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Peptide-drug conjugate for Her2-targeted drug delivery

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PEPTIDE-DRUG CONJUGATE FOR HER2-TARGETED DRUG DELIVERY

by

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PEPTIDE-DRUG CONJUGATE FOR HER2-TARGETED DRUG DELIVERY

Abstract

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Recent strategies for anticancer drug design have been focused on utilizing antibody as a drug or targeted moiety for targeted drug delivery. Antibody−drug conjugates (ADCs) have become a promising new class of targeted therapeutic agents for treatment of cancer. ADCs are designed to preferentially direct a cytotoxic drug to a cell-surface antigen recognized by an antibody. However, there are some challenges in developing ADCs, such as limited solid tumor penetration, high manufacturing costs and antibody-drug stoichiometry. Smaller molecules such as peptides have been shown to specifically bind to cancer related targets. These peptides can be used to form peptide-drug conjugates (PDCs) to overcome above-mentioned drawbacks presented by ADCs.

In this study, it was hypothesized that novel synthesized PDCs can be a strategy for breast cancer therapy. HER2 specific binding peptides, MARAKE and MARSGL, were modified by addition of a cysteine at C-terminus. The modified peptides were coupled with monomethylauristatin E (MMAE) by using maleimidocaproyl (MC) as a non-cleavable linker to form peptide-drug conjugates (YW1, YW2) and maleimidocaproyl-valine-citrulline (MC-VC) as a cleavable linker to form peptide-drug conjugates (YW3 and YW4). The peptides, peptide-drug conjugates and MC-MMAE, MC-VC-MMAE were characterized using ESI-MS and purified by using high-performance liquid chromatography (HPLC). Cellular uptake study was performed to determine binding specificity and internalization of two HER2 specific peptides and cysteine-modified peptides (MARAKEC, MARSGLC). In vitro cell viability assay was conducted to assess the cytotoxicity and determine the targeting specificity as well as the potency of the peptide-drug conjugates.

The purity of each compound was greater than 90%. Internalization of both HER2 specific binding peptides and cysteine-modified peptides were significantly higher than random peptides in HER2 over-expressed cell lines, MDA-MB361 and ZR75, while negligible uptake in HER2 negative cell line, HEK293. MC linked PDCs showed similar cytotoxicity as peptide in all cell lines; while MC-VC linked PDCs have higher cytotoxicity than MMAE in HER2 positive cell line and significant lower cytotoxicity than MMAE in normal cell line HEK293. However, PDCs with MC link

do not show significant difference in cytotoxicity compared to the peptide in all cell lines.

In conclusion, specificity of HER2 binding for both peptides was preserved after modification with cysteine. The derivation of MMAE to link drug and peptide played a crucial role in the anticancer activity. Peptide-MMAE conjugates with cleavable linker showed a promising targeting capability for delivery of MMAE to HER2 overexpressed cancer cells.

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CHAPTER 1: INTRODUCTION

1.1. Cancer Therapy and Anti-cancer Drugs

According to the World Health Organization (WHO) statistics, 3/5 of death in the world is attributable to cancer, diabetes, cardiovascular disease, and chronic respiratory system disease; of these four categories of diseases, cancer is one of the leading causes of death.

Several approaches to cancer treatment have been used for cancer therapy, including surgery, radiation therapy, chemotherapy, immunotherapy, targeted therapy, hormone therapy, stem cell transplant, and precision medicine. Among them, chemotherapy, hormone therapy, immunotherapy, and target therapy use anticancer drug. Anti-cancer drugs can also be classified into four types based on the mechanism and site of action, including the direct effects on DNA, drugs that disrupt its structure and function; interference with DNA synthesis; antimitotic drugs; and drug mechanism based on tumor biology.

1.1.1. DNA-damaging Drugs

1.1.1.1. *Alkylating Agents*

Alkylating agents are a class of cytotoxic drugs that possess highly reactive chemical properties. These molecules contain one to two alkyls groups, which can be transformed into electron-deficient intermediate. These intermediate products

covalently combine with DNA electrons in an alkylation reaction, resulting in the loss of function of DNA in cell metabolism; thereby the cell composition is affected, resulting in variation of cell division and, ultimately, cell death.

1.1.1.2. ***Platinum Compounds***

Cisplatin is a nonspecific drug that when enters the cell cycle after internalization into tumor cells and hydrolyzes the target molecule, such as cisplatin, carboplatin, and oxaliplatin. This hydrolyzed complex interacts with DNA and forms a chelate ring that results in the partial denaturation and loss of replicative capacity of the DNA [1]. Cisplatin also reacts with cellular proteins and RNA and forms inactive molecules like glutathione and cysteine. Because of the rapid speed of proliferation and synthesis in cancers, platinum compounds have a higher cytotoxic effect on cancer cells than normal cells.

1.1.1.3. ***Bleomycin***

Bleomycin is a glycopeptide antibiotic produced by *Streptomyces verticillus* that causes DNA strand break by binding to DNA and inhibiting thymidine entry into DNA. The breaking of DNA strands leads to the inhibition of DNA synthesis and cell proliferation [2].

1.1.2. Drug Interference with DNA Synthesis

Drug interference in DNA synthesis is also called as anti-metabolites, as it occurs by the inhibition of DNA synthesis of folic acid, purine, pyridine, and pyridine metabolism, thereby inhibiting the survival and replication of tumor cells, leading to

tumor cell death. This kind of drug can be classified further into folic acid antagonists.

1.1.3. Antimitotic Drugs

Mitosis is a vulnerable stage in the survival of carcinoma cells, and therefore it can be used to design drugs for anticancer intervention. Microtubule and associated proteins play a vital role in the process of mitosis; microtubule-targeted drugs may interfere with microtubule dynamics to activate the spindle assembly checkpoint (SAC), induce mitotic arrest, and cause cell death ultimately [3]. There are two types of mitosis drugs according to the mechanism involved, namely, microtubule-destabilizing agents and microtubule-stabilizing agents [4]. Destabilizing agents inhibit the polymerization of microtubules in high concentration, and most of them bind to the taxoid-binding domain or vinca domain [5]. Microtubule-stabilizing agents enhance microtubule polymerization, stabilize microtubules, and prevent Ca^{2+} and subsequent disassembly [6].

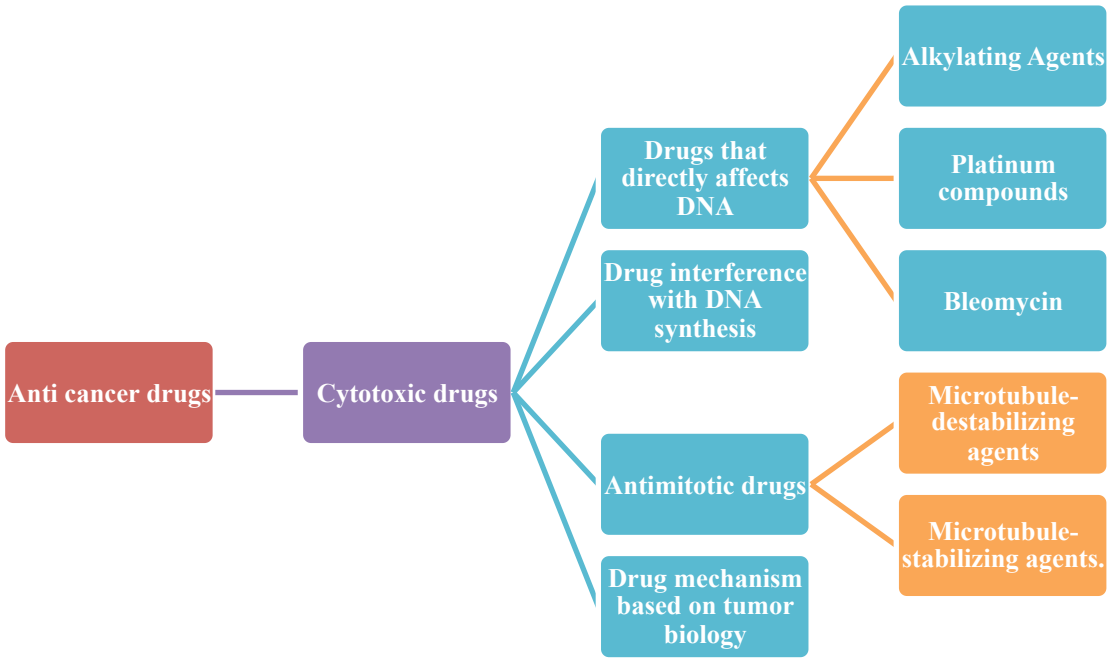

Figure 1.1 Four categories of anticancer drugs

1.2. Drug Delivery in Cancer

Anti-cancer drugs kill rapidly proliferating cells, which is not limited to cancer cells only, resulting in side-effects such as hair loss, reduced blood cells, and stomach irritation. However, discontinuation of drug therapy may result in cancer recurrence, resulting in rapid proliferation of cells again [7]. In order to completely eradicate cancer cells and reduce undesired side-effects, the strategy to deliver drugs to a target tumor site has been proposed.

1.2.1. Passive Target Delivery System

Passive targeting delivery system is designed to deliver the drug to the systemic circulation by using the body's natural response to specific physicochemical

characteristics. Rapid growing tumors generate new vessels or reroute the existing vessels in order to adequate supply of nutrients and oxygen, [8] which causes imbalance of angiogenic regulator, resulting in tumor vessel disorganization and expansion, with a gap junction between the endothelial cells, thereby causing enhanced permeability of the tumor vessel [9]. Because of the impaired clearance system in the tumor tissues, macromolecules and lipids stay in the tumor interstitial space for a long time [10]. This phenomenon is termed as tumor-selective enhanced permeation and retention (EPR) effect of nanoparticles. The EPR effect has been widely used to design passive-target delivery system because it meets the four requirements of effective target drug delivery system, that is, retain, evade, target, and release [11].

For obtaining abundant supply of nutrition and oxygen for the new vessels generated, the rapidly growing tumor cells use glycolysis to obtain extra energy to maintain their rapid metabolic rate, resulting in an acidic tumor microenvironment [12]. Several approaches such as the use of pH-sensitive liposome have been used to design pH-sensitive drug carriers that have stable physiologic pH (7.4), but which degrade and release in the target tumor tissue with lower pH value [13].

pH-sensitive nanoparticles are devised to combine EPR effect and pH-trigger release. The loaded drug accumulated in tumor via the EPR effect while remained stable during blood circulation and released the encapsulated medications into acidic environment provided by the solid tumor [14].

1.2.2. Active Target Delivery System

Active target delivery is designed to overcome the limited specificity of the passive delivery system. This delivery system is based on the ligand–receptor interaction that happens between delivery system and target when the delivery system meets the target cells where there is efficient interaction between the ligands and receptors [7]. For example, epidermal growth factor receptors (EGFR) are proteins overexpressed on the surface of cells that can be used for targeting delivery via specific ligand receptor interaction mechanism. To achieve efficient ligand–receptor interaction, several factors should be considered: the extent and homogeneity of target cell antigen or receptor expression, the availability of cell surface receptor on the cell surface, the rate of drug release, and internalization.

1.3. Therapeutic Monoclonal Antibody

Monoclonal antibody is developed and designed to target overexpressed antigen on or near the surface of malignant cells [15]. Therapeutic monoclonal antibody can be used as a treatment option in several diseases such as oncology, inflammation, cardiovascular diseases, and infectious diseases. Antibody exerts two important functions, including binding and modulating antigen and binding complement and immune-effector cells [16]. Tumor-targeting monoclonal antibody can be grouped into six classes based on the functional consideration: (1) mAbs that inhibits the intrinsic proliferation signal transduction pathway; (2) mAbs that activate the cell surface cytotoxic receptors and then triggers apoptotic demise; (3) mAbs that binds

tumor-associated antigens (TAAs) and exerts dependent antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC) through innate immunity; (4) immunoconjugates mAbs; (5) trifunctional or bispecific mAbs; and (6) mAbs that interfere with nutrient transfer between tumor cells and stroma [17][18][19][20].

Although, in recent years, oncology therapeutic mAbs are developed at a fast speed in preclinical and clinical trials, it remains common that antibodies do not alter tumor growth when treated with xenograft mice model. To improve the therapeutic effect, several approaches based on targeting cytotoxic agents on malignant tumor cells by conjugation with antibody have been developed, including antibody toxin-fusion proteins, antibody-drug conjugates (ADC), antibody enzyme conjugates, and antibody radionuclide conjugate [21].

1.4. Antibody-drug Conjugates

1.4.1. Pharmacological Mechanism of Antibody-drug Conjugate

Antibody-drug conjugates are composed of three basic parts: highly selective antibody, linker, and cytotoxic small molecule drug. The structure of a typical ADC is shown in Figure 1.

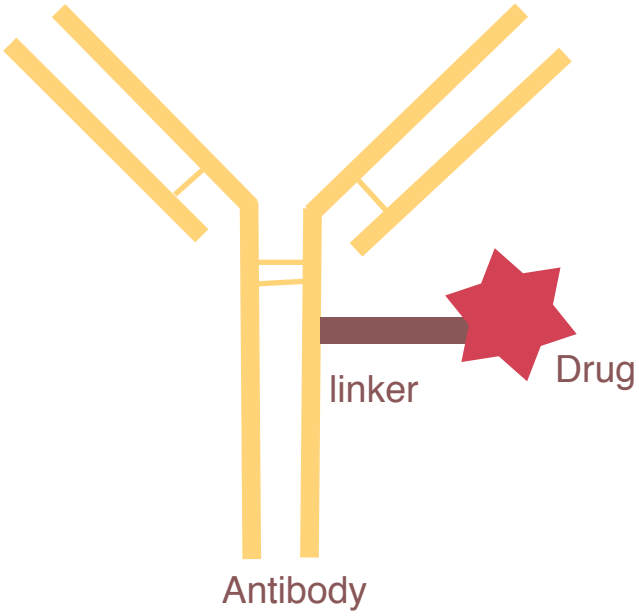

Figure 1.2 Structure of typical antibody-drug conjugate

The objective to accomplish a well-designed ADC is that an ADC preferentially delivers the cytotoxic small molecule drug only to tumor cells rather than to normal cells in order to improve the therapeutic index [22][23]. The mechanism by which ADCs disrupt tumor cells is by binding the specific mAbs to an antigen that is highly expressed only on the surface of tumor cells. After binding to the antigen, an ADC-antigen complex is formed, which is internalized into cells, and the cytotoxic drug is released, leading to cell death [23]. A typical mechanism of antibody-drug conjugates is shown in Figure 2.

