

**THE PREPARATION AND CHARACTERIZATION
OF PRO-APOPTOTIC PEPTIDE ALA-VAL-PRO-ILE AND
ITS DERIVATIVES**

A Thesis
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by
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ABSTRACT

The tetra-peptide sequence alanine-valine-proline-isoleucine (AVPI) is derived from a known inhibitor of apoptosis inhibitor proteins (IAPs) called Smac (second mitochondria-derived activator of caspases). Ala-Val-Pro-Ile can be further utilized as an anti-cancer agent by inhibiting the activities of apoptosis inhibitors so caspases can trigger apoptosis of cancer cells. AVPI, however, has poorly cell-penetration property thus limiting its ability to be utilized as a therapeutic agent for cancer treatments. We conjugated the AVPI molecule to a newly developed cell-penetrating peptide (CPP) called PepB to circumvent the situation of limited cellular availability. Solid Phase Peptide Synthesis (SPPS) methods have been utilized to prepared AVPI peptide derivatives. Key characterizations involve reverse-phase high-performance liquid chromatography (RP-HPLC), mass spectrometry, optical and fluorescence microscopy.

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CHAPTER 1

INTRODUCTION

1.1 Cancer Statistics and Facts

Cancer is a group of related diseases involves abnormal cell growth with the latent to invade or disperse to other parts of the body. A research from WHO (World Health Organization) shows that cancer is one of the major causation of morbidity and mortality worldwide, with an estimation of 14.1 million new cases, and 8.2 million people dying of cancer in 2014. Another study conducted by National Cancer Institute illustrated 1.7 million people in the United States in the year of 2017 had been diagnosed with cancer, also estimated 600,920 people died of cancer. The data demonstrated one of two hundred people in the United States had been diagnosed with cancer last year, and every 53 seconds there was a patient died of cancer. Breast cancer turned out to be the most common cancer diagnosis in 2017 which had 268,670 new cases, and the following was lung cancer with 234,030 new cases. In 2018, in the United States, there is approximately 609,640 people will die of cancer. Lung and bronchus cancer will have 154,054 people died, which is the most cause of cancer death. The most popular cancer treatments involve in: surgery, radiation therapy, immunotherapy and chemotherapy. Surgery is to remove cancer cells or tumors from a patient's body, which is painful and will take time to heal the operation.

Radiation therapy is to use high dose radiation to slow cancer cell grow or kill cancer cells, which can influence healthy cells around cancer cells and can also cause hair loss. Immunotherapy is to use drug or antibody to boost your immune system to against cancer, and patients might feel pain or swelling after treatment. Chemotherapy is to use chemical drug to kill cancer cells, however, there will be some drawbacks. For instance, Paclitaxel is used for breast, ovarian and lung cancer but its water-insoluble (0.7 mg/mL) so polyethoxylated castor oil (Cremophor EL) is always utilized to increase solubility. However, this will cause severe hypersensitivity^[1]. In this case, it is great utility to develop anti-cancer treatment since there is no effective way to cure cancer at present.

1.2 Drug Delivery Limitations

The development of peptides and proteins as therapeutic agents is, more often than not, deterred by the limited cellular incorporation abilities. Some bioactive peptidic (and proteinaceous) molecules are easily eliminated during blood circulation and have difficulty entering cells through the lipid bilayer, so overcoming the uptake is crucial in peptide drug delivery^[2]. Peptide therapeutic drugs, in addition, suffer from non-specific incorporating and side effects leading to the death of healthy cells^[3]. The cellular lipid bilayer (barrier) protects living cells by selective exclusion of macromolecules while allowing specific small molecules to pass through the barrier^[4]. This barrier leads to molecular engineering design challenges, since certain bioactive peptides can have their targets inside the cells and getting the therapeutic molecule to the target is a prerequisite

for modulating cellular pathways. Therefore, if we want to target cancer cell pathways inside the cell, it is necessary to design active non-viral drug systems to allow the anti-cancer therapeutics to enter the cancer cells and perform their function.

1.3 AVPI Cancer Killing Ability

Cell apoptosis is known as programmed cell death; the process is vital to sustain the life of organisms. If cells fail to undergo apoptosis, many diseases will occur: abnormal inhibition of apoptosis is a hallmark of cancer^[5]. Understanding of the specific apoptotic pathways that are involved in many diseases, such as cancer and neurodegenerative disorders, is necessary^[6].

Smac is known as a second mitochondria-derived activator of caspases, which is also called as DIABLO^[7]. Smac is a precursor of mitochondrial protein which has 239 amino acids. Its N-terminal sequence is the active domain for Smac function, the 55 residues of amino terminal work as the mitochondria targeting sequence and it will be taken off after import^[8]. Smac/DIABLO is the protein produced by mitochondria and can be released into the cytoplasm in response to apoptotic stimulus^[9]. There are two major pathways which can lead to apoptosis: intrinsic and extrinsic. Inhibitors of apoptosis are proteins which mainly affect the intrinsic pathway that inhibits programmed cell death. Smac can facilitate apoptosis by antagonizing inhibitor of apoptosis proteins (IAPs) by interacting with IAPs through its N-terminal AVPI. The AVPI sequence can promote apoptosis, so there is great utility in mimicking Smac^[10].

1.4 Cell Penetrating Peptides (CPPs)

The tetra-peptide sequence Alanine-Valine-Proline-Isoleucine (AVPI) is an inhibitor of the apoptosis inhibitor, which has the ability to kill cancer cells. AVPI, however, has poor cell-penetration properties due to its increased hydrophobicity levels^[11]. However, this can be solved by conjugating AVPI with cell-penetrating peptides (CPPs). CPPs are short peptides which can transfer active conjugates (cargo) into a cell and can penetrate the cell membrane both in vivo and in vitro without using any receptors and without causing severe damage to the membranes. The two major uptake mechanisms are endocytosis and direct penetration^[10]. AVPI can be connected with CPPs, which can ferry various cargos across the cellular membrane.

PepB1 is a short cell penetrating peptide with eight residues and can improve the intracellular delivery of various bioactive macromolecules^[12]. At the same time, repeat PepB1 sequence to get PepB2 (two times repeat) can get a better secondary structure and can also have a higher penetration efficiency. To overcome the poor penetrative ability and low delivery efficiency of AVPI, we want to conjugate PepB and AVPI to synthesize AVPI-PepB. The peptide can facilitate cell uptake of AVPI, thereby achieving treatment for cancer by promoting apoptosis. Additionally, scrambled AVPI such as APVI can also be synthesized as control groups. In this case, if we conjugate and compare scrambled APVI-PepB to synthesis unscrambled AVPI-PepB, then we can see if scrambled AVPI-PepB will be uptaken by cells and leads to an anti-cancer effect. On the other hand, if scrambled APVI-PepB sequences are unable to kill cancer cells, then we can conclude that only the unscrambled AVPI tetra-peptide sequence has the anti-cancer ability.

