionic phospholipids of cancer cell membranes and the cationic peptides would play a vital role in the peptide-membrane interaction (Hoskin, Ramamoorthy 2008).

A recently discovered AMP temporin-PEa, generated through modification on a natural-derived peptide temporin-PE, has been reported to represent strong antiproliferation activity on lung cancer cells and low cytotoxicity on mammalian cells (Sang, Wu, Xi, Ma, Wang, Zhou, Burrows, Chen 2018). This enables temporin-PEa to become a proper candidate with high therapeutic effects for further drug development. However, the molecular mechanisms by which this peptide induces tumor cell death still remain poorly understood.

Phosphatidylserine (PS), accounting for 3-9% of the total amount of phospholipids, is negatively charged and normally exists on the inner leaflet of the cell membrane, and could be transferred to the outer membrane according to apoptosis or mutations in cancer cells (Birge et al 2016, Shklyar, Levy-Adam, Mishnaevski, Kurant 2013). Based on the literature, the outer membrane exposure of PS could be mediated by Tumour Necrosis Factor (TNF), which

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is significantly important for cellular homeostasis, and is a master regulator in cell necroptosis (Parvy, Yu, Dostalova, Kondo, Kurjan, Bulet, Lemaitre, Vidal, Cordero 2019, Kalliolias, Ivashkiv 2016). Necroptosis is a regulated cell death program without caspase activation, and it is mainly mediated through Receptor-Interacting Protein Kinase 1/3 (RIPK1/3) and Mixed Lineage Kinase Domain-Like (MLKL)(Gong et al 2019). Necroptosis plays a pivotal role in oncogenesis, cancer metastasis, and cancer immunity (Seehawer 2018). In the necroptosis signaling pathway, the interaction of TNF-α and TNFR would induce a downstream signaling cascade, followed by energy depletion due to reactive oxygen species (ROS) over-generation, and ultimately leads to cell death (**Figure 1**) (Christofferson, Yuan 2010, Vanden Berghe 2014).

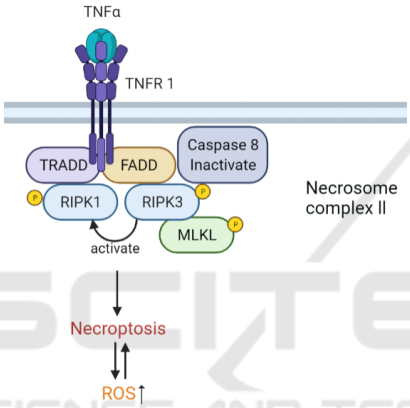

Figure 1: Schematic of TNF-α-induced necroptosis pathway and ROS accumulation.

TRADD: TNF receptor-associated death domain; FADD: Fas-associated cell death domain.

Therefore, I hypothesize that temporin-PEa exhibits certain antiproliferation activity on NSCLC via the TNF-α-induced necroptosis pathway and ROS over-generation. In this study, the molecular mechanisms of temporin-PEa inducing NSCLC cell death are investigated. Also, the bioactivities of temporin-PEa and two other modified peptides are studied, in both *in vitro* and *in vivo* occasions.

2 MATERIALS AND METHODS

2.1 Peptides Design and Synthesis

In order to improve the cell-penetrating activity and better investigate the intracellular mechanism of temporin-PEa, two analogues are designed. The RGD peptide (Arg-Gly-Asp) and the TAT (GRKKRRQRRR) peptide have been reported to effectively deliver drugs as the cell-penetrating peptide (CPP) in treating lung cancers, as described previously (Duan et al 2017, Diao et al 2012). Therefore, RGD is added to the N-terminus of the template peptide, generating the novel peptide RGD-temporin-PEa (RGD-PEa). Meanwhile, the other analogue is designed by linking the TAT sequence to the C-terminus of the original peptide, namely TAT-PEa. The specific sequences are listed in **Table 1**.

Table 1: Anticancer peptide sequences.