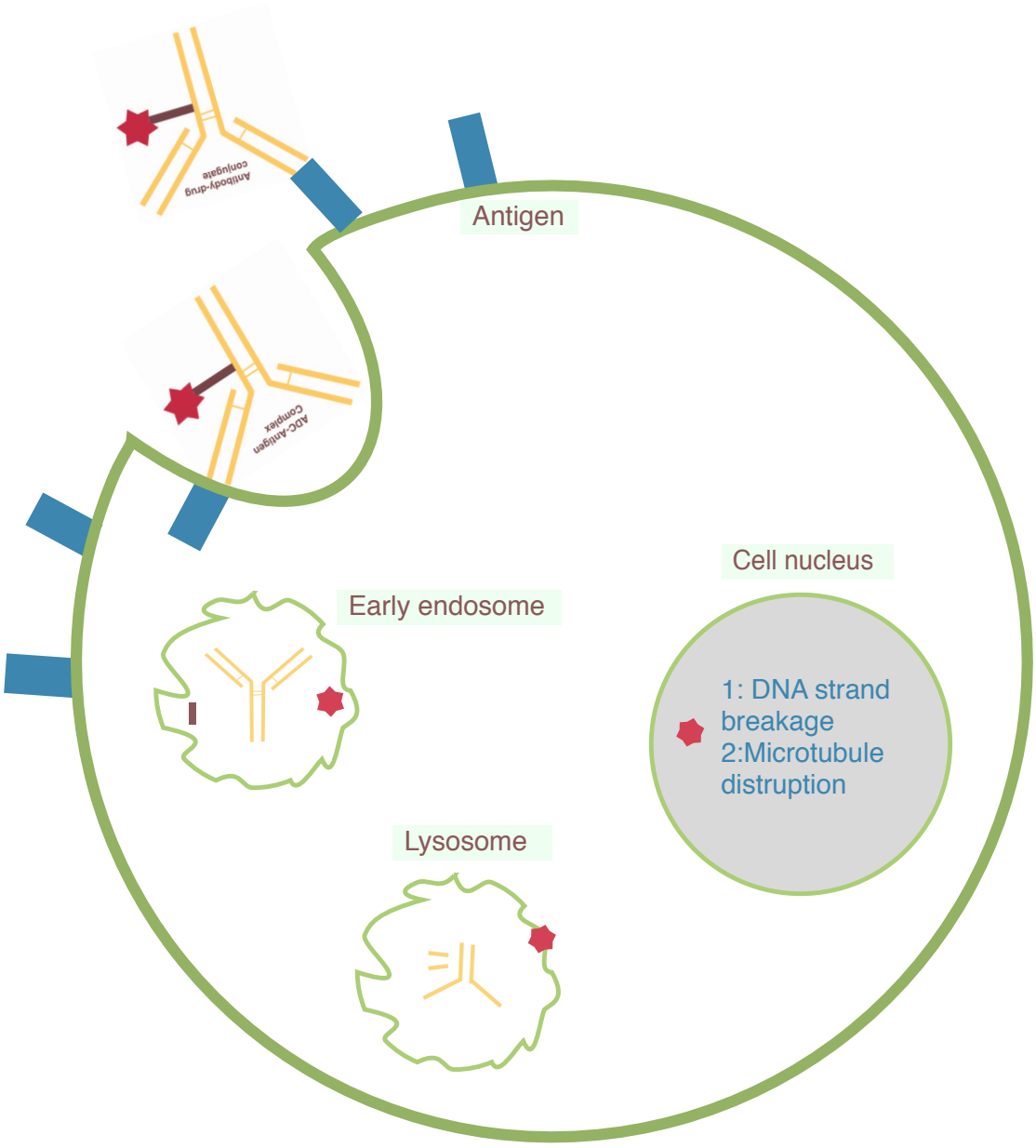

Figure 1.3 Typical mechanism of antibody-drug conjugates internalization

1.4.2. Design of Antibody-drug Conjugate

Based on the mechanism described above, three key procedures need to be paid attention to when designing an ideal ADC: (1) antibody targets appropriate antigens and forms an ADC–antigen complex, (2) the stable linker prior to reaching the target

and releasing cytotoxic drug after internalization, and (3) the highly potent cytotoxic drug should exert its pharmacological effect after reaching the target. . The selection of an appropriate target, a stable linker, and a highly potent drug are discussed with reference to the design principle of ADC as follows.

1.4.2.1. ***Target***

An appropriate target selection should fulfill two criteria: high selectivity on tumor cells and high internalization efficiency.

Most of the antigens express the same level on both normal cells and cancer levels, which makes it difficult for the related antibody to recognize the cancer cells. Therefore, an ideal target antigen should be expressed in higher copy numbers on the tumor cells (overexpress); this overexpressed characteristic, in part, contributes to ADC efficacy and tolerability [23][24]. For example, two ADCs—brentuximabvedotin (ADCETRIS) and trastuzumabemtansine (T-DM1)—have been shown to achieve distinct clinical activities with overexpressed target antigens on the tumor cells. ADCETRIS targeted antigen CD30 that is expressed on normal cells and strongly expresses in systemic anaplastic large-cell lymphoma (ALCL) and Reed–Sternberg cells of Hodgkin lymphoma (HL) tumor cells [25]. Similarly, T-DM1 targeted antigen, HER2, with normal expression in normal tissues, is highly overexpressed in some breast cancer tumors [26].

After binding to the antigen, the ADC conjugates with the antigen and forms an ADC–antigen complex, which is then internalized into the tumor cells—this process

is called as a receptor-mediated endocytosis. The extent and speed of internalization of an ADC is depended on the nature of cell-surface receptors. Some receptors, like transferrin receptors, continuously internalize without requiring binding with ligands, whereas, receptors like epidermal growth receptor accelerate the internalization rate after accumulate binding with their corresponding ligands [26]. In some cases, the internalization of an ADC is much more efficient than that of an unconjugated antibody [27][28].

1.4.2.2. ***Linker***

A linker that connects monoclonal antibody with a cytotoxic drug binds through covalent linkage. The stability of a linker is important because it stays stable in the blood circulation while preventing damage to the non-target tissue, followed by release of the drug after reaching the target site [22]. There are two main types of linkers, including cleavable linker and non-cleavable linker; cleavable linker releases drugs by enzymatic reactions or hydrolysis after internalization, while non-cleavable linker releases drug through the degradation of antibody in the lysosome after ADC–antigen complex internalization [23][24]. Table 1.1 lists some release mechanisms of important linkers.

Table 1.1 Release mechanisms of ADC linkers

Linker	**Release mechanism**
Hydrazone	Serum stable, degrade in acidic compartment within cytoplasm
Peptide	Enzymatically hydrolyzed by lysosomal protease like *cathepsin* B
Disulfide	Cleavage through disulfide exchange with an intracellular thiol
Thioether	Nonreducible, intracellular proteolytic degradation

To optimize the solubility, the drug antibody ratio (DAR) and reduce the drug resistance (the protein that transports cytotoxic agents out of the cells) of ADCs, several strategies have been proposed including induce bystander effect and the use of polar linkers.

Solid tumors express antigen heterogeneously, whereas, a positive antigen-specific ADCs only kills antigen-positive cells, which is ineffective in eradicating tumors. Therefore, an effective ADC should kill not only antigen-positive cells but also the surrounding cells through the bystander effect. Bystander effect depends on the charge of the linker-drug derivation released from ADCs, for example, the ADC brentuximabvedotin release neutral-charge MMAE, cross bio-membranes, and kill bystander epithelial cells, whereas ADCs based on MMAF release a charged metabolite, which is difficult to pass through bio-membranes and kill the neighboring cells. Reducible cleavable disulfide linkers can cross bio-membranes, while non-reducible thio-ether linkers cannot, which indicates that the ADCs with disulfide

linker have bystander cytotoxicity, while ADCs with thio-ether linker do not.

MDR1 is a protein that transports hydrophobic cytotoxic agents out of cells, and, as a result, hydrophobic agents like ADCs with non-charged or non-polar maytansinoid linkers prove to have lower efficiency to MDR-positive cells. Development of an ADC with a polar or charged linker would not only overcome the adverse effect of MDR but also improve the solubility and therapeutic index. Because reducing hydrophobicity can slow down the clearance and enhance pharmacokinetics [25].

1.4.2.3. ***Cytotoxic Drug***

Low-potent cytotoxic agents such as doxorubicin and mitomycin used in the early ADC developments were found to be inefficient in inhibit solid tumor growth and showed lower accumulation in the target cells [29]. The reason for the failure of the conventional cytotoxic agent ADC could be attributed to only a limited number of antigens are expressed on the tumor surface, and the amount of drugs delivered to the tumor cell surface is low for a low drug to antibody ratio (DAR 3.5:4). Hence, only a limited number of novel highly potent cytotoxic agents with IC_{50} of 10^{-10}-10^{-12}M have been chosen to develop ADCs. Most of them target DNA or microtubule. The drugs targeting DNA are cytotoxic for both proliferating and non-proliferating cells, while drugs targeting microtubules are cytotoxic only for the proliferating cells [30]. When cytotoxic agents are chosen for developing ADCs, several factors should be considered, such as (1) most of the cytotoxic agents are hydrophobic, which can

induce antibody aggregation, and therefore lead to increased clearance rates and shortened validity [31]. (2) When a drug structure is modified for the linkage of the conjugate, the potency may decrease, and water solubility and stability may change [32]. Table 1.2 lists the current ADCs in clinical trials.

Auristatins and maytansionid are two typical groups of cytotoxic agents used in ADC developments. Auristatins have two derivatives: monomethyl auristatin E (MMAE) and monomethyl auristatin F (MMAF), which derived from the natural antimitotic agent dolastatin 10 and exerted their effects by inhibiting tubulin assembly.

In maytansinoid ADC, drugs used in the clinical trial are DM1 and DM4, which derived from natural benzoansamacrolide product maytansine, and it acts on tubulin. Due to its high toxicity, it has failed in clinical trials as an independent anticancer drug, but its stability and acceptable solubility make it a candidate for use as cytotoxic agent in ADC [33].

Other cytotoxic agents that have been used to develop ADCs include indolino-benzodiazepine, irinotecan derivatives, duocarmycins, pyrrolobenzodiazepines (PBDs), and tubulysins. Tubulysins are antimitotic peptides that inhibit microtubule polymerization during mitosis, which results in cell death. Duocarmycins is a DNA groove-alkylating agent. It binds to the minor groove of DNA and destroys it at a specific site [34][35]. PBDs, natural anticancer antibiotics that bind to the minor groove of DNA and destroy DNA in a specific sequence, are

now fast becoming as popular as ADCs development candidates for at least 10 ADCs based on PBDs have entered clinical trials.

Table 1.2 ADC drugs in clinical stage

Drug	Target	Linker	Cytotoxic class	Clinical stage
Pinatuzumabvedotin	CD22	VC	MMAE	Phase II (stopped)
Indusatumabvedotin	GCC	VC	MMAE	Phase II (stopped)
Vorsetuzumabmafodotin	CD70	MC	MMAF	Phase I (stopped)
Trastuzumabemtansine	HER2	SMCC	DM1	Entered market in 2013
Mirvetuximabsoravtansine	FOLR1	sulfo-SPDB	DM4	Phase III
Anetumabravtansine	Mesothelin	SPDB	DM4	Phase II
SAR428926	LAMP1	SPDB	DM4	Phase II
ADCT-301	CD25	PEG8-va	SG3199	Phase I
Rovalpituzumabtesirine	DLL3	PEG8-va	SG3199	Phase III
IMGN779	CD33	sulfo-SPDB	DGN462	Phase I
Gemtuzumabozogamicin	CD33	Hydrazone	CM1	Approved in Japan
Inotuzumabozogamicin	CD22	Hydrazone	CM1	Pre-registration
Trastuzumabduocarmazine	HER2++	vc–seco	DUBA	Phase I
RC48-ADC	HER2	VC	MMAE	Phase I
Sacituzumabgovitecan	TROP2	CL2A	SN38	Phase III
Vadastuximabtalirine	CD33 Engineered	VA	SGD1882	Phase III

1.4.3. Other Optimized Approaches of Antibody-drug Conjugates

Apart from selecting an appropriate antibody, linker, and cytotoxic agents, several additional approaches have been proposed to optimize ADC based on increased tumor penetration and antibody binding to inhibit immune checkpoints.

Due to the poor penetration into solid tumors by antibody, there is a limit (0.001–0.01% of injected amount per g) to which the cytotoxic agents can reach and target tumor cells *in vivo* [36]. Based on this limitation of ADCs, several new designs have been generated for the next generation ADCs, including (1) non-internalizing mAbs that link cytotoxic agents with disulfide linkers that are cleaved in a specific tumor extracellular microenvironment [37] and (2) Non-lgG scaffold includes designed Ankyrin Repeat Proteins (DARPins) [38]. Both design approaches demonstrated improved pharmacological and therapeutic performance.

Another attractive way to improve the potency of ADCs is by combining it with immune-oncology antibodies. Antibodies that target immune checkpoint inhibitors like programmed cell death protein 1 (PD1), programmed cell death 1 ligand 1 (PDL1), and immune responses like cytotoxic T lymphocyte 4 (CTLA4) have surprising efficiency on a series of tumor types. Checkpoint inhibitors activate immune responses without antigen-specific, seems to be active in a variety of tumors mutations, meanwhile, some cytotoxic agents used in ADCs have been shown to induce the death of immunogenic cells and induce activation and maturation of

dendritic cells. Hence, a combination therapy by immune-checkpoint inhibitors with ADCs that increase the immune response may have spectacular effect on oncology therapy [39].