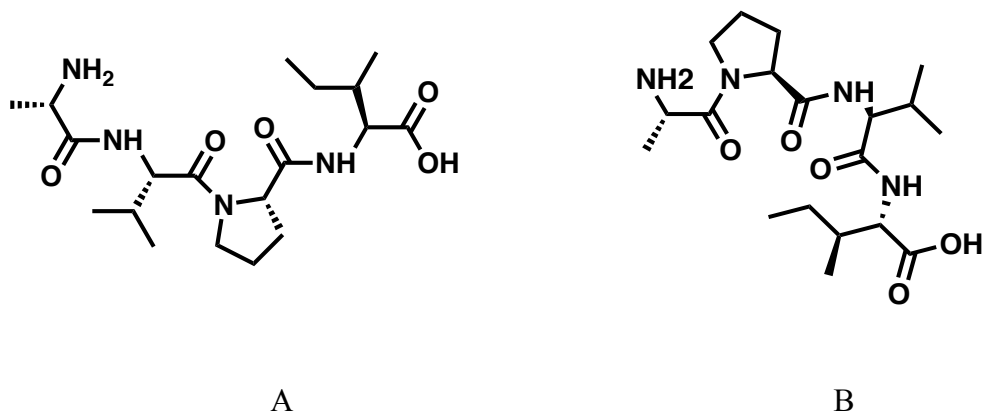


Figure 1. Molecular Structures of Key Peptides. (A) AVPI Chemical Structure; (B) APVI Chemical Structure.

1.5 Solid phase peptide synthesis (SPPS)

Cell-penetrating peptides are small peptides with a few amino acid residues, which means that they can be easily synthesized with the solid phase peptide synthesis (SPPS) method^[13]. Fmoc group (9-Fluorenylmethoxycarbonyl) protected amino acids were utilized in synthesizing peptides. The whole synthesis process happened in a glass peptide synthesis vessel. All the peptides were prepared on Rink Amide AM Resin inside the peptide synthesizer. The amine-protecting Fmoc group was removed using 4-methyl piperidine^[14]. One amino acid was coupling every time. Kaiser test was needed to confirm Fmoc group deprotection and Fmoc amino acid conjugation. Ninhydrin can react with the deprotected N-terminal amine group and change to a dark blue color. To confirm Fmoc group deprotection, the beads need to turn to dark blue, to the contrary, if the beads were remaining yellow, it is indicating the conjugation reaction was completed (no free

amine)^[15]. At the same time, UV spectrometer was also utilized to get an absorption spectrum of the peptides, a sharp peak showed at around 301 nm demonstrated Fmoc group present ^[16].

CHAPTER 2

GOALS AND AIMS

2.1 Project Goals

In this study, AVPI and its derivatives will be synthesized using the Fmoc SPPS method. PepB as a CPP with eight residues, as well as PepB2 and (two times repeat of PepB1) will also be synthesized. The secondary structure of PepB is beta-sheet with a random coil, which is a structural type that increases cellular uptake ability. Rink amide AM resin will be utilized as peptide conjugated beads, and the synthesis process is start from C-terminus to N-terminus. Each individual peptide chain will be conjugated with a dye -- carboxy rhodamine also known as TAMRA. The Fmoc group of Fmoc-Lys (Dde)-OH will be deprotected and have carboxy rhodamine added. Seven peptides chains had been synthesized, which are TAMRA-AVPI; TAMRA-APVI; Ac-K(TAMRA)-AVPI; Ac-K(TAMRA)-APVI; Ac-K(TAMRA)-AVPI-PepB1 and Ac-K(TAMRA)-AVPI-PepB2. The four AVPI derivatives TAMRA-AVPI; TAMRA-APVI; Ac-K(TAMRA)-AVPI and Ac-K(TAMRA)-APVI will be synthesized as control groups, which purpose is to determine whether AVPI without CPPs will be uptaken by cancer cells or not. After all the amino acid had been conjugated, the peptides need to be cleaved from the rink amide AM beads to get crude peptide products. The purpose of cleaving procedure was to remove the peptide from Rink Amide resin, and side-chain protecting groups can be moved at the same

time. The crude peptides need to be purified and isolated. Diethyl ether wash was applied to remove for further chemistry. The most common chemistry was disulfide bonds which can be formed after global deprotection. Reverse-phase high-performance liquid chromatography (RP-HPLC) was applied to get purified peptides. Lyophilization - the freeze-drying process can remove water from a peptide product after it is frozen and exposed to vacuum. The method was applied to get floppy products and conducive to following measurement. To confirm the structure of the synthetic peptides mass spectroscopy will be implemented.

2.2 Aim 1: Synthesis of TAMRA-AVPI; TAMRA-APVI; Ac-K(TAMRA)-AVPI; Ac-K(TAMRA)-APVI; Ac-K(TAMRA)-AVPI-PepB1 and Ac-K(TAMRA)-AVPI-PepB2 with Fmoc SPPS method.

Fmoc SPPS method will be utilized to synthesize TAMRA-AVPI; TAMRA-APVI; Ac-K(TAMRA)-AVPI; Ac-K(TAMRA)-APVI; Ac-K(TAMRA)-AVPI-PepB1, and Ac-K(TAMRA)-AVPI-PepB2 peptides onto rink amide AM resin beads. The four AVPI derivatives peptide chain: TAMRA-AVPI; Ac-K(TAMRA)-AVPI; TAMRA-APVI; and Ac-K(TAMRA)-APVI will be synthesized as positive and negative control group. The process involves swelling the resin beads with dichloromethane (DCM), deprotecting the Fmoc group, conjugating the amino acids, and a cleaving procedure. Before cleaving, the peptides are conjugated carboxy rhodamine (TAMRA). The purpose of coupling a dye is that fluorescence dye can be detected by fluorescence microscope in further cellular uptake experiments.