Peptide	Sequence
Temporin-PEa	FLYIVAKLLSGLL-NH_2
RGD-PEa	RGD- FLYIVAKLLSGLL-NH_2
PEa-TAT	FLYIVAKLLSGLL-GRKKRRQRRR-NH_2

*-NH_2: C-terminal amidation

Temporin-PEa and the other modified peptides will be synthesized using a Tribute 2-channel automated peptide synthesizer through solid-phase peptide synthesis. Briefly, the process will employ resin with rink amide and standard Fmoc protection chemistry, and will be performed in the PS4 peptide synthesizer. Then the crude peptides will be purified by the RP HPLC and then characterized by MALDI-TOF MS.

2.2 Cell Lines, Cell Culture, and Chemicals

Human NSCLC cell lines A549, NCI-H157 will be obtained and cultured in the medium of DMEM/F12 with 10% FBS and 1% penicillin-streptomycin. Human microvascular epithelial cell line HMEC-1 will be cultured in a full-growth MCDB-131 medium with 10 mM L-Glutamine as well as 10 ng/mL of epidermal growth factor. The specific cell lines are shown in **Table 2**.

Table 2: Cell lines applied in this study.

Cell line	Cell Type
NCI-H157	Human Non-small cell lung cancer cell
A549	Human Non-small cell lung cancer cell
HMEC-1	Human microvascular epithelial cell

2.3 MTT Cell Proliferation Assay

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) cell viability assay is utilized to evaluate the antiproliferation activity of temporin-PEa, and its analogues.

Firstly, the cells with the amount of 5× 103 cells per well will be plated in the 96-well plate and cultured under 5% CO2 at 37°C for 24 h. The cells will be then starved by the medium without FBS for 6 h, followed by treatment with three peptides gradient dilutions (10-9-10-4 M) for 24 h. Meanwhile, PBS solution will be used in the vehicle control group, and 1% Triton X-100 will be utilized as the positive control. After incubation for another 24 h, a total amount of 10 μL MTT with the concentration of 5 mg/mL will be added to each well and co-incubated for 4 h at the conventional incubator. Finally, after removing the medium, a certain amount of DMSO will be added to each well, and the optical density (OD) value of each well will be subsequently measured by a Synergy HT plate reader at the wavelength of 490 nm. After the experiment, the half-maximal inhibitory concentration (IC50) values of three peptides will be determined by GraphPad Prism Software. Also, the Geometric Mean (GM) of each peptide inhibiting cancer cell growth is calculated.

2.4 Lactate Dehydrogenase Release (LDH) Assay

LDH will release from the damaged cells, and the cell membrane integrity will be measured to estimate the cytotoxicity of the three peptides on normal cells. The degree of LDH release from destroyed cells after peptide treatment will be determined by the Pierce LDH Cytotoxicity Assay Kit. In brief, cells with a density of 5 × 103 each well will be seeded into 96-well plates and incubated for 24 h. The cells will be treated with a range of doses (10-9-10-4 M) of the three peptides or PBS solution for 24 h incubation as sample groups and negative control group, respectively. Also, in the positive control group, cells will be mixed with 1% Triton X-100 and then incubated at 37°C under 5% CO2 for 30 min to acquire a relatively maximum LDH release. After the incubation, the cell supernatant of each group will be transferred to another 96-well plate, and the reaction buffer will be added with incubation for about half an hour at room temperature. At last, the stop solution will be added, and the OD value will be determined at the wavelength of 490 nm. Also, the therapeutic index (TI) of each peptide will be calculated as the ratio of the IC50 value for the HMEC-1 cell line to the GM value for two NSCLC cell lines.

2.5 Haemolysis Assay

The haemolytic effect of three peptides will be determined using defibrinated horse red blood cells. A total volume of 100 μL peptide serial dilutions, ranging from 1 to 128 μM, will be incubated with an equal volume of 4% erythrocytes for 120 min at 37°C. The positive control and negative control groups will contain the same volume of 2% Triton X-100 and PBS solution, respectively. Then, the OD value of each well will be measured at 550 nm.

2.6 Annexin V-PI Assay

Cancer cells (1×106) will be treated with peptide solution at half IC50, IC50, and 2×IC50 concentrations. Z-VAD-FMK, the pan-caspase inhibitor, followed by TNF-α, and PBS solution will be utilized in the positive control the negative control group, respectively. The Annexin V-FITC/PI assay will be conducted based on the protocol, as reported previously (Yu et al 2019). Briefly, after incubation for 24 h, cells will be rinsed with PBS solution, trypsinized, and resuspended in the binding buffer. Then, the cell suspension will be incubated with Annexin V-FITC and propidium iodide (PI) for 5 min at room temperature in the dark. The cells will then get analyzed for morphological changes and DNA content by flow cytometry with a ZE5 Cell Analyzer.