1.5. Peptide-drug Conjugates

Peptide-drug conjugates (PDCs), like ADCs, are another class of prodrugs. Targeting delivery of a drug to particular tumor-surface receptors is achieved by conjugating a drug to a specific sequence peptide. Peptide-drug conjugates are composed of three parts: drug, linker, and peptide (Figure 2). Due to the short length of peptide, PDCs are biodegradable and can avoid undesired immunogenic responses [40],[41]. The diversity of sequence made the diversity application of PDCs, while different sequences elicit different hydrophobicity and ionization, which could influence bioavailability both *in vitro* and *in vivo*. Other than ADCs, the low molecular weight of peptide allows PDCs high purification by using HPLC. Based on the difference in the characteristics of peptides, PDCs can be separated into two categories: Cell-targeting peptide drug conjugates and cell-penetrating peptide-drug conjugates.

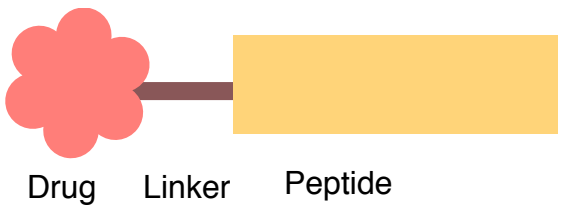

Figure 1.4 Structure of peptide-drug conjugate

1.5.1. Cell-targeting Peptide-drug Conjugates

Tumor targeting peptide-drug conjugates are designed by using peptide with binding specificity to deliver the drug to particular tumor cells or tissues. The tumor targeting peptides were generated to target receptors expressed on the surface of the tumor vascular endothelial cell and cancer cell, or target to the tumor's extracellular matrix [42]. The following sessions list some categories of cell-targeting peptides that have been used to developed peptide-drug conjugates.

1.5.1.1. *IntegrinTtargeted Peptide–drug Conjugates*

Integrins are a family of proteins that are widely used as receptors because they are widely expressed in numerous cells. They play vital roles in physiological development, maintenance, repair of tissues, and pathological process in diseases, especially in cancer [43]. In the past two decades, integrin-targeted peptide-drug conjugates have been developed based on the wide distribution of $\alpha_v\beta_3$ targeting Arg-Gly-Asp (RGD) motif. Our group previously reported the potential of using modified RGD peptide amphiphiles to deliver hydrophobic drug like methotrexate, paclitaxel [42][44][45][46]. The Wadih group [47] conjugated RGD4c to doxorubicin, displaying enhancement of tumor inhibition and less toxicity of PDC to the liver and the heart compared with free doxorubicin. Another research group proved that when conjugated to integrin-targeting peptides, doxorubicin is preferentially released in tumor cells, which explains the unknown problem that the reported effect stems from the PDCs or the released doxorubicin. Except doxorubicin (DOX), paclitaxel (PTX)

and camptothecin (CPT) have also been used to conjugate with integrin-targeting peptides, both of which reveal high antitumor effect than free drugs in *in vitro* studies [48][49][50].

1.5.1.2. ***Hormone Analog Peptide-drug Conjugates***

Hormone receptors like gonadotropin-releasing hormone (GnRH) receptor and somatostatin receptors are particularly overexpressed on the membrane of tumor cells [51]. Gonadotropin-releasing hormone (GnRH) receptor also named luteinizing-hormone-releasing hormone (LHRH) receptor is not expressed in critical organs. LHRH are being developed as conjugated to selectively deliver cytotoxic agents to cells expressing LHRH receptors [52]. Liu et al. conjugated luteinizing hormone-releasing hormone (LHRH) agonist AEZS-108 with anti-cancer agent, doxorubicin to targeted deliver doxorubicin selectively to prostate cancer CTCs, which has entered into clinical phase I study [53]. Tomas Vanek successfully developed targeted delivery system by conjugating mitotic inhibitor Paclitaxel and GnRH analog peptide (pGlu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly·NH_2) [54].

Somatostatin, also known as growth hormone- inhibiting hormone, is a cyclic polypeptide that controls endogenous inhibitory [55]. Somatostatin is broadly distributed in the nervous system, regulates cell proliferation and division by directly activating somatostatin receptors [56]. The somatostatin belongs to G protein-coupled receptors family, and there are five subtypes of somatostatin have been identified.

Among them, somatostatin receptor-2 is preferential overexpressed in many tumors and tumor blood vessels. Hence, most of the somatostatin analog conjugates were developed to target to somatostatin receptor 2 site [57]. Huang et al. produced paclitaxel conjugate with somatostatin analog octreotide via a succinate linker[58]. This taxol octreotide conjugate was found to trigger apoptosis of somatostatin expression cells MCF-7 [58].

1.5.1.3. *Other Cell-targeting Peptide-drug Conjugates*

In addition to RGD peptides and hormone analog peptides, other cell-targeting peptides were studied to develop new peptide-drug conjugates. EphA2 is tumor-targeting receptors that observed overexpressed on the surface of specific solid tumor cells including breast, prostate, and pancreatic. Ahmed F. Salem et al. generated peptide-drug conjugate by coupling EphA2-targeting peptide 123B9 with paclitaxel [59]. In breast cancer models, EphA2-targeting PDCs were found to efficiently inhibit carcinoma metastasized to the lung. PDCs that target to HER2 receptors was performed, Tai group utilize HER2 targeting peptide KCCYSL linked to anticancer agent TGX-221 via self-cyclizing linkage. The conjugate turned out to specified bind to the HER2 receptor expressed on prostate cancer cells surface and provide a promising treatment strategy for prostate cancer [60].

1.5.2. Cell-penetrating Peptide-drug Conjugates

Cell penetration peptides (CPPs) are types of peptides that are able to cross the

mammalian cell membrane through the receptor-independently way. The rapid speed that CPPs enter into cells made CPPs attractive cargo to deliver therapeutic agents like a drug, DNA to the various situation and biological system [61]. Marco Lelle group synthesized two doxorubicin cell-penetrating peptide conjugates through hydrazone linker. The cellular uptake and distribution were assessed in MCF-7 and HT29 cell lines, and the increased toxicity on breast cancers has been observed on both two PDCs compared to HT29 cells [62]. Duan group developed two paclitaxel (PTX) cell-penetrating peptide conjugates PTX-TAT and PTX-LMWP. The enhanced antitumor potency was observed in tumor-bearing mice [63]. The anticancer agent gambogic acid (GA) has been developed to link with cell penetration peptide TAT. The improved solubility and induced EJ bladder cell uptake, toxicity and apoptosis were observed, which provide a promising used in bladder cancer therapy [64].

1.6. Anticancer Peptides (ACPs)

In addition to conjugating anti-tumor cytotoxic drug with targeting antibody or peptide, anticancer peptide has become new treatment option for the molecular target anticancer drug development. Anticancer peptides are mostly derived from antimicrobial peptides (AMPs), which are a part of the immune defense mechanism of several organisms. The reason that AMPs can be used as ACPs for cancer therapy is because of the same membrane characteristic (negative cell surface charge) of the cancer cell and bacterial cells [65][66]. ACPs can be divided into two categories: membranolytic and non-membranolytic [67].

The selectivity and toxicity of peptide with membranolytic mechanism are modulated by target membrane feature [68]. The first difference between cancer cells and normal cells is the negative charge in the membrane disperse anionic molecules like phospholipid phosphatidylserine (PS), sialylated gangliosides, and O-glycosylated mucins in the membranes of cancer cells, resulting in a contrasting negative charge compared to the normal mammalian cell. Another difference between cancer cells and normal cells is in the content of cholesterol, as normal cell membranes have a high content of cholesterol in order to protect the cell by modulating the cell fluidity and block the entry of cationic peptides while most cancer cell surface cannot [69].

By this membranolytic mechanism, the peptide action events include permeation, swelling of mitochondria, and apoptosis [69]. Peptides regulate the tumor vasculature by blocking the receptors that on the angiogenic endothelial cells. The formation of tumor vasculature is perturbed via this non-membranolytic mechanism [70][71].

There are a total of 202 anticancer peptides recorded in the APD databases. Table 3 shows some of the anticancer peptides that possess anticancer activity. Peptides can also be divided into two main groups based on the tumor type: solid and hematological tumors.

Table 1.3 List of anti-cancer peptides

Peptide name	Sequence	Anticancer activity	Tumor type	Cancer cell
D-K6L9	LKLLKKLLK KLLKLL	Necrosis via membrane depolarization	Solid, Hematological	Human prostate
NRC-03	GRRKRKWL RRIGKGVKIIGG AALDHL	ROS production and cell membrane lysis with possible pore formation in mitochondria	Solid	Human breast
NRC-07	RWGKWFKK ATHVGKHVGKA ALTAYL	ROS production and cell membrane lysis with possible pore formation in mitochondria and	Solid	Human breast
Polybia-MPI	IDWKKLLDA AKQIL	Cell membrane disruption with probable pore formation	Solid	Human bladder and prostate
Hepcidin-TH2-3	QSHLSLCRW CCNCCRSNKGC	Cell membrane lysis	Solid	Human cervix, hepatocellular carcinoma, fibrosarcoma
Temporin-1CEa	FVDLKKIANI INSIF	ROS production, membrane disruption, calcium release,	Solid	Human breast
Epinecidin-1	GFIFHIIKGLF HAGKMIHGLV	Cell membrane lysis mediated by necrosis inhibitory activity	Solid	Human lung, cervix, hepatocellular carcinoma

PTP7	FLGALFKALSKLL	Apoptosis induction	Solid	Human lung, prostate, breast and hepatocellular carcinoma
BEPT II	RAALAVVLGRGGPR	Apoptosis induction	Solid	Human prostate
ERα17p	PLMIKRSKKNSLALSLT	Apoptosis induction and massive necrosis	Solid	Human breast
R7-KLA	RRRRRRRGGKLAKLAKKLAKLAK	Plasma membrane permeabilization by pore formation	Hematological	Human acute T cell leukemia
BIM $SAHB_A$	IWIAQELRXIGDXFNAYYARR	Apoptotic resistance	Hematological	Human histiocytic lymphoma, chronic myelogenous leukemia, acute myeloid leukemia
SK84	AAGAATTCAGCCAGCTAGGTGACTTG	Membrane disruption	Hematological	Human leukemia, liver and breast
LfcinB	FKCRRWQWRMKKLGAPSITCVRRAF	Apoptosis by direct disruption of the mitochondrial membrane	Hematological	Human acute lymphoblastic T leukemia, acute T cell leukemia

1.6.1. Lytic Peptide

Lytic peptides are a group of bioactive cationic peptides that act on the surface of cell membranes and have strong anti-cancer activities [72]. As the multidrug Resistance (MDR) resulted in reduced efficiency in the cancer cells when treated with the cytotoxic agents, the development of lytic peptide is attractive for its ability to

bypass MDR mechanism by acting on the cell membrane [73].

K6L9, a typical lytic peptide, induces cytolytic effect through these two steps: (1) membrane disruption and necrosis of tumor cells and (2) decrease in the tumor vessel density. It can inhibit the metastases for both solid tumor and hematological tumor when they are used in treatment *in vivo* [74]. By targeting with phospholipid phosphatidylserine (PS) expressed on the neoplastic cells, the peptide colocalizes with the negatively charged phospholipid and then elicits membrane-depolarizing lytic activity.

Consider the lower extracellular pH in the solid tumor site as compared to the pH in the normal tissue site, the pH-sensitive lytic peptide has raised interest. Liang et al. [80] designed a pH-sensitive lytic peptide by modifying a known lytic peptide LL-1 (FLGALWKALSKLL) with histidine (H) amino acid, and then found more pH-sensitive activity on histidine-containing peptides LL-1a, LL-1b, and LL-1c (FLGALWHALSKLL, FLGALWKALSHLL, FLGALWHALSHLL) [73]. Another advantage of histidine-containing peptides represents improved stability and half-lives (up to 11 h) [72].

1.6.2. Hybrid peptide

Although lytic peptide has its selectivity for tumor cells, a new strategy has been proposed, wherein antigen-targeting peptide is combined with lytic peptide to form a hybrid peptide for use in assessing the better selectivity and cytotoxic activities *in*

vitro and *in vivo*. In 2013, Kawakami et Al. [75]designed a HER2-lytic hybrid peptide by linking the HER2-binding moiety peptide KCCYSL and a newly designed lytic peptide KL**L**LK**L**L**KK**LLK**L**LKKK (bold letters indicate D-amino acids) and achieved significant inhibition of HER2 receptors and block the HER2 signal in the HER2 overexpressing cell lines. *In vivo* studies also proved the efficiency of HER2-lytic hybrid peptides [75]. In 2016, the Kawakami group [76] prepared a new hybrid peptide based on the EGFR binding moiety, that is, EGFR-lytic hybrid peptide, which elicited cytotoxic effect on the pathophysiology of esophageal squamous cell carcinoma. Table 1.4 shows some of the hybrid peptides that have been developed. It is worth suggesting that conjugating hybrid peptide with cytotoxic drug may help reverse drug assistance and induce efficiency of cytotoxic agents.

Table 1.4 List of hybrid peptides

Target	Target peptide	Lytic peptide	Hybrid peptide
EGFR	YHWYGYTPQNVI	KL**L**LK**L**L**KK**LLK**L**LKKK	YHWYGYTPQNVIGGGKL**L**LK**L**L**KK**LLK**L**LKKK
HER2	KCCYSL	KL**L**LK**L**L**KK**LLK**L**LKKK	KCCYSLGGG KL**L**LK**L**L**KK**LLK**L**LKKK
αvβ3	CIRTPKISKPIKFELSG	SKKPVPIIYCNRRSGKCQRM	GSKKPVPIIYCNRRSGKCQRMGSIRTPKISKPIKFELSG [78]
TfR	HAIYPRH	MPKKKPTPIQLNP	HAIYPRHGGCGMPKKKPTPIQLNP [79]

1.7. Human Epidermal Growth Factor Receptor 2

The ErbB family is a group of proteins containing four receptor tyrosine kinases, including epidermal growth factor receptor (EGFR), HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). The ErbB receptors play an important role in human cancers because they are expressed in multifarious tissues of epithelial and neuronal origin [77]. After ligand binding with the receptor, the pathways will be activated. Consequently, the cancer cells show higher proliferation, survival, migration, and differentiation potential [78][79].