2.3 Aim 2: Characterization of synthetic AVPI derivatives

After peptide synthesizing, peptide isolation with Reverse-phase high-performance liquid chromatography (RP-HPLC) is needed. For peptide isolation, air dry the products, then ether wash, centrifuge, and air dry the crude peptide. The gradient method of HPLC will be used to purify the peptide and separate the sample. The HPLC equipment comprises a Waters 2998 photodiode array detector and a Waters 2545 quaternary gradient module pump and a Waters Fraction Collector. The pump is used to provide a gradient for the two mobile phases - 1 % TFA/water and 1% TFA/ acetonitrile. In a gradient method HPLC, the strategy is to use multi-segment linear gradients to obtain optimum resolution to get peptides separation. Lyophilization was used to get floppy products. The final products were analyzed by mass spectrometry, electrospray ionization (ESI) and matrix-assisted laser desorption/ ionization (MALDI) will be used as the ionization method. Mass spectrometry is an instrument to determine peptides' molecular weight. The samples in mass spectrometry will be converted to gaseous molecules and ionized by different ionization methods such as ESI and MALDI. After ionization, a sample will reach a mass filter and be separated according to their mass-to-charge (m/z) ratio and can be detected by the detector. Every different peptide has its molecular weight as their signature, and according to different m/z , the sequence is right or not can be determined.

CHAPTER 3

MATERIALS AND METHODS

3.1. Peptide Synthesis Materials

Sulfuric acid (H_2SO_4), hydrogen peroxide (H_2O_2), 4-methyl piperidine, 1, 8-Diazabicyclo[5.4.0]undec-7-ene (DBU), triisopropylsilane (TIPS), diethyl ether were purchased from Acros (NJ, USA). 1-hydroxybenzotriazole monohydrate (HOBt), O-(Benzotriazol-1-yl)-N,N,N, N-tetramethyluronium hexafluorophosphate (HBTU), N,N-Diisopropylethylamine (DIPEA), Fmoc-Ala-OH, Fmoc-Lys(Dde)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Ile-OH, Fmoc-Pro-OH, Fmoc-Val-OH, Rink Amide AM resin were purchased from AAPPTec (Louisville, KY, USA). Ninhydrin, potassium cyanide (KCN), phenol, ethanol, carboxy rhodamine(TAMRA), Trifluoroacetic acid (TFA), dichloromethane (DCM), N,N-dimethylformamide (DMF) were purchased from the Fisher Scientific (Fair Lawn, NJ, USA), deionized water.

3.2 Peptide Synthesis Methods

3.2.1 Preparation and Cleaning of the Peptide Synthesis Vessel

25 mL of H₂SO₄ was transferred to a cylinder, another 15 mL of H₂O₂ was also added to make a Piranha solution. A peptide synthesis vessel was then assembled and the Piranha solution was transferred into it. The solution was then left for 1 hour in order to clean out any (organic) chemical residues from previous uses.

3.2.2. Fmoc Solid Phase Peptide Synthesis

The Rink Amide AM resin beads were then placed in to a clean peptide synthesis vessel and swelled in DCM for 1 hour. The resin beads were then deprotected in a 10 mL deprotection solution (4-methyl piperidine 2: DBU 2: DMF 96, v/v/v) 3 times, for 20 minutes each, thus totaling an hour. Coupled Fmoc protected amino acids (3 eq.) with the coupling reagents: HOBT (3 eq.), HBTU (3 eq.), and DIPEA (6 eq.) were then dissolved in 10 mL DMF. After adding coupling reagents to the synthesis vessel, the vessel was shaken for at least 4 hours. For each amino acid conjugation, they were deprotected and coupled once. Between each deprotection and conjugation procedure they were each washed 3 times in a 10 mL DMF, 5 minutes each, for 15 minutes in total.

3.2.3 Reagent Preparation for the Kaiser Test

The Kaiser Test comprises three reagents: (A) ninhydrin in ethanol, (B) phenol in ethanol and (C) potassium cyanide in pyridine. In order to prepare reagent A, 0.5 g ninhydrin was dissolved in 10 mL ethanol. For reagent B, a 20 g phenol was dissolved in

10mL of ethanol. To prepare for reagent C, a 2 mL a 0.001 M solution of potassium cyanide (KCN) was diluted with 100 mL of pyridine.

3.2.4 Fmoc Group Coupling Confirm by Kaiser Test

A Kaiser Test (ninhydrin free amine test) or UV spectroscopy can be used before deprotection and conjugation step in order to determine if the amino acids were added successfully or Fmoc group deprotected successfully. When applying for a Kaiser Test, 10-15 rink amide AM resin beads were put in a 2 mL glass vial then one drop of reagent A, B and C was added. The glass vial was then placed in a 100 °C oven. The results were then checked after five minutes.

3.2.5 Fmoc Group Coupling Confirm by UV Spectroscopy

A TECAN UV spectrometer was set up first, followed by an absorbance wavelength scan for the cuvette from 280-310 nm, the absorbance value was then acquired after every 2 nm. Then a solution of 20 % 4-methyl piperidine in DMF solution was prepared (v/v), the solution acquired was then used as a blank. 10-15 beads were placed into the cuvette every time followed by the addition of 800 µL 20 % 4-methyl piperidine solution. The peptides could be released into a 20 % 4-methyl piperidine solution immediately, which exhibited a sharp peak off 301 nm that represented the presence of Fmoc group, otherwise meaning that the peptides were deprotected (no Fmoc group).

3.2.6 Acetyl tail to Peptide Conjugation

For Ac-K(TAMRA)-AVPI; Ac-K(TAMRA)-APVI; Ac-K(TAMRA)-AVPI-PepB1 and Ac-K(TAMRA)-AVPI-PepB2, after all the amino acids were conjugated to the peptides, an acetyl tail (3 eq.) was added to the end of the peptides. The conjugation reagents were the same as the amino acid conjugation reagents (acetic acid 3 eq., HOBT 3 eq., HBTU 3 eq., and DIPEA 6 eq. in 10 mL DMF).

3.2.7 Carboxy Rhodamine (TAMRA) to Peptide Conjugation

For Ac-K(TAMRA)-AVPI; Ac-K(TAMRA)-APVI; Ac-AVPI-K(TAMRA)-PepB1 and Ac-AVPI-K(TAMRA)-PepB2, after all the amino acids and acetyl tail had been coupled, Dde group on Lysine was deprotected with a deprotection solution (hydrazine 2, DMF 98, v/v), the peptides were then conjugated with carboxy rhodamine (TEMRA) (2eq). The conjugation reagents were the same as the amino acids conjugation reagents.

3.2.8 Peptide Cleaving

After all the coupling procedure were completed, the raw products were washed 3 times with 10 mL DMF for 5 minutes per wash, for a total of 15 minutes. After which the products were again washed 3 times with pure ethanol to remove all the organic phase. The

peptides were then cleaved from the resin by shaking for 2 hours in a solution of TIPS, nano-pure water and TFA (2.5: 2.5: 96, v/v/v). After all this the cleaving procedure was repeated 2 times and the peptides were collected with the same 50 mL conical centrifuge tube. The crude peptides were then air-dried.