2.7 Western Blot Analysis

Harvested cells will be lysed with 400 μl of lysis buffer. Protein concentration will be determined according to the Bradford method. A certain amount of protein extraction will be loaded into SDS gels to separate the protein components. After the membrane transfer, the blotted membrane will be blocked with skim milk for 60 min at room temperature and incubated with the appropriate primary antibody for 10 h at 4˚C. At last, HRP will be coupled with appropriate secondary antibodies. After 60 min, signals from the combined antibodies will be detected by using the chemiluminescence kit.

2.8 ROS Accumulation Detection

The ROS assay kit will be used in this section. A total amount of cells with a density of 5×105 per dish (10 cm) will be incubated for 24 h, and then will be treated with the peptide solution at half IC50, IC50, and 2×IC50 for a specific time length. Ten microliters of the ROS probe H2DCFDA will be added to the cells suspension after PBS-rinsing, and incubated for

at 37˚C, 5% CO2 for half an hour in the dark. Then, cells will be treated with trypsin and instantly analyzed for DCF fluorescence intensity by flow cytometry. Hydrogen peroxide (H2O2) dilution and PBS will be used as the positive control and negative control respectively.

2.9 In Vivo Antiproliferation Assay

The cell line that peptides exhibit a stronger anticancer level will be chosen to conduct the in vivo assay. Also, the peptide with the highest TI value and no significant haemolytic activity will be selected for the in vivo assay.

In vivo antiproliferation will be detected by subcutaneous injection of a total amount of 2×106 cancer cells in the right flank of the seven-week-old BALB/c-nu/nu nude mice, based on the Guide for the Care and Use of Laboratory Animals published by the US NIH. When tumor volumes reach 200 mm3, mice will be divided into six groups, including a negative control group treated with PBS solution, a positive control group treated with Cisplatin (DDP), and three doses of sample groups treated with a high, medium, and low concentration of peptide solutions, respectively. Moreover, mice with no tumor will be treated as the blank group.

Mice of peptide groups will receive intratumoral injections on a daily basis, with a volume of 20 μL of peptide solutions with different concentrations for two weeks. Additionally, mice in the negative control group will be daily treated with 20 μL PBS solution, while the mice in the positive control group will receive the seven-day intraperitoneal injections with 200 μL DDP and then will be continuously fed until day 14. Tumour growth inhibition will be then detected by tumor size measurement twice a week using a digital caliper.

2.10 Statistical Analysis

Each experiment will be repeated five times. And the statistical significance of data acquired will be analyzed with the student's T-Test or one-way ANOVA on GraphPad Prism software at ($p < 0.05$).

3 RESULTS

3.1 Possible Results on the Mechanisms of the Peptide Temporin-Pea Inducing Lung Cancer Cell Death (The Overview of Four Possible Results is Demonstrated in Table 3).

Possible Result 1: Temporin-PEa induces lung cancer cell death via the TNF-α necroptosis pathway and ROS over-generation.

Based on western blot analysis, there is a significant up-regulation of the expression level of TNF-α, phosphorylated MLKL (pMLKL), pRIPK1, and pRIPK3, and no significant changes in the level of the procaspase-8 as well as the cleaved caspase-8 expression. Also, ROS accumulation is detected in the peptide-treated cells.

Possible Result 2: Temporin-PEa induces lung cancer cell death via the TNF-α necroptosis pathway, but not ROS over-generation.

Based on western blot analysis, there is a significant up-regulation of the expression level of TNF-α, pMLKL, pRIPK1, and pRIPK3, and no significant changes in the level of the procaspase-8 as well as the cleaved caspase-8 expression. However, ROS accumulation is not detected.

Possible Result 3: Temporin-PEa induces lung cancer cell death through ROS over-generation, but not the TNF-α necroptosis pathway.

After the treatment of the peptide, the expression levels of TNF-α, pMLKL, pRIPK1, and pRIPK3 have not been increased significantly, and/or there are significant changes in the expression level of procaspase-8 and cleaved caspase-8. However, ROS accumulation is detected.