The human epidermal growth receptor 2 oncogene encodes a receptor tyrosine kinase that is overexpressed in >20% of human breast cancer cases [80]. HER2 has become a suitable target candidate for molecular target cancer therapy. Humanized recombinant monoclonal antibodies that inhibit HER2 receptors have also been developed, including trastuzumab and pertuzumab, which have been used for the treatment of HER2-positive breast cancers [81]

Except antibodies, peptides that target HER2 receptor tyrosine kinase have also been developed. Table 5 shows the HER2-targeting peptides derived from the phage display library.

Table 1.5 Sequence of HER2-targeting peptides

Peptide sequence	Library	Reference
AC#SLQDPNC#DWWGHYC#G (H8)c	ACX6CX6CG	[88]
ACGLQGYGCWGMYGKCG (H30)c	ACX6CX6CG	[88]
CVGVLPSQDAIGIC (L-26-19)d	Ph.D.-12	[89]
CGPLPVDWYWC (L-26-24)d	Ph.D.-12	[89]
CEWKFDPGLGQARC (N-12-1)e	Ph.D.-12	[89]
CDYMTDGRAASKIC (N-12-2)e	Ph.D.-12	[89]
KCCYSL	6mer	[90]
MARSGL, MARAKE, MSRTMS	6mer	[91]
LTVSPWY	Ph.D.-7	[92][93]
WTGWCLNPEESTWGFCTGSF (EC-1)	20mer	[94]

1.8. Statement of the Problem

In the background section, we have listed novel target-delivery system and among them, the HER2-targeted antibody-drug conjugate Kadcyla (trastuzumab emtansine) was developed and approved by FDA in 2014. However, the poor penetration in solid tumor, the high manufacturing cost, and long development cycle limits the extensive development of antibody–drug conjugates. Compared to the antibody, peptides with flexible structure and low molecular weight are becoming a promising target agent for application in the target delivery system.

1.9. Hypothesis

Designed HER2-targeting peptide–drug conjugates and hybrid peptides could exhibit highly selective cytotoxicity for HER2 overexpressed breast cancer cells.

1.10. Specific Aims

The objective of this research is to design the potent peptide–drug conjugates and

hybrid peptides that provide a prospective chemotherapy strategy for breast cancer patients. In order to achieve this objective, the following specific aims have been established:

1. To synthesize HER2-targeted peptides using solid phase synthesis method on Wang resin.
2. To study the *in vitro* binding specificity of Her2-targeted peptides and cysteine-modified peptides.
3. To design and synthesize peptide–drug conjugates with a non-cleavable linker maleimidocaproyl (MC) and MMAE.
4. To design and synthesize peptide–drug conjugates with a cleavable linker maleimidocaproyl-valine-citrulline-p-aminobenzoyloxycarbonyl (MC-VC-PAB) and MMAE
5. To synthesize hybrid peptides with a cytolytic peptide Melittin (GIGAVLKVLTTGLPALISWIKRKRQQ).
6. To purify and characterize peptides and peptide–drug conjugates.
7. To study the cytotoxic potency of synthetic peptide–drug conjugates and hybrid peptides.

CHAPTER 2: SYNTHESIS AND CHARACTERIZATION OF PEPTIDES

2.1. Introduction

A typical peptide synthesis requires the formation of amide bond, provided and formed by the amino and carboxyl groups of two amino acids. However, numerous possibilities of combinations of amino acids made the synthesis of peptide more complicated than forming a simple amino acid. Solid-phase peptide synthesis (SPPS) technique was developed in 1962 by Bruce Merrifield in order to provide an effective, rapid, and simplified method for the preparation of peptides and small proteins. The basic concept of SPPS is the step-wise construction of a peptide chain attached to an insoluble polymer substrate. The insoluble polymers used for SPPS also named resins. Different resins can be used to synthesize various peptides. For example, Wang resin is conventional resin for Fmoc/tBu SPPS; DHPP Resin is developed especially for peptides with C-terminal proline; PDDM-resin is applied for peptides containing C-terminal Cys or His. 2-Chlorotrityl is an acid-labile resin. This resin is suitable for synthesizing peptides containing a C-terminal Cys or Pro. [82]. The insoluble polymer resin attached to the carboxyl group of the terminal amino acid conjugates to the next amino acids protected by the Fmoc group. After coupling, the Fmoc acid protection group is removed from the chain in order to provide a free amino group for coupling the next amino acid. The peptide chain is extended by the duplication of synthesis

cycle. The polymer resin and the side-chain protection group must be removed prior to the cleavage of resulting peptide by using a 95% solution of trifluoroacetic acid (TFA).

The purpose of this chapter is to utilize solid phase peptide synthesis technique to synthesis HER2 targeting peptides for binding specificity study and PDC synthesis.