3.3 Characterization of Synthetic AVPI and Its Derivatives

3.3.1 Peptide Isolation

Transferred 20 mL of diethyl ether to each 50 mL centrifuge tube containing the dried peptides. Sonicated the 50 mL centrifuge tubes for 20 minutes in an ice bath, after which they were centrifuged for another 20 minutes at 4 °C and at between 5000-6000 rcf. The ether wash was repeated 2 times and centrifuged 2 times, then the products were air dried and stored below 4 °C (e.g., inside the refrigerator).

3.3.2 Peptide Purification

The peptides were dissolved with 15 mL of 0.1% TFA/water or until the peptides are fully dissolved. The dissolved peptides were filtered, first, through a 0.45 µm filter and then a 0.22 µm filter. The HPLC instrumentation comprises a preparative Phenomenex Luna C5 reverse-phase column, a Waters 2545 Quaternary Gradient Module Pump, a Waters 2998 Photo Array Detector and a Waters Fraction Collector III. The reverse-phase column was placed in a Fisher 40 °C water bath. The whole HPLC system was then washed

with DI water for 30 minutes to make sure the system was clean and equilibrated. After the water wash step, the sample was separated 5 mL per injection, each requiring 30 minutes. A gradient method was utilized to isolate the peptide. The pumping device generated a gradient mobile phase between 0.1 % TFA/water and 0.1 % TFA/acetonitrile. After the HPLC analysis, the samples were collected into 15 mL conical centrifuge tubes.

3.3.3 Peptide Lyophilization

The collected peptide solutions were stored at a -80 °C until frozen. These tubes were then put into a lyophilizer jar and lyophilized for, at least, 72 hours until a dried product was recovered.

3.3.4 Mass Spectrometry for Peptide Structure Identification

The HPLC-purified samples were sent out to the University of Maryland to be analyzed via MALDI FT-ICR or analyzed via ESI method at Temple University.

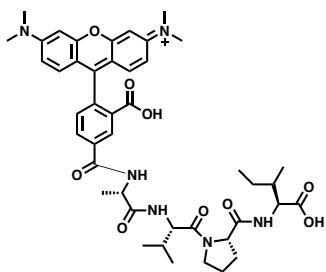
3.4 Statistical Analysis

All the results are presented as mean \pm standard deviation. Data sets were grouped and analyzed with JMP 13 Statistical Software. One-way ANOVA followed by post-hoc Fisher LSD and Tukey HSD analyses were conducted. A p-value < 0.05 is regarded as statistically significant.

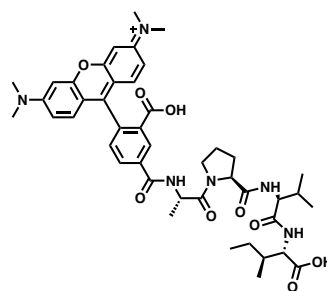
CHAPTER 4

RESULTS

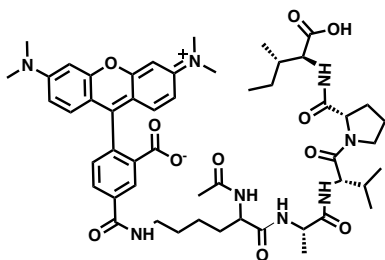
4.1 Chemical Structures of AVPI and Its Derivatives



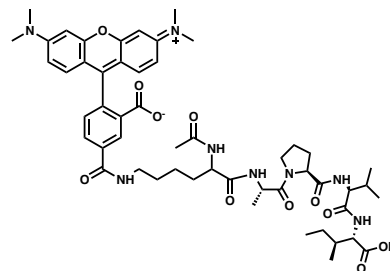
(A) TAMRA-AVPI



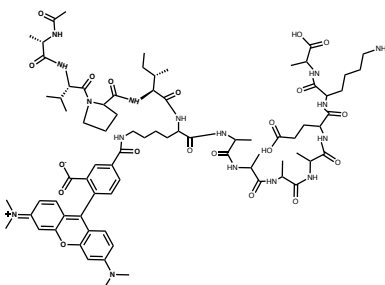
(B) TAMRA-APVI



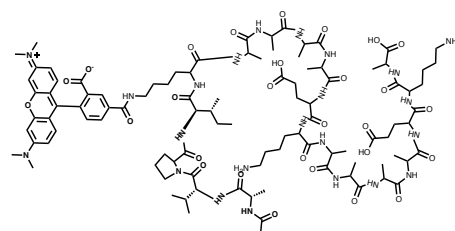
(C) Ac-K(TAMRA)-AVPI



(D) Ac-K(TAMRA)-APVI



(E) Ac-AVPI-K(TAMRA)-PepB1



(F) Ac-AVPI-K(TAMRA)-PepB2

Figure 2. Chemical Structures of Peptides. (A) TAMRA-AVPI; (B) TAMRA-APVI; (C) Ac-K(TAMRA)-AVPI; (D) Ac-K(TAMRA)-APVI; (E) Ac-AVPI-K(TAMRA)-PepB1 and (F) Ac-AVPI-K(TAMRA)-PepB2.

4.2 Fmoc Protected Amino Acid Conjugation and Deprotection Confirmed by Kaiser Test Reagents

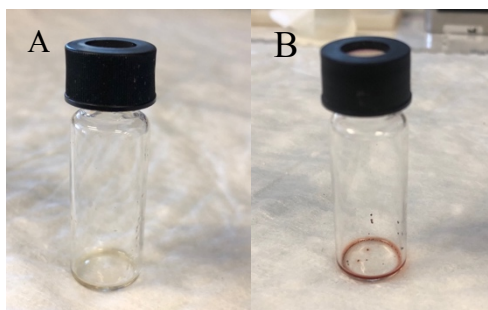


Figure 3. Fmoc protected amino acid conjugation and deprotection confirmed by Kaiser test reagents. (A) First Fmoc-Ala-OH of PepB1 Conjugation, no color change for the beads; (B) First Fmoc-Ala-OH of PepB1 Deprotection, all beads change to dark blue.