Table 3: Possible results on the mechanisms of the peptide temporin-PEa inducing lung cancer cell death.

	Result 1	Result 2	Result 3	Result 4
TNF-α	+	+	-	-
pRIPK 1/3	+	+	-	-
pMLKL	+	+	-	-
Procaspase 8	-	-	+	+
Cleaved caspase 8	-	-	+	+
ROS over-generation	+	-	+	-

Note. "+" represents a significant increase from the negative control. "-" represents there is no significant difference from the negative control.

Possible Result 4: Temporin-PEa induces lung cancer cell death via other signalling pathways, instead of the TNF-α necroptosis pathway and ROS over-generation.

After the treatment of the peptide, the expression level of TNF-α, phosphorylated RIPK1, phosphorylated RIPK3, and phosphorylated MLKL has not been increased significantly, and/or there are significant changes in procaspase-8 and cleaved caspase-8 expression. Also, ROS accumulation is not detected.

3.2 Possible Results on the Therapeutic Index (TI) of the Peptides (The Overview of Four Possible Results is Demonstrated in Table 4).

Possible Result 5: RGD-PEa shows the highest TI value, while PEa-TAT and temporin-PEa show the second and the third TI value respectively.

According to the peptide antiproliferation activity on lung cancer cells and cytotoxicity to normal cells, the peptide RGD-PEa exhibits the highest TI value among the three peptides, while peptide PEa-TAT shows the second TI value, and temporin-PEa shows the lowest.

Possible Result 6: RGD-PEa shows the highest TI value, while temporin-PEa and PEa-TAT show the second and the third antiproliferation activity respectively.

Based on the antiproliferation activity and cytotoxicity of the peptide, RGD-PEa exhibits the highest TI value among the three peptides, while temporin-PEa shows the medium TI value, and PEa-TAT shows the lowest.

Possible Result 7: PEa-TAT shows the highest TI value, and RGD-PEa and temporin-PEa show the second and the third TI value respectively.

Based on the antiproliferation activity and cytotoxicity of the peptide, PEa-TAT exhibits the highest TI value among the three peptides, while peptide RGD-PEa shows the second TI value, and temporin-PEa shows the lowest.

Possible Result 8: PEa-TAT shows the highest TI value, and temporin-PEa, as well as RGD-PEa shows the second and the third TI value respectively.

According to the peptide antiproliferation activity on lung cancer cells and cytotoxicity to normal cells, the peptide PEa-TAT exhibits the highest TI value among the three peptides, while temporin-PEa shows the medium level of TI value, and RGD-PEa shows the lowest.

Table 4: Possible results on the TI values of the three peptides.

	Result 5	Result 6	Result 7	Result 8
RGD-PEa	+++	+++	++	+
PEa-TAT	++	+	+++	+++
Temporin-PEa	+	++	+	++

Note. "+" represents the peptide with the lowest TI value, "++" represents the peptide with the medium TI value, and "+++" represents the peptide that shows the highest TI value

3.3 Possible Results on The Haemolytic Activity of The Peptides (The Overview of Four Possible Results is Demonstrated in Table 5).

Possible Result 9: None of the peptides shows significant haemolytic activity.

Compared with the negative control, all of the three peptides display no significant haemolytic activity on mammalian red blood cells.

Possible Result 10: RGD-PEa and temporin-PEa both show no significant haemolytic activity, while PEa-TAT shows significant haemolytic activity.

Compared with the negative control, the peptide RGD-PEa and temporin-PEa both display no significant haemolytic activity on red blood cells. However, PEa-TAT exhibits significant haemolytic activity.

Possible Result 11: PEa-TAT and temporin-PEa both show no significant haemolytic activity, while RGD-PEa shows significant haemolytic activity.

Compared with the negative control, the peptide RGD-PEa and temporin-PEa both display no significant haemolytic activity. However, PEa-TAT exhibits significant haemolytic activity.

Possible Result 12: Both PEa-TAT and RGD-PEa show significant haemolytic activity, and only temporin-PEa shows no significance with the negative control.

Both of the modified peptides exhibit significant haemolytic activity, and only the original peptide temporin-PEa shows no significance with the negative control group.