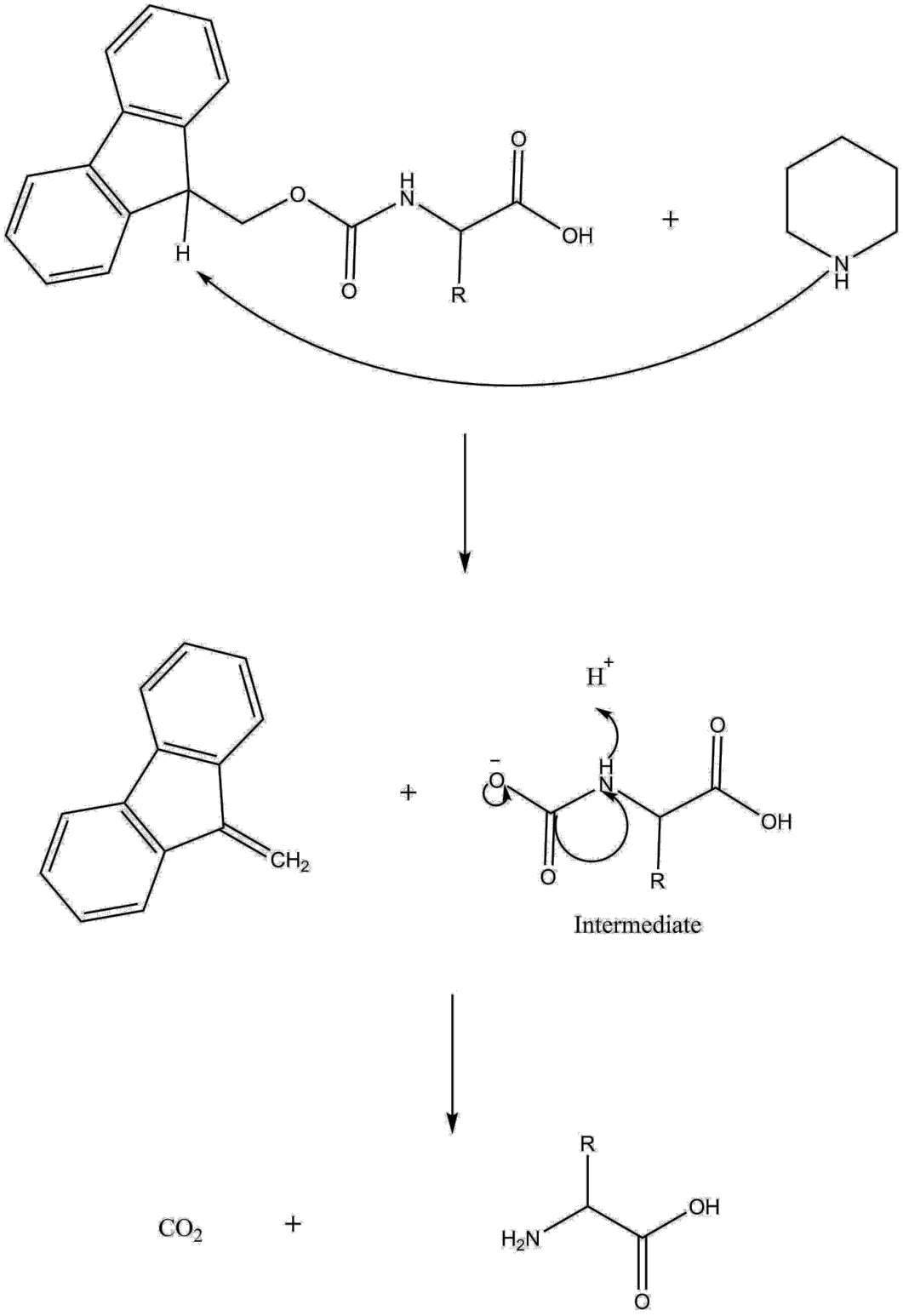

Figure 2.1 Mechanism of piperidine de-protection

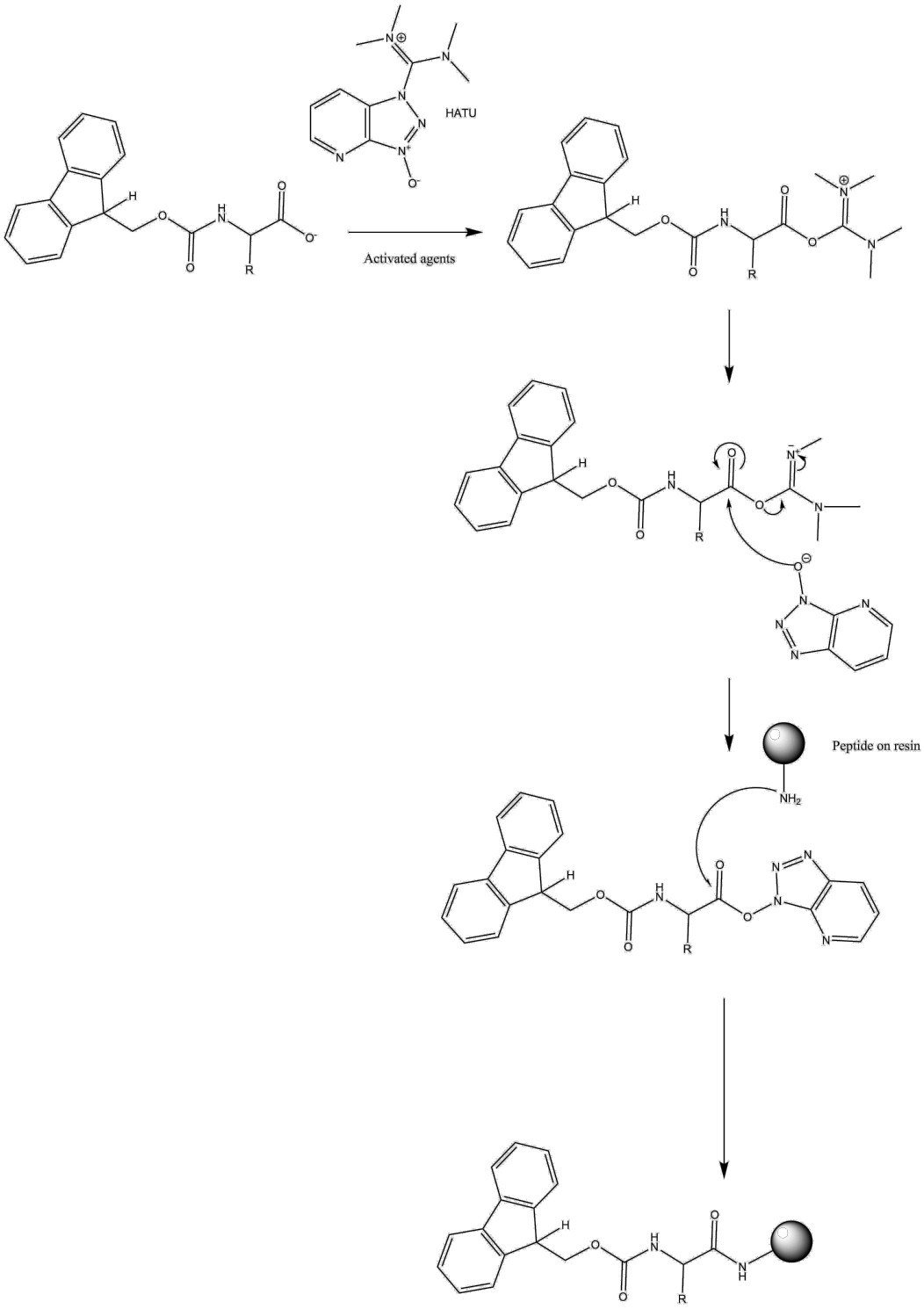

Figure 2.2 Mechanism of coupling

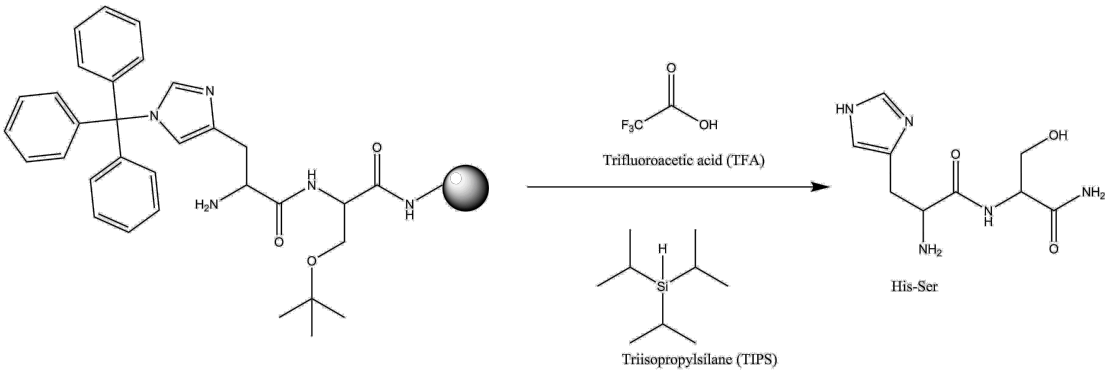

Figure 2.3 Mechanism of cleavage

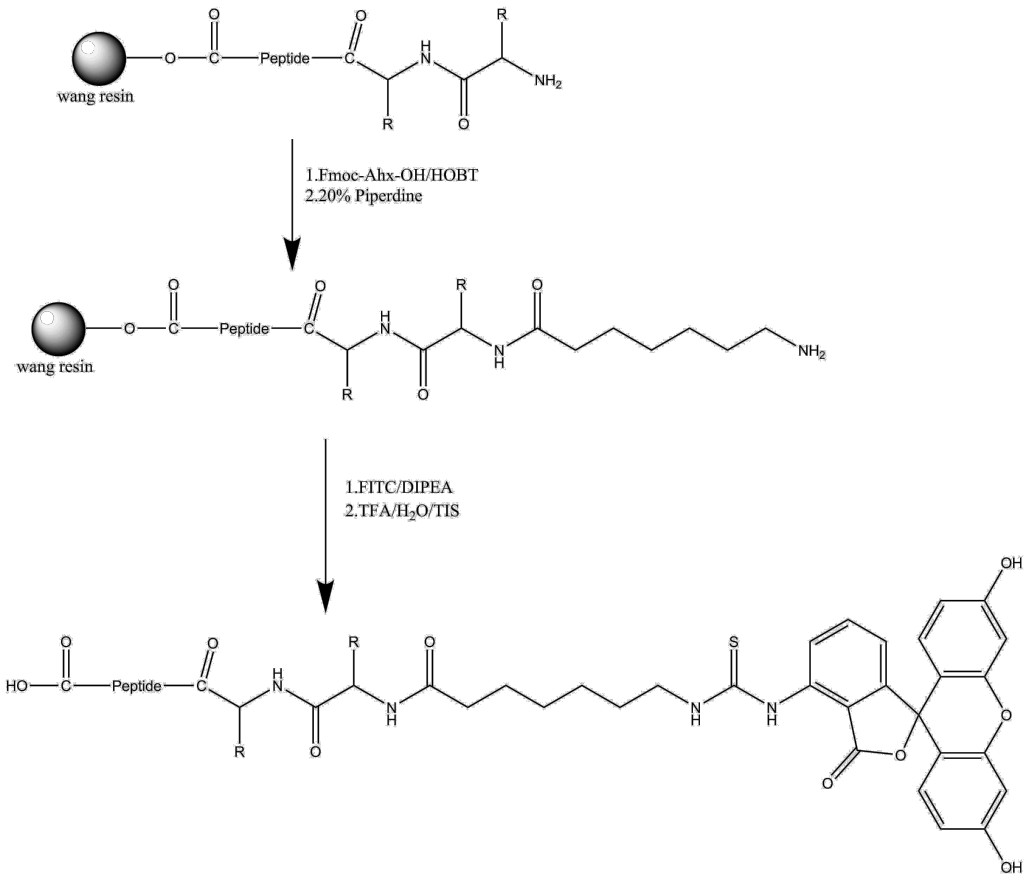

Figure 2.4 Mechanism of synthesis FITC-labeled peptide

2.2. Materials

Fmoc-Wang resins, N-hydroxybenzotriazole (HOBT), *N,N,N′,N′*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uranium hexafluorophosphate (HBTU), triisopropylsilane (TIS) were obtained from Chem-Impex International Ltd. (Wood Dalw, IL, USA). Acetonitrile (ACN), dichloromethane (DCM), and 5-FITC were purchased from Fisher Scientific (Pittsburgh, PA, USA). Diisopropylethylame (DIPEA), tert-butyl methyl ether, trifluoro acetic acid (TFA), and N,N'-dimethylformamide (DMF) were obtained from Axros Organics (New Jersey, USA). All amino acids used in the SPPS were purchased from Anaspec Int. (Fremont, CA)

2.3. Methods

2.3.1. Synthesis of Peptides

Peptides were synthesized by SPPS with Fmoc chemistry on Wang resin (0.2 mmol) and coupled amino acids (1 mmol) with DIPEA (1.8 mmol), HBTU (1 mmol), and HOBT (1 mmol). After coupling for 2.5 h at the room temperature, Kaiser test was performed to ensure the completion of the reaction. Fmoc group deprotection solution was prepared with 20% piperidine in DMF. Peptide cleavage cocktail was performed with phenol (0.33 g), deionized water (330 µL), TIPS (60 µL), and TFA (5.7 mL). After shaking for 3 h at the room temperature, the TFA solution was cooled and collected by using nitrogen. The peptide was then precipitated by adding cold tert-butyl methyl ether. The resultant peptide precipitate was washed three times with

cold ether, dissolved in water, and then lyophilized overnight.

2.3.2. Synthesis of FITC-labeled Peptides

The 6-aminohexanoic acid protected with Fmoc was coupled to peptide with resin by using the same method as that for regular amino acid. 5-FITC (0.3 mmol) was conjugated with peptide (0.1 mmol) in anhydrous DMF solution catalyzed with DIPEA (1.2 mmol). The reaction was performed at the room temperature for 12 h in dark. The FITC-labeled peptide was removed from the resin by using the same cocktail as mentioned above and following the same washing, precipitating, and lyophilizing procedures used in 2.3.1. Dried FITC-labeled peptide was stored at -20°C for further use.

2.3.3. HPLC Analysis and Purification

The peptides and FITC-labeled peptides were analyzed by reverse-phase high-pressure liquid chromatography (HPLC) using the Agilent 1100 System with Agilent Zorbax SB-C18 (4.6 x 150 mm, 3.5 μm column) at 210 nm and 230 wavelengths. The mobile phase solvents were water (solvent A) and acetonitrile (solvent B), and both the solvents were used with 0.1% TFA. Elution was performed with a liner gradient for 30 min from 5% to 95% solvent B at the flow rate of 1 mL/min. The peptide was purified by collecting the eluting peak, lyophilizing, and then storing at -20°C for further use.

2.3.4. Characterization of Peptides

The peptides were characterized by using the Electro Spray Ionization Mass

Spectrometry (ESI-MS; AB Sciex PI 3000 Ion Trap Mass Spectrometer, SCIEX, CA, USA) . The samples were prepared in acetonitrile and water mixtures and were infused at the speed of 10 μL/min. Ion source parameters are listed as follows: Nebulizer Gas (NEB) 8 Psi, IonSpray Voltage 5500 V; TEM （Temperature）300°C; CUR （Curtain Gas） 8 Psi.

2.4. Results and Discussions

Peptides were successfully synthesized using SPPS technique. To make the coupling reaction more efficient, a potent coupling agent, a mixture of HOBT, HBTU, and DIPEA, was used. Fluorescent-dye FITC was conjugated with the peptides for confocal studies. The peptide cannot be directly linked to the FITC because of the unstable abilities of conjugates. To prolong the stability of the conjugates, a spacer, 6-amino hexanoic acid, was attached to the N-terminus of the peptide and then conjugating FICT on the spacer.

The HPLC peaks of all peptides were observed at around 8–9 min (Figures 2.15 to 2.18). HPLC peaks of FITC-conjugated peptides were observed at 15–16 min (Figures 2.19 to 2.22). Different peaks were collected to identify and confirm the molecular weights of peptides and FITC-labeled by MS. All the peptides were characterized using ESI MS. The MS results revealed the expected molecular weight of the peptides and FITC-labeled peptides. The MS confirmed the formation of all peptides and FITC-labeled peptides molecules as shown in Figures 2.5 to 2.14 and table 2.1.

HER2 targeting peptides and cysteine modified HER2 targeting peptides were successfully synthesized for the further peptide binding specificity study and peptide-drug conjugates synthesis.

The HER2 targeting peptide has been reviewed in 1.7. Among the peptides listed above, the peptide MARAKE and MARSGL have been chosen to develop peptide-drug conjugates. These 6-mer peptides with published binding constant (KD) value 10 nM [83], were ideal candidates to for PDC synthesis. The equilibrium binding constant (K_D) is generally used to determine the binding affinity between the ligand and the receptor. Binding affinities are used to determine the strength of binding of a single molecule to its receptors. The KD value relates to the concentration of antibody. The lower KD value represents the higher affinity of the antibody or antibody mimic. Peptides cannot be used directly to conjugate to drugs without linkage.

In our study, the reaction between peptides and linker rely on a thiol group reacting with the maleimide group, which will be further discussed in Chapter 4. Thiol group was provided by cysteine in the peptide, while maleimide group comes from maleimidocaproyl to form the linker. As described above, cysteine modified peptides (MARAKEC, MARSGLC) have been synthesized for PDC synthesis.

To further prove the binding specificity of the chosen HER2 targeting peptide and cysteine modified HER2 targeting peptides, FITC-labeled peptides were synthesized for immunofluorescence in-vitro study. Scramble peptide (MAKRAE) and

FITC-labeled scramble peptide were developed as the negative control in immunofluorescence study.

All peptides were successfully synthesized by SPPS. FITC was conjugated to the peptides at the N-terminus. All peptides molecules were characterized by MS and purified by RP-HPLC for use in further experiments.

Table 2.1 Sequence of synthesized peptides

Peptides	Structure	HPLC Purity	MW
ER1	MARAKE	94.00%	704.84
FITC-ER1	MARAKE-FITC	96.00%	1206.00
ER1C	MARAKEC	95.00%	807.99
FITC-ER1C	MARAKEC-FITC	99.90%	1309.99
ER2	MARSGL	97.00%	633.76
FITC-ER2	MARSGL-FITC	99.80%	1135.76
ER2C	MARSGLC	96.00%	736.91
FITC-ER2C	MARSGLC-FITC	96.10%	1238.00
RA1	MAKRAE	94.00%	704.84
FITC-RA1	MAKRAE-FITC	95.10%	1206.00