4.3 Fmoc Protected Amino Acid Conjugation and Deprotection Confirmed by UV Spectrometry

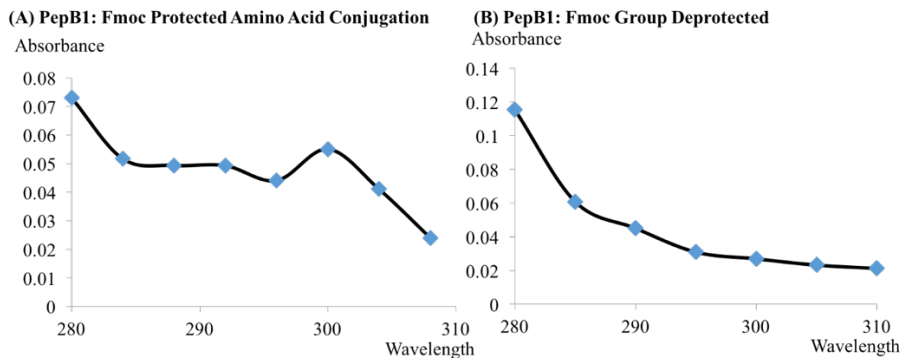


Figure 4. Fmoc Protected Amino Acid Conjugation and Deprotection Confirmed by UV Spectrometer. (A) Profile after Fmoc-amino acid conjugation, which

shows a sharp peak at 301 nm (Fmoc); (B) profile after deprotection showing no peak at 301 nm.

4.4 Isolated Peptide Product Solutions

The purified peptides are all magenta in color (Figure 5) due to the conjugation of a rhodamine-based dye.

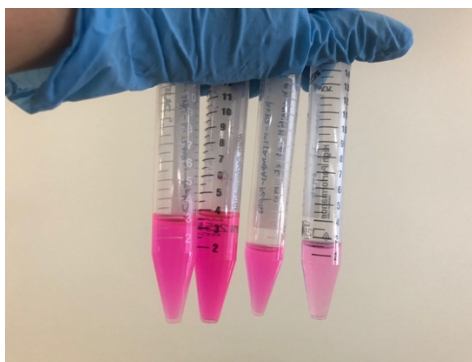


Figure 5. Purified Peptide Product Solutions. Left to right: TAMRA-AVPI, 100 μ M Ac-K(TAMRA)-AVPI, 100 μ M Ac-AVPI-PepB2, 100 μ M Ac-AVPI-PepB1.

4.5 HPLC Results of AVPI and Its Derivatives

The peptides were isolated and purified via HPLC (High Performance Liquid Chromatography). The fraction-collected samples were combined based on retention profiles and then lyophilized with proper labeling schemes (e.g., sample name, number of runs, date).

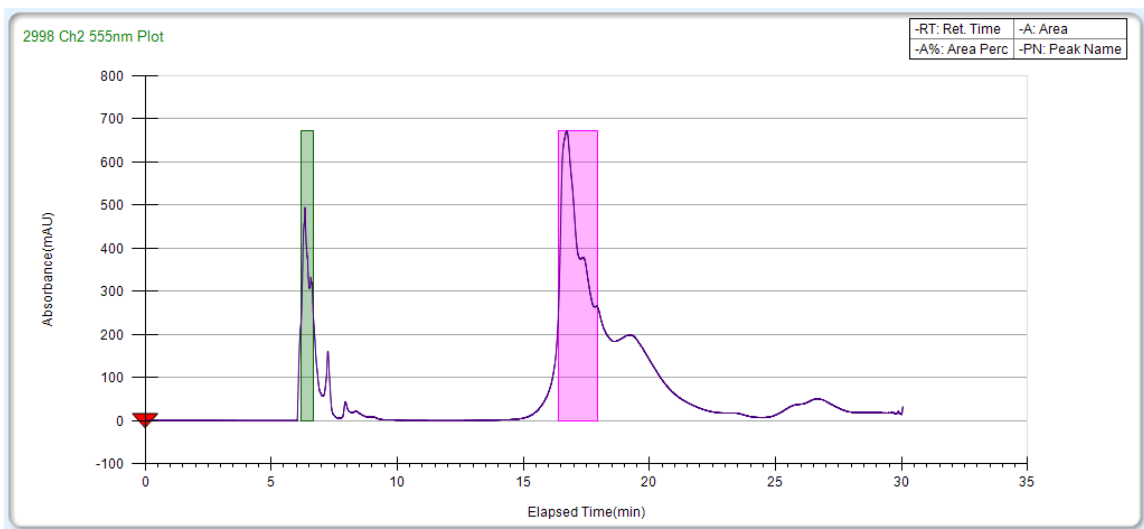


Figure 6. HPLC Separation of AVPI-PepB1 at 555 nm, mobile phase: 0.1 % TFA water and 0.1 % TFA methanol, using the gradient method.

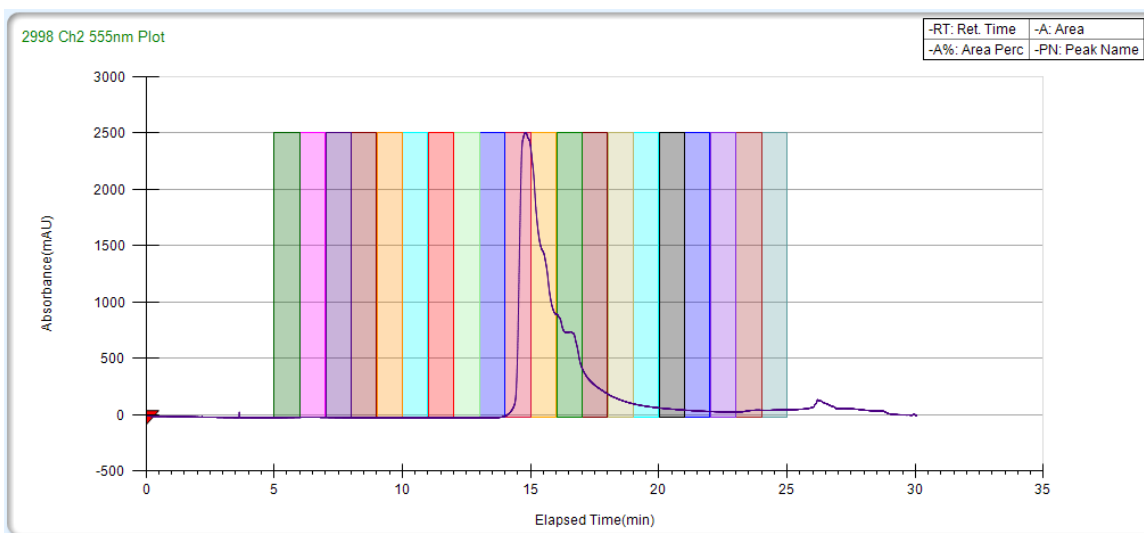


Figure 7. HPLC Separation of Ac-K(TAMRA)-AVPI at 555 nm, mobile phase: 0.1 % TFA water and 0.1 % TFA acetonitrile, using the gradient method.