Table 5: Possible results on the haemolytic activity of three peptides.

	Result 9	Result 10	Result 11	Result 12
RGD-PEa	-	-	+	+
PEa-TAT	-	+	-	+
Temporin-PEa	-	-	-	-

Note. "+" represents a significant difference in haemolytic activity from the negative control. "-" represents there is no significant difference from the negative control.

3.4 Possible Results on the *In Vivo* Antiproliferation Activity of the Peptides (The Overview of Four Possible Results is Shown in Table 6).

Possible Result 13: The chosen peptide represents stronger antiproliferation activity on the NCI-H157 cancer cell line, and induces significant lung cancer cell death *in vivo*.

In the *in vitro* detection, the chosen peptide shows stronger antiproliferation activity on the NCI-H157 cancer cell line, which is used to establish *in vivo* models. Also, the peptide induces significant lung cancer cell death in the animal model.

Possible Result 14: The chosen peptide represents stronger antiproliferation activity on the NCI-H157 cancer cell line, but does not show significant *in vivo* antiproliferation activity on lung cancer.

In the *in vitro* detection, the chosen peptide shows stronger antiproliferation activity on the NCI-H157 cancer cell line, which is used to establish *in vivo* models. However, the peptide does not induce significant lung cancer cell death in the animal model.

Possible Result 15: The chosen peptide represents stronger antiproliferation activity on the A549 cancer cell line, and induces significant lung cancer cell death *in vivo*.

In the *in vitro* detection, the chosen peptide shows stronger antiproliferation activity on the A549 cancer cell line, which is used to establish *in vivo* models. Also, the peptide induces significant lung cancer cell death in the animal model.

Possible Result 16: The chosen peptide represents stronger antiproliferation activity on the A549 cancer cell line, but does not show significant *in vivo* antiproliferation activity on lung cancer.

In the *in vitro* detection, the chosen peptide shows stronger antiproliferation activity on the A549 cancer cell line, which is used to establish *in vivo* models. However, the peptide does not induce significant lung cancer cell death in the animal model.

3.5 Additional Possible Results on the Temporin-Pea and Modification Peptides Different from Previous Researches

Possible Results 17: Compared with temporin-PEa, modified peptides RGD-PEa and PEa-TAT both show lower TI values.

Temporin-PEa shows the highest TI value, while the other two modification strategies decrease the PI value of the original peptide.

Possible Results 18: The chosen peptide shows no significant difference in antiproliferation activity between the two cancer cell lines.

In the *in vitro* detection, the chosen peptide shows no significant difference in the anticancer activity between NCI-H157 and A549 cancer cell lines. In this case, cell line NCI-H157 is used for mechanism study and *in vivo* detection.

Table 6: Possible results on the *in vivo* antiproliferation activity of the chosen peptide.

	Result 13	Result 14	Result 15	Result 16
Cell line for xenograft model	NCI-H157	NCI-H157	A549	A549
***In vivo* antiproliferation**	+	-	+	-

Note. "+" represents a significant difference in antiproliferation activity from the negative control. "-" represents there is no significant difference from the negative control.

4 DISCUSSION

Previous studies report that AMPs show certain anticancer activity in a cell-line-dependent manner (Tornesello, Borrelli, Buonaguro, Buonaguro, Tornesello 2020). An AMP temporin-PEa has been reported to present strong antiproliferation activity on human lung cancer cells (Sang, Wu, Xi, Ma, Wang, Zhou, Burrows, Chen 2018). To unravel the molecular mechanisms of this peptide-inducing lung cancer cell death, this study further investigates whether temporin-PEa would trigger the TNF-α necroptosis signaling pathway and ROS accumulation. Also, in order to assess potential target modification strategies to improve the therapeutic value of the peptide, the *in vitro* bioactivity assays are designed, and the peptide with no significant haemolytic activity and relatively higher TI value is used for xenograft animal model detection.