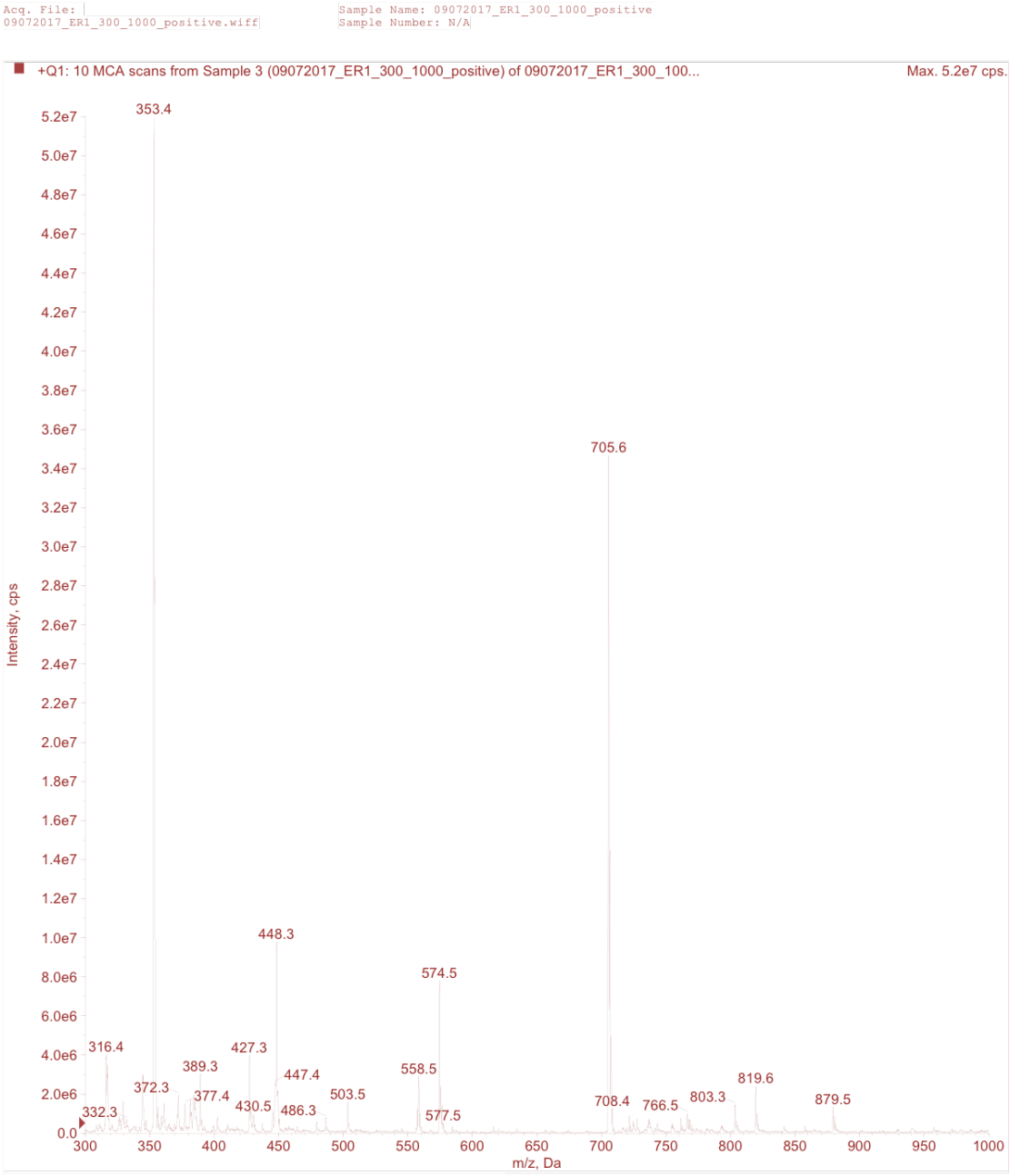

Figure 2.5 The ESI-MS spectrum of peptide ER1

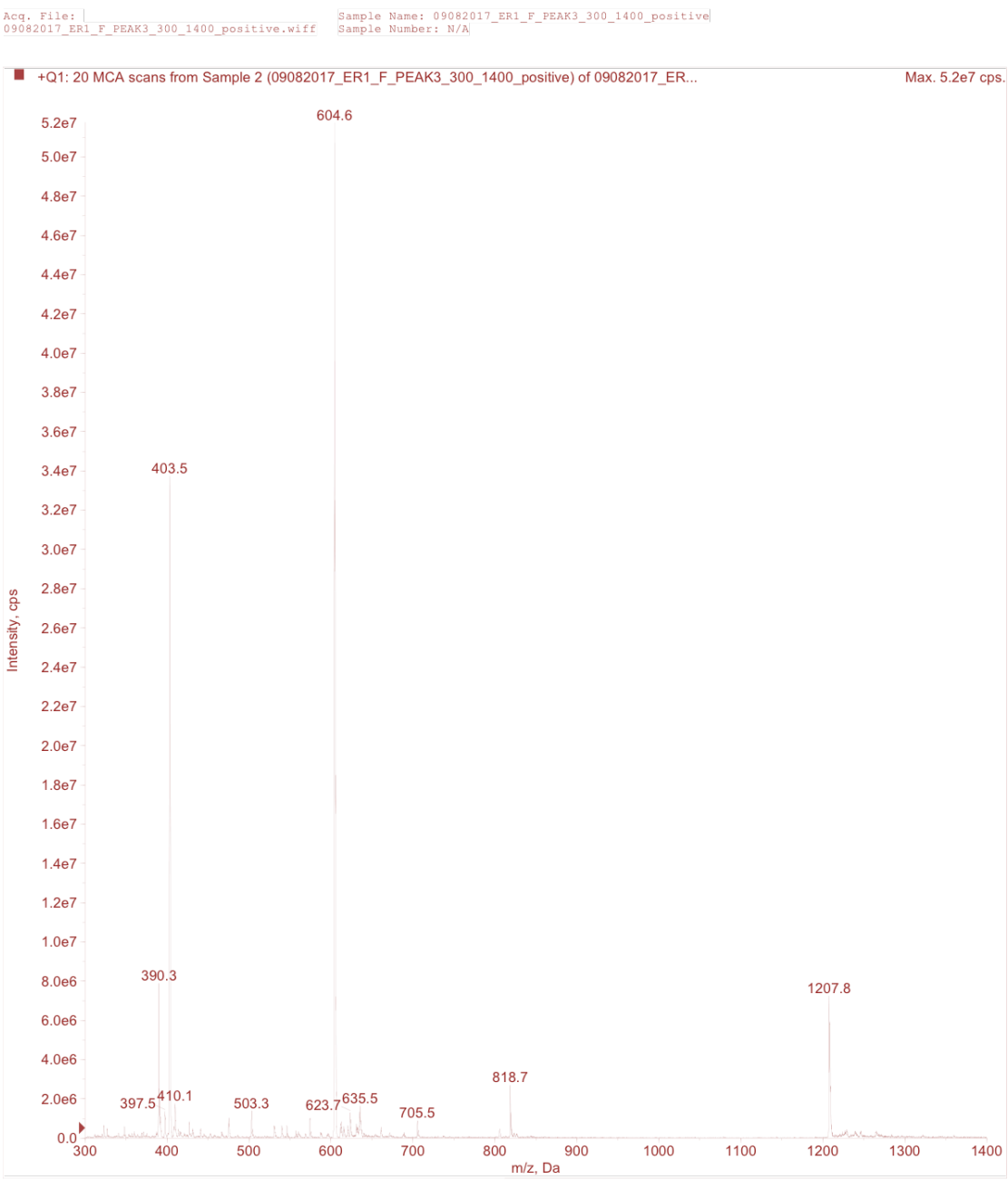

Figure 2.6 The ESI-MS spectrum of FITC-labeled peptide ER1

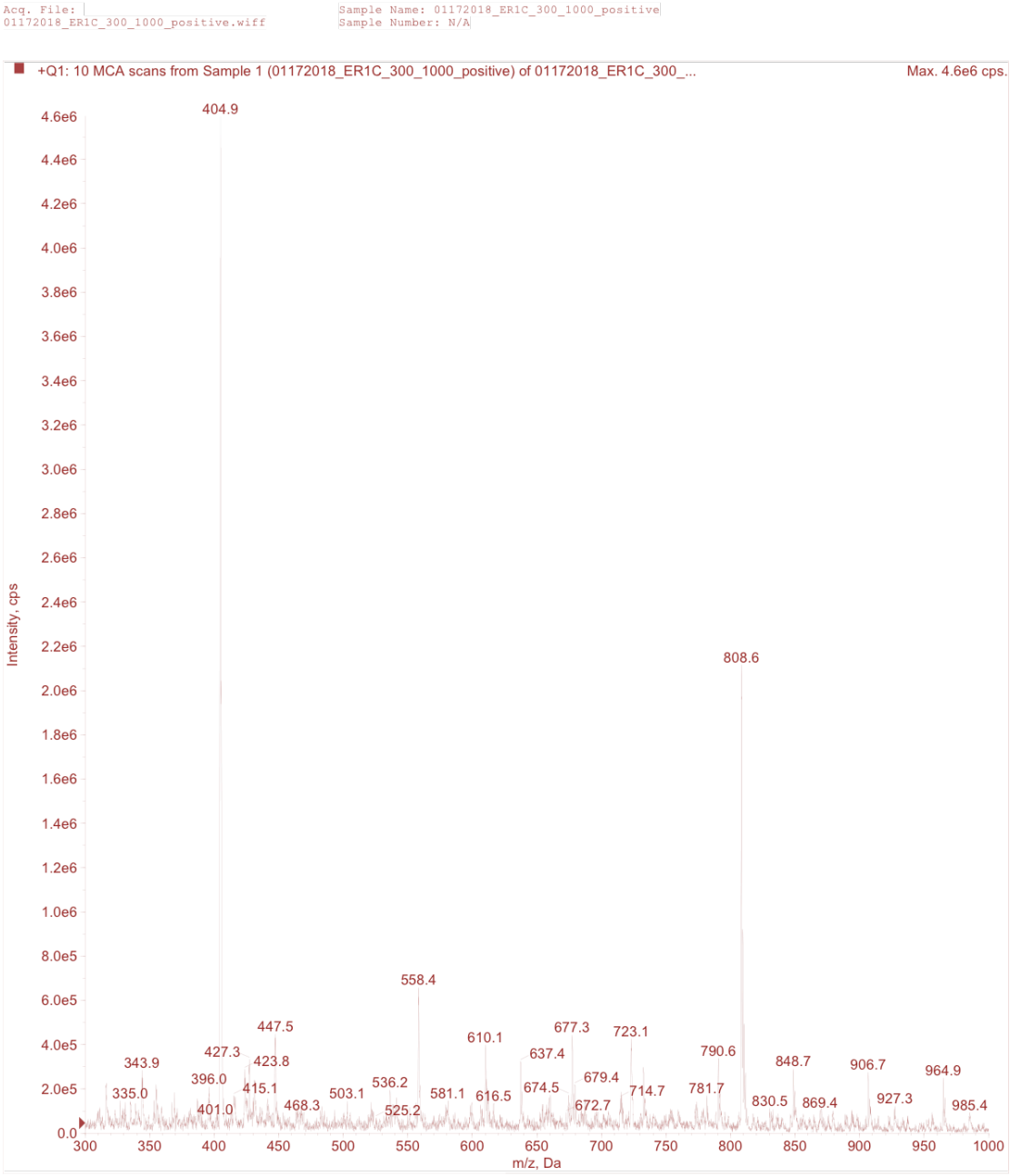

Figure 2.7 The ESI-MS spectrum of peptide ER1C

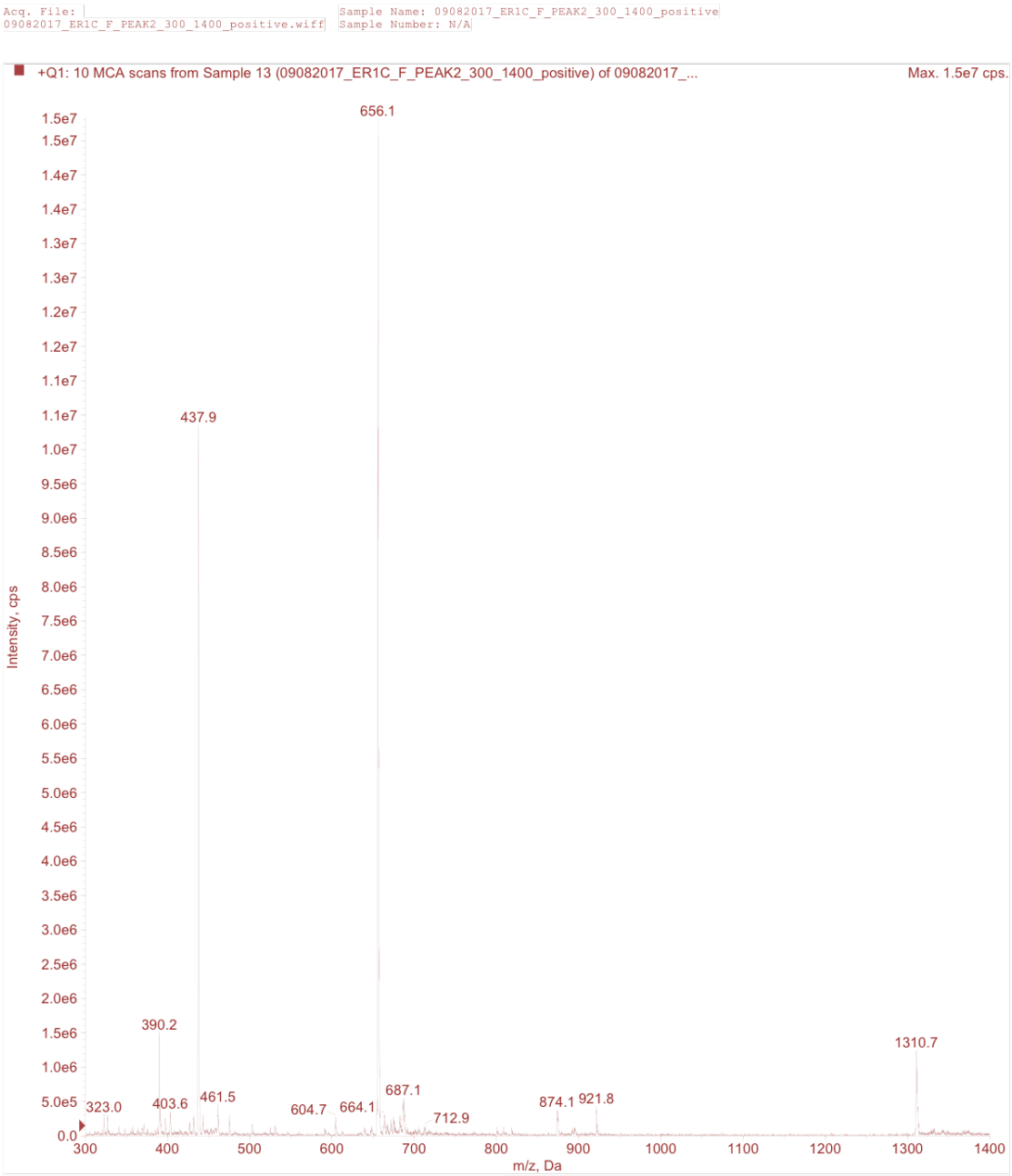

Figure 2.8 The ESI-MS spectrum of FITC-labeled peptide ER1C

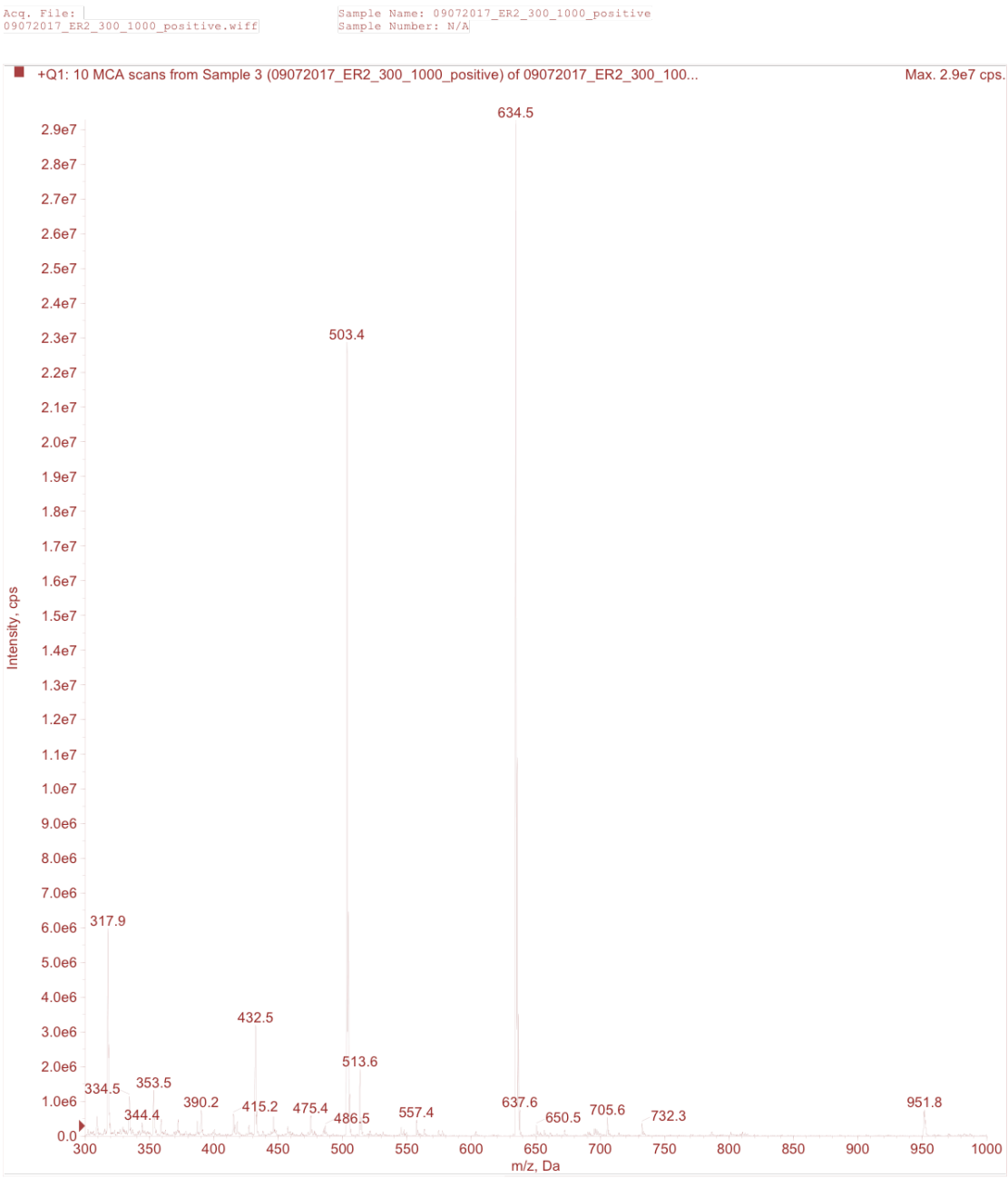

Figure 2.9 The ESI-MS spectrum of peptide ER

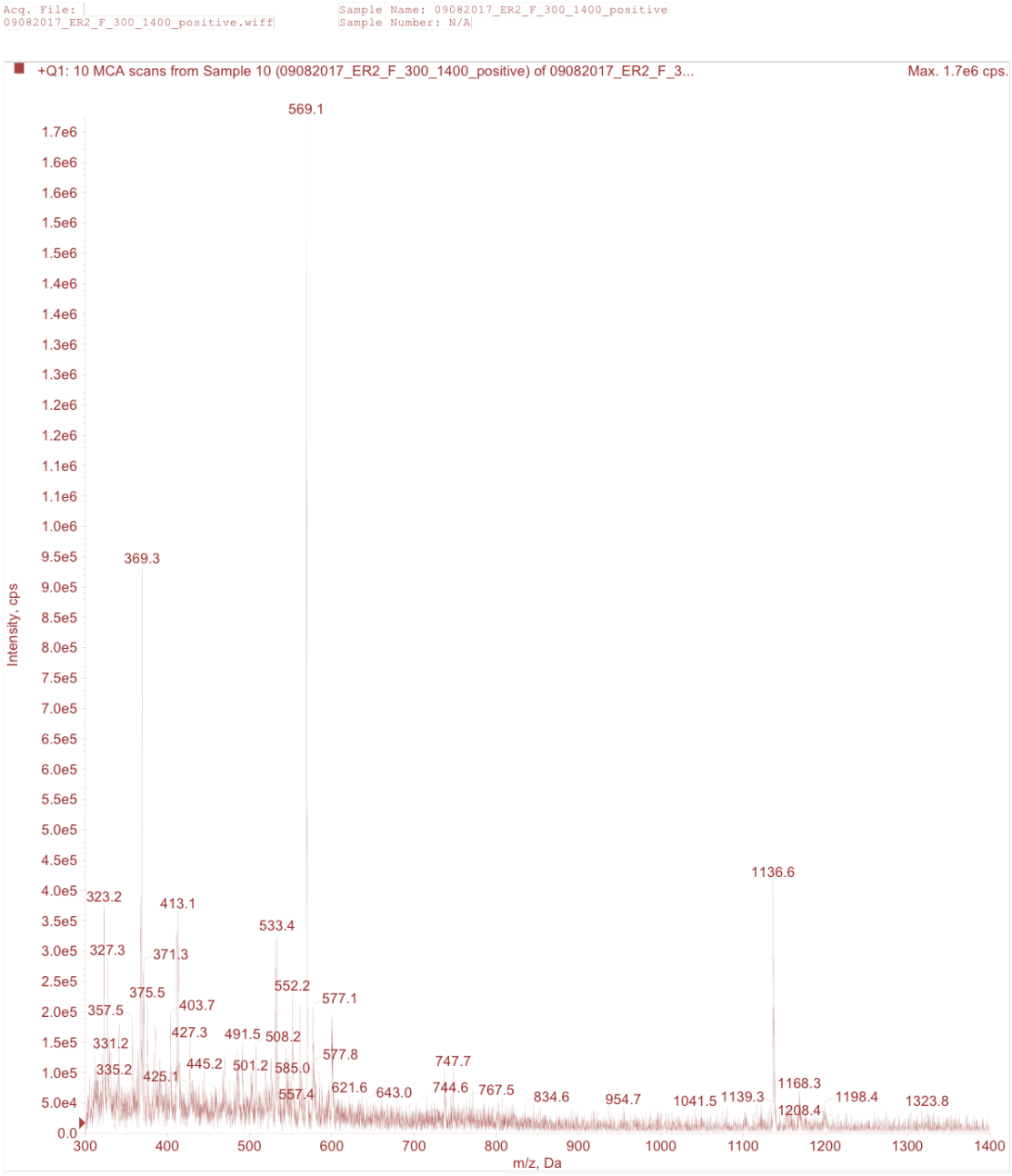

Figure 2.10 The ESI-MS spectrum of FITC-labeled peptide ER2