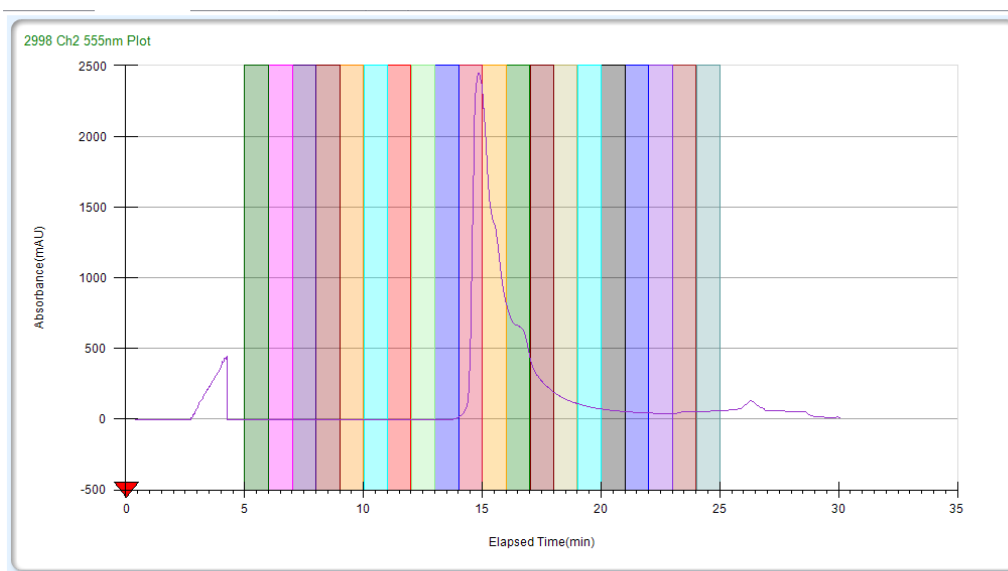


Figure 8. HPLC Separation of Ac-K(TAMRA)-APVI at 555 nm, mobile phase: 0.1 % TFA water and 0.1 % TFA acetonitrile, using the gradient method.

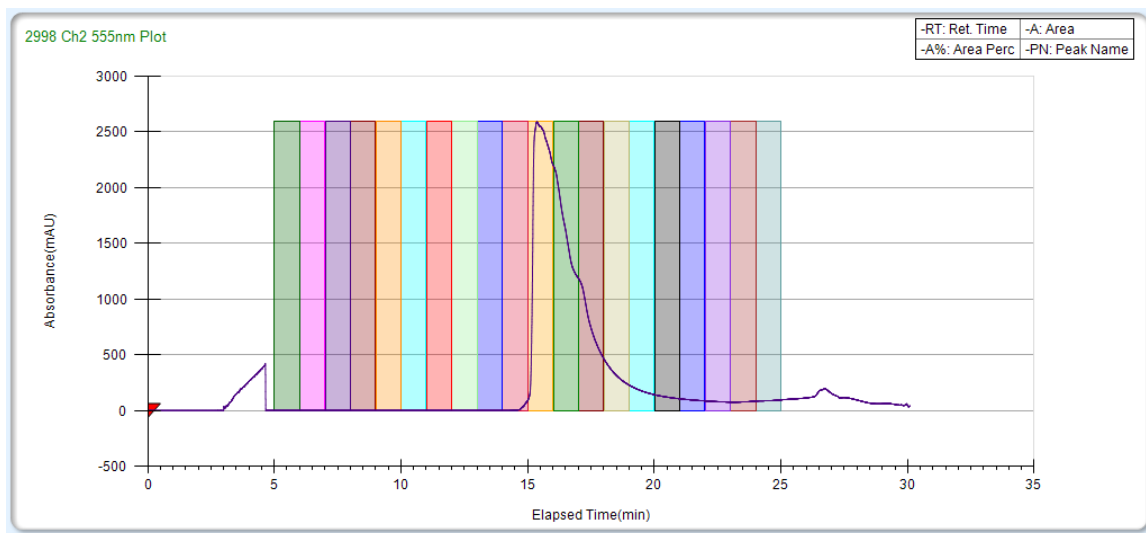


Figure 9. HPLC Separation of TAMRA-AVPI at 555 nm, mobile phase: 0.1 % TFA water and 0.1 % TFA acetonitrile, using the gradient method.

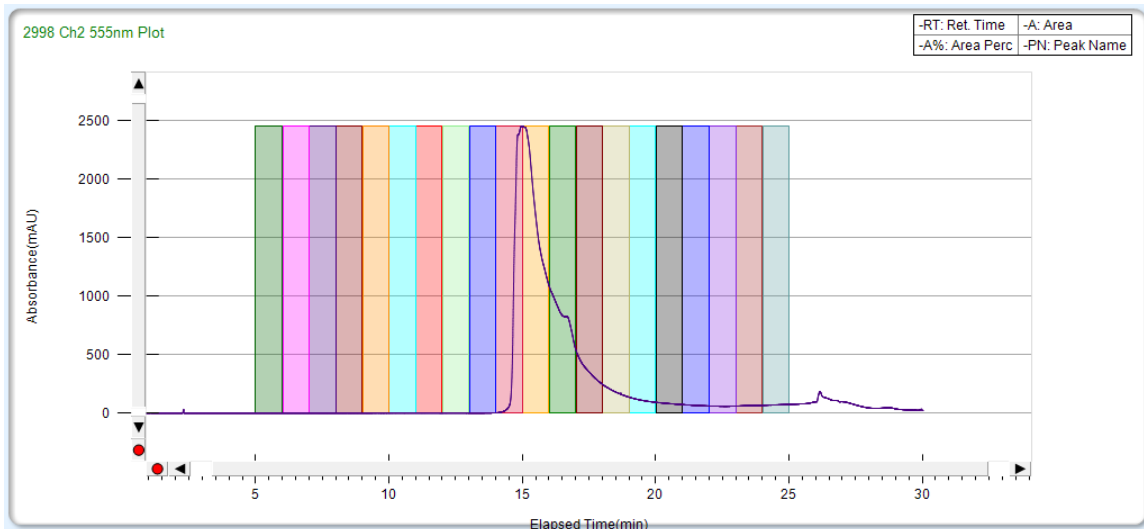


Figure 10. HPLC Separation of TAMRA-APVI at 555 nm, mobile phase: 0.1 % TFA water and 0.1 % TFA acetonitrile, using the gradient method.