Possible Result 1 shows that temporin-PEa would induce lung cancer cell death through the TNF-α necroptosis pathway and ROS accumulation, which fully supports the hypothesis of this study. According to the literature, there are few studies on the exploration of temporin-peptide inducing cancer cell death, and the mechanisms include membrane destruction, intracellular Ca^{2+} leakage, and inducing apoptosis pathway (Wang et al 2013, Shaheen et al 2018). Here, temporin-PEa would trigger the TNF-α necroptosis pathway and ROS accumulation, which could provide a new research direction for the mechanism study on the anticancer activity of AMPs from the temporin family. This might also provide a new strategy for novel anticancer drug development based on the structure of temporin-PEa. Moreover, further investigation should be conducted on the specific intracellular target of the peptide for target drug delivery system design.

ROS accumulation, which always occurs downstream of the necroptosis pathway, can lead to energy depletion and initiate oxidative stress-induced cancer cell death (Moloney 2018). In Possible Result 2, ROS accumulation is not detected, which might indicate that there are other mechanisms as the downstream of TNF-α necroptosis pathway, including ER (endoplasmic reticulum) stress, mitochondria membrane disruption, and Ca^{2+} leakage (Kim, Kim 2018, Wang, Zhou, Li, Li, Tian, Wang 2013).

The negative results of temporin-PEa not triggering the TNF-α necroptosis pathway are described in Possible Results 3 and 4. Possible Result 3 might indicate that lung cancer cell death is induced by other signaling pathways, such as the apoptosis pathway or FasL-mediated necroptosis pathway, which might trigger ROS over-generation as well (Otani 2018, Sauler, Bazan, Lee 2019). This could be further verified by flow cytometry and western blot detection. In Possible Result 4, neither TNF-α necroptosis nor ROS over-generation is detected, which contradicts the hypothesis and probably means that temporin-PEa could directly break the cancer cell membrane instead of triggering the intracellular pathway. Based on the two results, further investigation should be done on the molecular mechanisms of temporin-PEa inducing lung cancer cell death.

In order to improve the therapeutic value of the original peptide, two modifications are conducted on temporin-PEa. Possible Results 5 and 7 show that both of the modified peptides have higher TI value than temporin-PEa, which is consistent with former studies and support the hypothesis of this study (Diao et al 2012, Hu, Chen, Huang, Chen 2018). This would indicate that both RGD and TAT motifs could increase the cell penetration activity and tumor selectivity of the peptide, and thus increase the anticancer activity while reducing the cytotoxicity to normal cells. RGD motif shows better efficacy in Possible Result 5, while TAT peptide behaves better in Possible Result 7. While in Possible Results 6 and 8, only one of the two modification strategies could improve the therapeutic efficacy.

Possible Result 9 demonstrates that all of the three peptides show no haemolytic activity, which enables them to be proper candidates for further clinical therapeutic development. Except for temporin-PEa, PEa-TAT and RGD-PEa show significant cytotoxicity or haemolytic activity in Possible Results 10 and 11 respectively. The modified peptide, which is harmful to the normal cells, cannot be utilized for *in vivo* experiments or further clinical research. Both of the modified peptides display significant haemolytic activity in Possible Result 12, which suggests that the novel peptide might generate new structures that change the properties of the original peptide and result in haemolysis. These two modification strategies are not suitable for temporin-PEa so that further modification approaches should be studied. Based on the Possible Results (5-12), the peptide with no haemolytic activity and relatively higher TI value is chosen for *in vivo* detection.

Possible Results 13-16 demonstrate the *in vivo* detection of the chosen peptide. The results that the peptide shows higher anticancer activity on the NCI-H157 cell line are listed in Possible Results 13 and 14. This is consistent with the former study, as temporin-PEa exhibits strong anticancer activity on

the NCI-H157 cell line. Possible Results 15 and 16 show that the chosen peptide has better anticancer activity on the A549 cancer cell line, which indicates that the peptide might behave differently in various lung cancer cell types. Also, Possible Results 14 and 16 demonstrate that the peptide would probably show no antiproliferation activity in the xenograft animal models, which might due to the biological barriers and poor drug delivery efficiency.

The Possible Result 17 contradicts the hypothesis and the current understanding of RGD and TAT peptides improving drug therapeutic value. This might indicate systemic errors of the experiment design, and these two modification strategies cannot promote the peptide cell-penetration ability or tumor selectivity. Thus, further studies on peptide structure-function relationship as well as peptide modifications should be carried out. In Possible Result 18, the peptide shows no significant difference between NCI-H157 and A549 cell lines, which would indicate that the choice of the two cancer cell lines might not be appropriate, and the antiproliferation activity of the peptide exhibiting on other lung cancer cell lines should be further exploited.