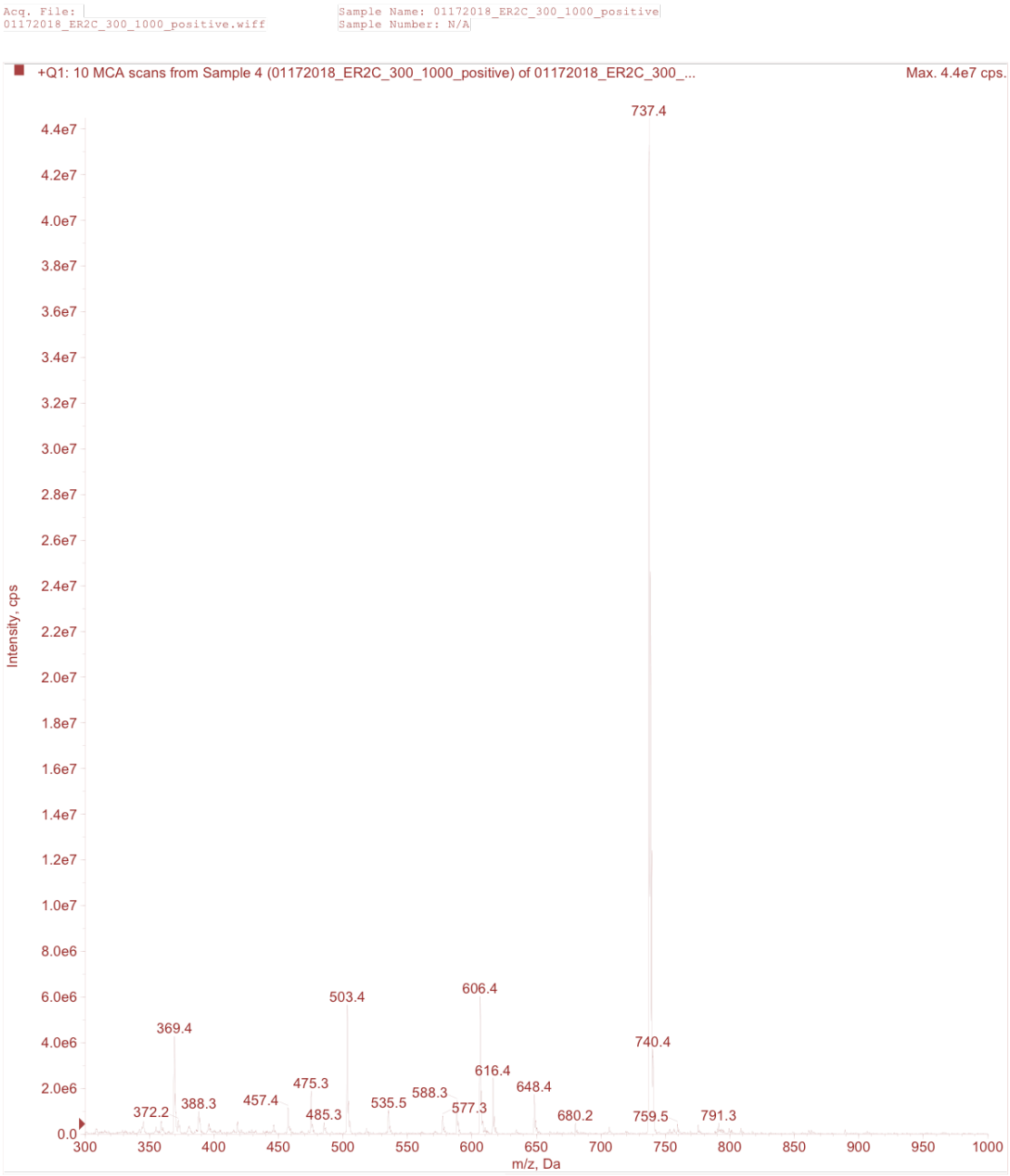

Figure 2.11 The ESI-MS spectrum of peptide ER2C

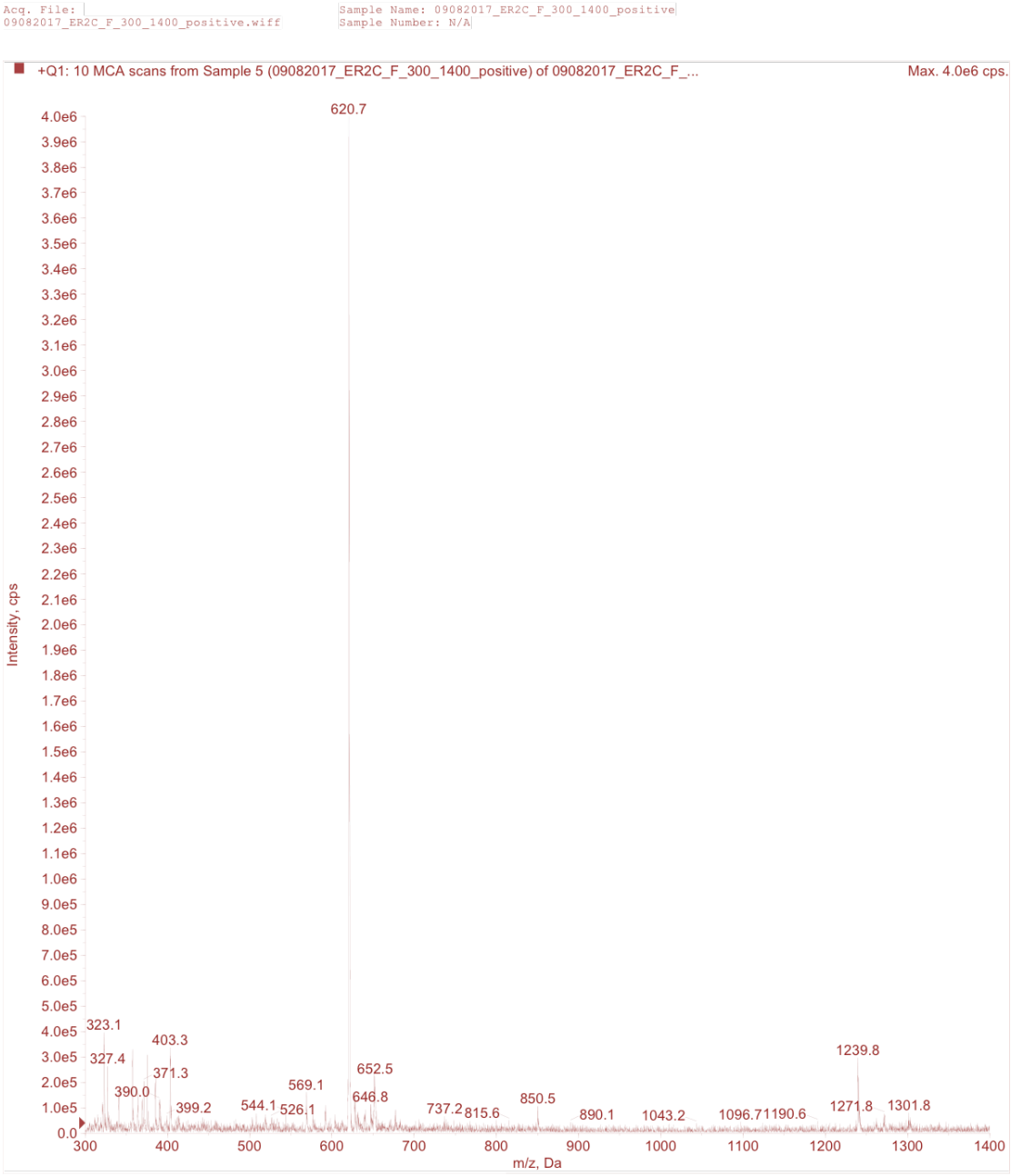

Figure 2.12 The ESI-MS spectrum of FITC-labeled peptide ER2C

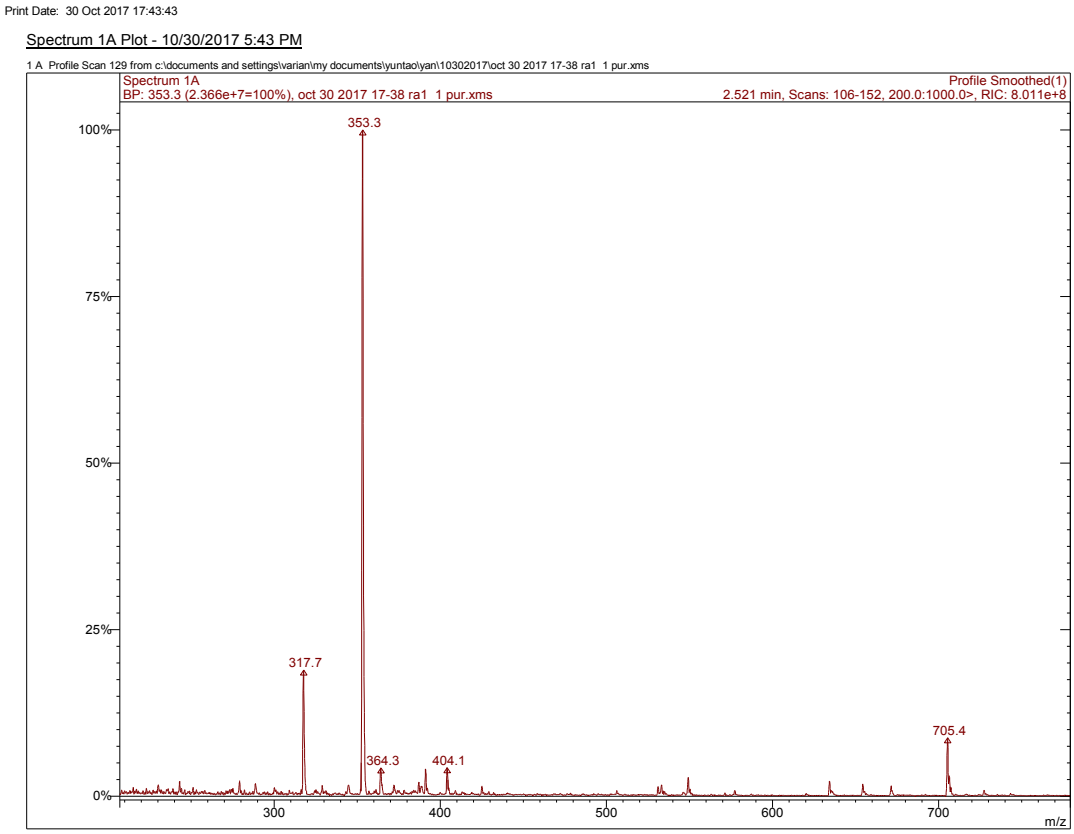

Figure 2.13 The ESI-MS spectrum of peptide RA

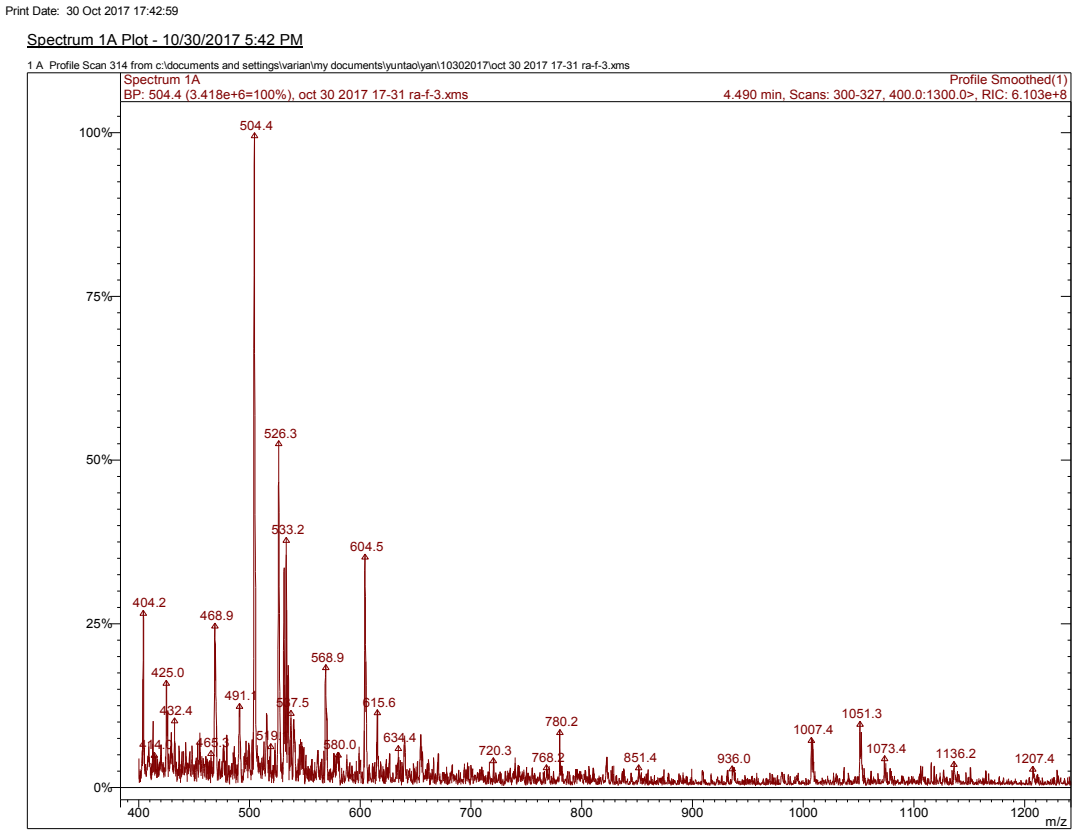

Figure 2.14 The ESI-MS spectrum of FITC-labeled peptide RA

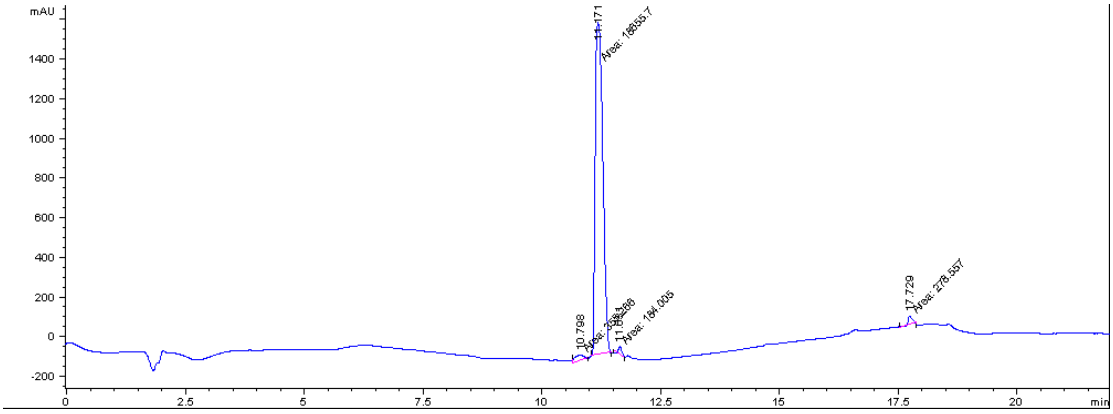

Figure 2.15 The HPLC chromatogram of peptide ER1

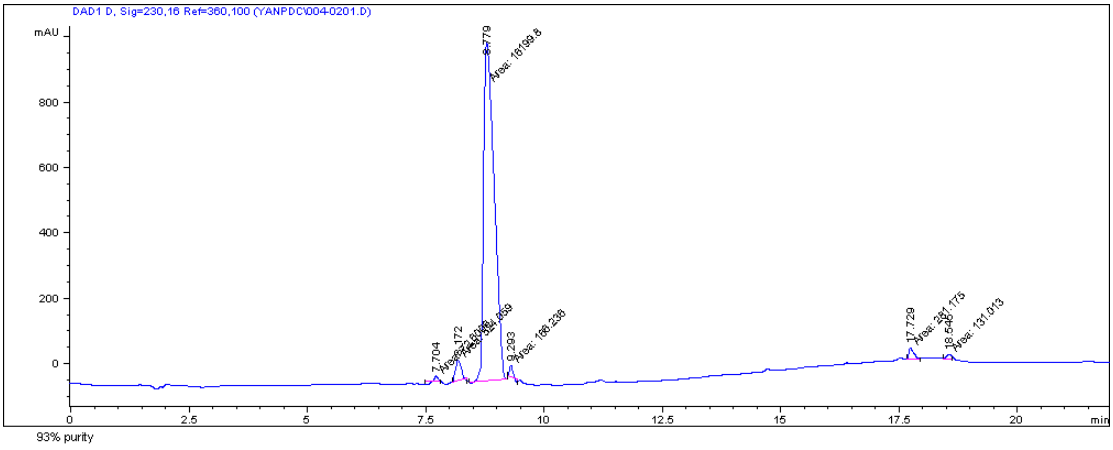