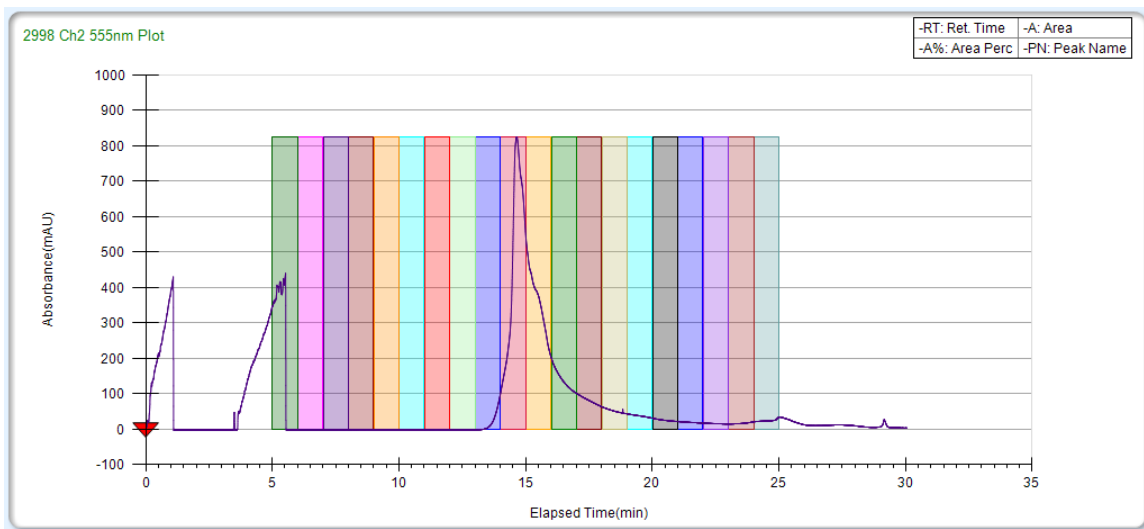


Figure 11. HPLC Separation of Ac-AVPI-K(TAMRA)-PepB2 at 555 nm, mobile phase: 0.1 % TFA water and 0.1 % TFA acetonitrile, using the gradient method.

4.6 Mass Spectroscopy of AVPI and Its Derivatives

Mass spectroscopy analysis was conducted to confirm the compound of synthetic peptides. For each synthetic peptide, they have their own signature - molecular weight, so the structure can be determined. The molecular weight of Ac-AVPI-K(TAMRA)-PepB1 is 1592.86. The molecular peak (m/z) in Figure 13 is 796.93, corresponding to Ac-AVPI-K-PepB1 (M+H)⁺ (Equally split of the Ac-AVPI-K-PepB1, $796.93 \times 2 = 1593.86$).

The molecular weight of Ac-K(TAMRA)-AVPI is 981.2 g/mol. The molecular peak (m/z) in Figure 14 is 980.72, corresponding to Ac-K(TAMRA)-AVPI (M+H)⁺. The molecular weight of TAMRA-AVPI is 812 g/mol. The molecular peak (m/z) in Figure 14 is 813, corresponding to TAMRA-AVPI (M+H)⁺. The molecular weight of TAMRA-APVI is 812 g/mol. The molecular peak (m/z) in Figure 14 is 810.451, corresponding to TAMRA-APVI (M+H)⁺.

The molecular weight of Ac-AVPI-K(TAMRA)-PepB2 is 2135.5 g/mol. The molecular peak (m/z) in Figure 14 is 2133.94, corresponding to Ac-K(TAMRA)-AVPI (M+H)⁺. The molecular weight of Ac- K(TAMRA)-APVI is 981.2 g/mol. The molecular peak (m/z) in Figure 14 is 980.73, corresponding to Ac- K(TAMRA)-APVI (M+H)⁺.

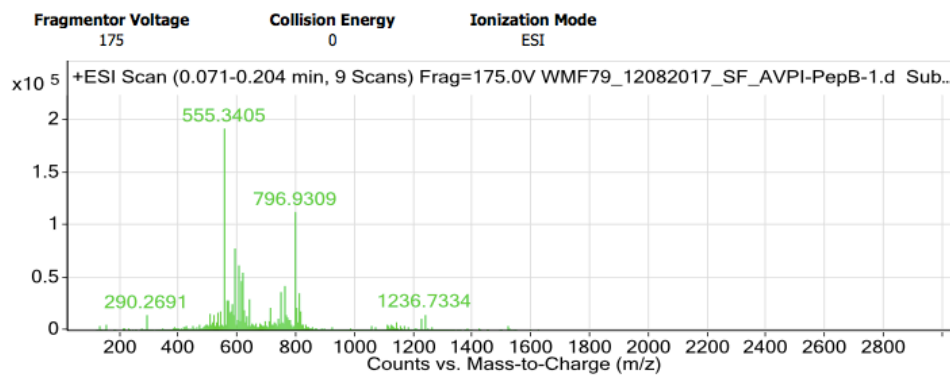


Figure 12 . Mass Spectrum of Synthetic Ac-AVPI-K(TAMRA)-PepB1

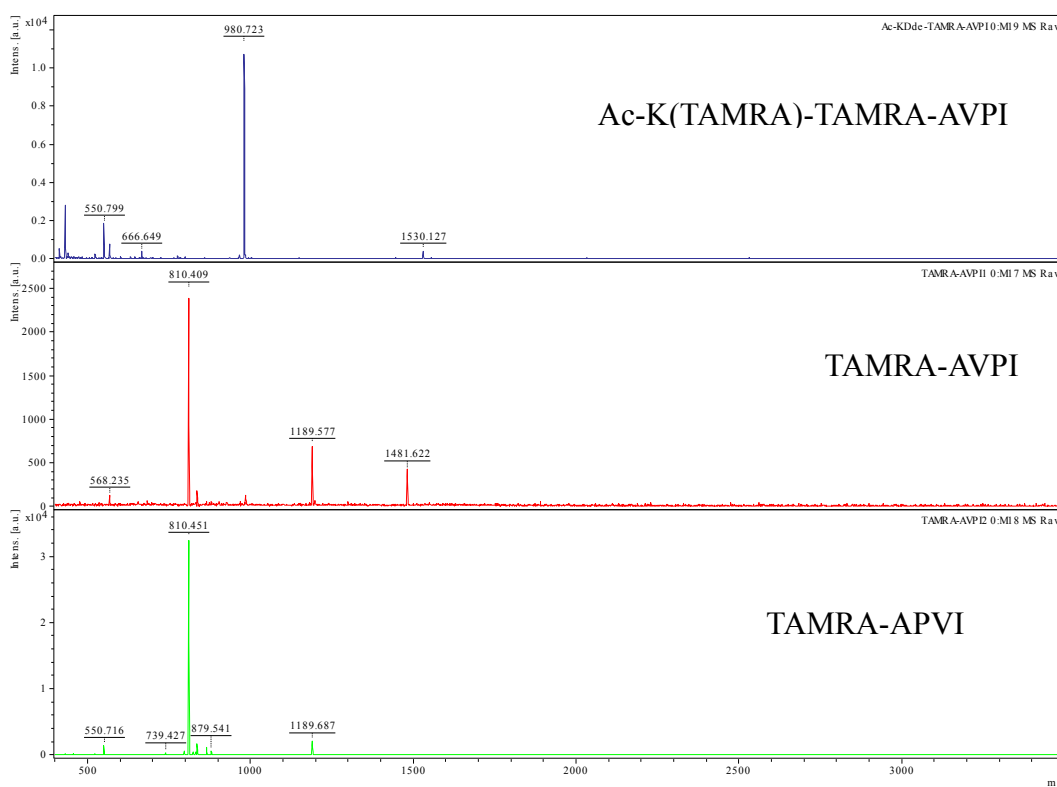


Figure 13 . Mass Spectra of Synthetic Ac-K(TAMRA)-TAMRA-AVPI (blue), TAMRA-AVPI (red) and TAMRA-APVI (green).