5 CONCLUSION

In summary, this study investigates the molecular mechanisms of the AMP temporin-PEa inducing lung cancer cell death, and target modifications on this peptide. The results of this study will test the hypothesis that whether the peptide would induce lung cancer cell death via the TNF-α necroptosis pathway and ROS over-generation, and whether the RGD and TAT motifs will enhance the therapeutic value of the peptide.

The possible results on the anticancer mechanism of temporin-PEa indicate that the peptide would trigger TNF-α necroptosis or other pathways followed by energy depletion processes. Also, the results might suggest that there would be a signaling network of AMPs triggering immunogenic cell death (ICD), which involves both death-receptor signaling pathways and the engagement of other organelles. Additionally, the possible results on CPP modifying temporin-PEa would provide potential peptide modification strategies for further anticancer drug development. However, novel drug delivery systems should get further studied to help the peptide overcome the biological barriers, and to reduce cytotoxicity to normal cells.

Researchers have started to explore the anticancer activity of AMPs in recent years, and the detailed understanding of the intracellular mechanisms of AMPs still remains largely unclear. Therefore, more studies on the AMPs anticancer mechanisms should be conducted to provide more therapeutic potentials for peptide biologics development.

REFERENCES

A.L. Tornesello, A. Borrelli, L. Buonaguro, F.M. Buonaguro, M.L. Tornesello, Antimicrobial Peptides as Anticancer Agents: Functional Properties and Biological Activities, Molecules 25(12) (2020).

B. Shklyar, F. Levy-Adam, K. Mishnaevski, E. Kurant, Caspase activity is required for engulfment of apoptotic cells, Mol Cell Biol 33(16) (2013) 3191-201.

C. Hu, X. Chen, Y. Huang, Y. Chen, Synergistic effect of the pro-apoptosis peptide kla-TAT and the cationic anticancer peptide HPRP-A1, Apoptosis 23(2) (2018) 132-142.

C. Kim, B. Kim, Anti-Cancer Natural Products and Their Bioactive Compounds Inducing ER Stress-Mediated Apoptosis: A Review, Nutrients 10(8) (2018).

C. Wang, L.L. Tian, S. Li, H.B. Li, Y. Zhou, H. Wang, Q.Z. Yang, L.J. Ma, D.J. Shang, Rapid cytotoxicity of antimicrobial peptide tempoprin-1CEa in breast cancer cells through membrane destruction and intracellular calcium mechanism, PLoS One 8(4) (2013) e60462.

C. Wang, Y. Zhou, S. Li, H. Li, L. Tian, H. Wang, D. Shang, Anticancer mechanisms of temporin-1CEa, an amphipathic alpha-helical antimicrobial peptide, in Bcap-37 human breast cancer cells, Life Sci 92(20-21) (2013) 1004-14.

D.E. Christofferson, J. Yuan, Necroptosis as an alternative form of programmed cell death, Curr Opin Cell Biol 22(2) (2010) 263-8.

D.W. Hoskin, A. Ramamoorthy, Studies on anticancer activities of antimicrobial peptides, Biochim Biophys Acta 1778(2) (2008) 357-75.

F. Shaheen, M. Nadeem-Ul-Haque, A. Ahmed, S.U. Simjee, A. Ganesan, A. Jabeen, Z.A. Shah, M.I. Choudhary, Synthesis of breast cancer targeting conjugate of temporin-SHa analog and its effect on pro- and anti-apoptotic protein expression in MCF-7 cells, Peptides 106 (2018) 68-82.

G. Liu, F. Pei, F. Yang, L. Li, A.D. Amin, S. Liu, J.R. Buchan, W.C. Cho, Role of Autophagy and Apoptosis in Non-Small-Cell Lung Cancer, Int J Mol Sci 18(2) (2017).

G.D. Kalliolias, L.B. Ivashkiv, TNF biology, pathogenic mechanisms and emerging therapeutic strategies, Nat Rev Rheumatol 12(1) (2016) 49-62.

J.N. Moloney, T.G. Cotter, ROS signalling in the biology of cancer, Semin Cell Dev Biol 80 (2018) 50-64.

J.P. Parvy, Y. Yu, A. Dostalova, S. Kondo, A. Kurjan, P. Bulet, B. Lemaitre, M. Vidal, J.B. Cordero, The antimicrobial peptide defensin cooperates with tumour necrosis factor to drive tumour cell death in Drosophila, Elife 8 (2019).

M. Sang, Q. Wu, X. Xi, C. Ma, L. Wang, M. Zhou, J.F. Burrows, T. Chen, Identification and target-modifications of temporin-PE: A novel antimicrobial peptide in the defensive skin secretions of the edible frog, Pelophylax kl. esculentus, Biochem Biophys Res Commun 495(4) (2018) 2539-2546.

M. Sauler, I.S. Bazan, P.J. Lee, Cell Death in the Lung: The Apoptosis-Necroptosis Axis, Annu Rev Physiol 81 (2019) 375-402.

M. Seehawer, F. Heinzmann, L. D'Artista, J. Harbig, P.F. Roux, L. Hoenicke, H. Dang, S. Klotz, L. Robinson, G. Dore, N. Rozenblum, T.W. Kang, R. Chawla, T. Buch, M. Vucur, M. Roth, J. Zuber, T. Luedde, B. Sipos, T. Longerich, M. Heikenwalder, X.W. Wang, O. Bischof, L. Zender, Necroptosis microenvironment directs lineage commitment in liver cancer, Nature 562(7725) (2018) 69-75.

R.B. Birge, S. Boeltz, S. Kumar, J. Carlson, J. Wanderley, D. Calianese, M. Barcinski, R.A. Brekken, X. Huang, J.T. Hutchins, B. Freimark, C. Empig, J. Mercer, A.J. Schroit, G. Schett, M. Herrmann, Phosphatidylserine is a global immunosuppressive signal in efferocytosis, infectious disease, and cancer, Cell Death Differ 23(6) (2016) 962-78.

R.L. Siegel, K.D. Miller, A. Jemal, Cancer Statistics, 2017, CA Cancer J Clin 67(1) (2017) 7-30.

S. Ran, A. Downes, P.E. Thorpe, Increased exposure of anionic phospholipids on the surface of tumor blood vessels, Cancer Res 62(21) (2002) 6132-40.

T. Otani, M. Matsuda, A. Mizokami, N. Kitagawa, H. Takeuchi, E. Jimi, T. Inai, M. Hirata, Osteocalcin triggers Fas/FasL-mediated necroptosis in adipocytes via activation of p300, Cell Death Dis 9(12) (2018) 1194.

T. Vanden Berghe, A. Linkermann, S. Jouan-Lanhouet, H. Walczak, P. Vandenabeele, Regulated necrosis: the expanding network of non-apoptotic cell death pathways, Nat Rev Mol Cell Biol 15(2) (2014) 135-47.

W.N. Yu, Y.J. Lai, J.W. Ma, C.T. Ho, S.W. Hung, Y.H. Chen, C.T. Chen, J.Y. Kao, T.D. Way, Citronellol Induces Necroptosis of Human Lung Cancer Cells via TNF-alpha Pathway and Reactive Oxygen Species Accumulation, In Vivo 33(4) (2019) 1193-1201.

Y. Diao, W. Han, H. Zhao, S. Zhu, X. Liu, X. Feng, J. Gu, C. Yao, S. Liu, C. Sun, F. Pan, Designed synthetic analogs of the alpha-helical peptide temporin-La with improved antitumor efficacies via charge modification and incorporation of the integrin alphavbeta3 homing domain, J Pept Sci 18(7) (2012) 476-86.

Y. Gong, Z. Fan, G. Luo, C. Yang, Q. Huang, K. Fan, H. Cheng, K. Jin, Q. Ni, X. Yu, C. Liu, The role of necroptosis in cancer biology and therapy, Mol Cancer 18(1) (2019) 100.

Z. Duan, C. Chen, J. Qin, Q. Liu, Q. Wang, X. Xu, J. Wang, Cell-penetrating peptide conjugates to enhance the antitumor effect of paclitaxel on drug-resistant lung cancer, Drug Deliv 24(1) (2017) 752-764.