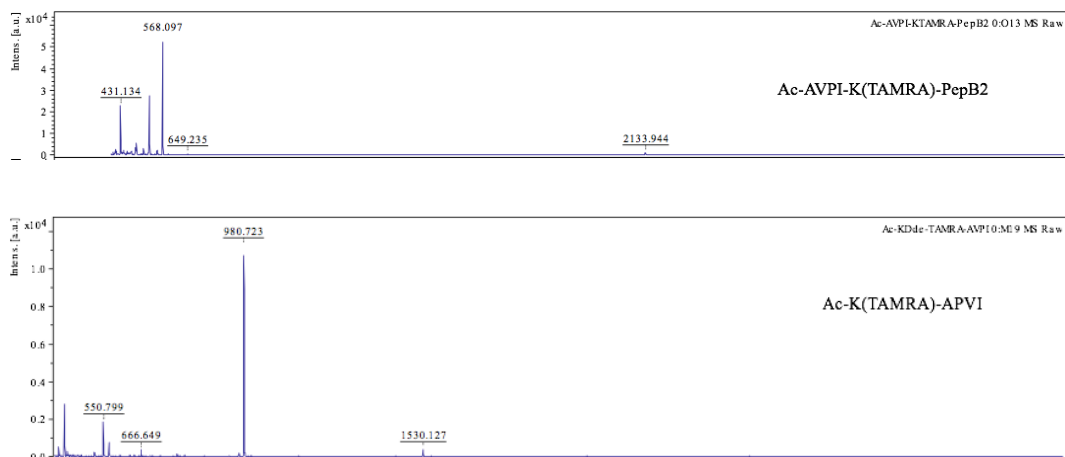


Figure 14 . Mass spectrum of synthetic Ac-AVPI-K(TAMRA)-PepB2 (upper) and Ac-K(TAMRA)-APVI (lower).

CHAPTER 5

DISCUSSIONS

The results of the Mass spectrometry reveal that I synthesized the peptides successfully with Fmoc solid phase peptides synthesis method. However, the throughput of peptides is not as high as we expected. The reason for this low throughput may be one of the following: (1) The resin beads have been taken out every time before conjugation and deprotection to check the reaction completed or not. (2) Peptides could be not totally cleaved from the beads, since before I figured this out, I left the cleaving solution inside a peptide synthesise vessel for 4 hours without shaking. (3) The peptides could be lost during ether wash procedure since I got rid of ether waste directly to the waste container. (4) In the HPLC isolation process, I didn't figure out the column is leaking before all my peptides had been purified, the water bath changed to pink.

All the peptides are prepared to be further cell viability experiments. The tetra peptide AVPI is a hydrophobic peptide which is impermeable to cells and this characteristic limits its utilization. AVPI can only become effective when it penetrates into cells. The purpose of synthesizing AVPI and its derivatives is to determine whether the novel therapeutic agents AVPI-CPPs (cell penetrating peptides) can penetrate cells, thereby allowing AVPI to accelerate cell apoptosis and produce an anti-cancer effect. The AVPI-

PepB (1, 2) and/or scrambled APVI derivatives can, therefore, be tested inducing cancer cell apoptosis and leading to cell deaths.

Efforts to improve the accuracy and efficacy in transporting anti-cancer drugs into cancer areas have been one of the most important issues in developing effective anti-cancer activity. Nowadays, different methods such as loading drugs into liposomes and the use of nanoparticles and degradable polymers have been used. Microspheres are fine particle dispersion system in which drug molecules are dispersed or adsorbed, which are smaller than erythrocytes and can pass through the circulatory system and be uptake by cells easily. Polypropylene carbonate (PPC) is a biodegradable aliphatic polyester and is degradable in vivo, the degraded products are H₂O and CO₂ which are environmentally friendly, so that it may be suitable for drug delivery^[17]. Lactic acid, the degradation product of Polylactic acid (PLA), can be entirely removed in vivo. PLA is also a promising biomaterial for drug delivery systems (DDS), it is biocompatible and biodegradable and it a controlled release system for peptides and proteins^[4]. In this case, AVPI can also be encapsulated into microspheres to increase cellular uptake. Meanwhile, encapsulating AVPI with microspheres as a novel DDS also be an efficient way of cancer treatment.

CHAPTER 6

CONCLUSIONS

The overall outcome of this thesis was to synthesis the tetra-peptide pro-apoptotic peptide Ala-Val-Pro-Ile (AVPI) and its derivatives. The specific molecules are TAMRA-AVPI; TAMRA-APVI; Ac-K(TAMRA)-AVPI; Ac-K(TAMRA)-APVI; Ac-AVPI-K(TAMRA)-PepB1 and Ac-AVPI-K(TAMRA)-PepB2. Based on mass spectrometry results, all the peptides appear to have been synthesized successfully. The solvent gradient method applied to the reverse-phase high-performance liquid chromatography (RP-HPLC) was an effective method to isolate and purified synthetic peptides.

CHAPTER 7

FUTURE DIRECTIONS

The pro-apoptosis peptide AVPI and its derivatives were prepared and characterized to be further cell viability assays. The purpose of the cell viability assays is to determine AVPI itself or AVPI-CPPs has cell entering ability and can kill cancer cells. At the same time, the optimum dosage to kill cancer cells needs to be figured out.

Polyesters such as PLA (poly lactic acid) are promising biomaterial for drug delivery systems (DDS) are biocompatible and biodegradable in vivo and can be used as controlled release system for peptides and proteins^[4]. In this case, AVPI and its derivatives can also be encapsulated into microspheres so the drug release profile can be at a constant rate. Basically, encapsulating AVPI with microspheres as a novel DDS can be an efficient delivery method for the controlled release treatment of cancerous tissue.

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