Figure 2.16 The HPLC chromatogram of peptide ER1C

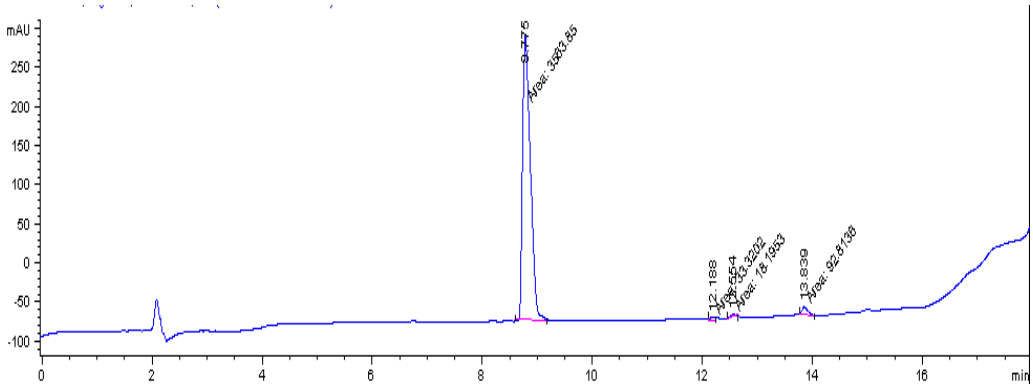

Figure 2.17 The HPLC chromatogram of peptide ER2

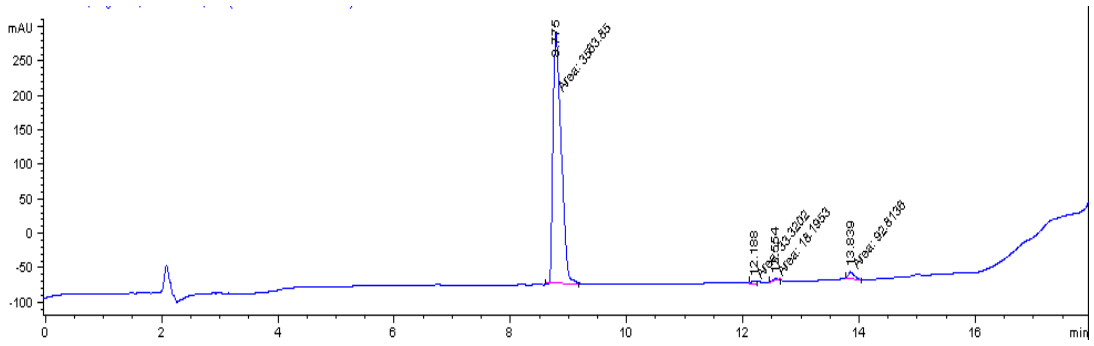

Figure 2.18 The HPLC chromatogram of peptide ER2C

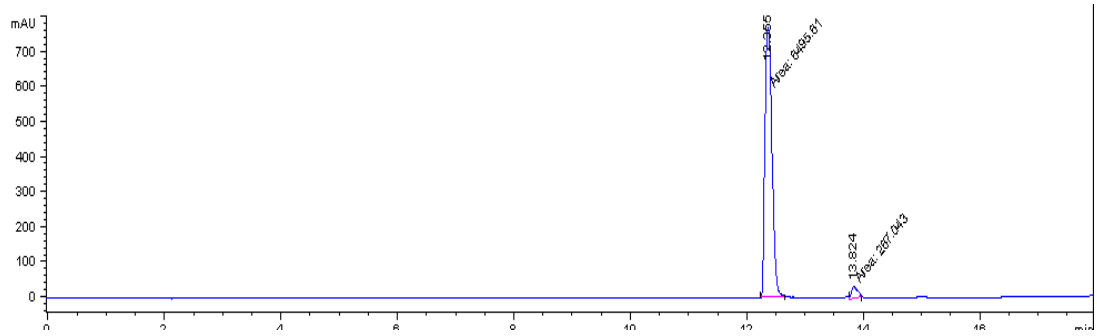

Figure 2.19 The HPLC chromatogram of FITC-labeled peptide ER1

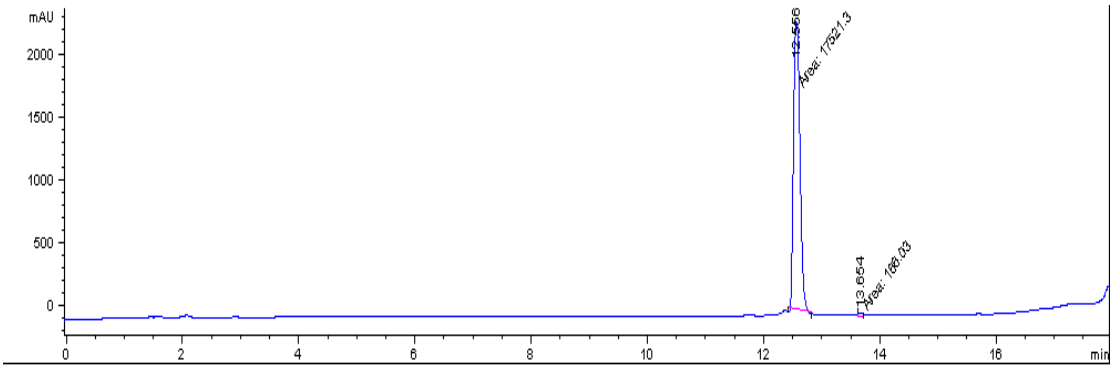

Figure 2.20 The HPLC chromatogram of FITC-labeled peptide ER1

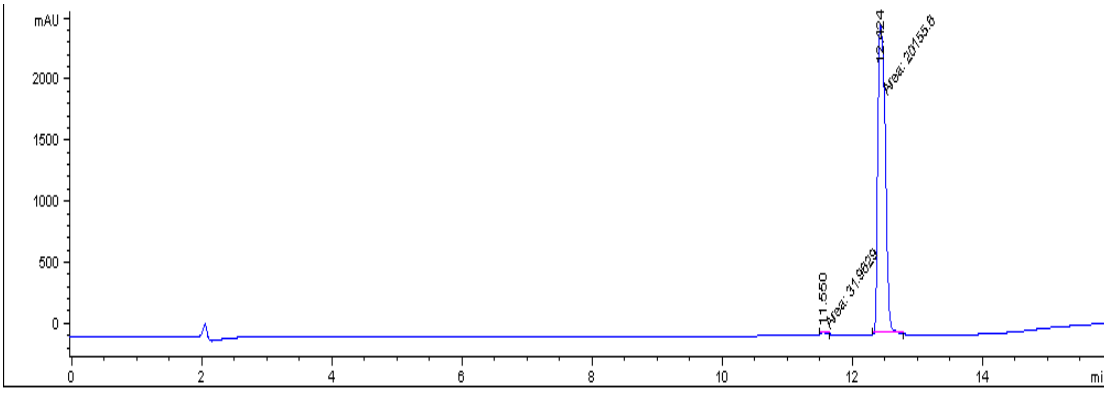

Figure 2.21 The HPLC chromatogram of FITC-labeled peptide ER2

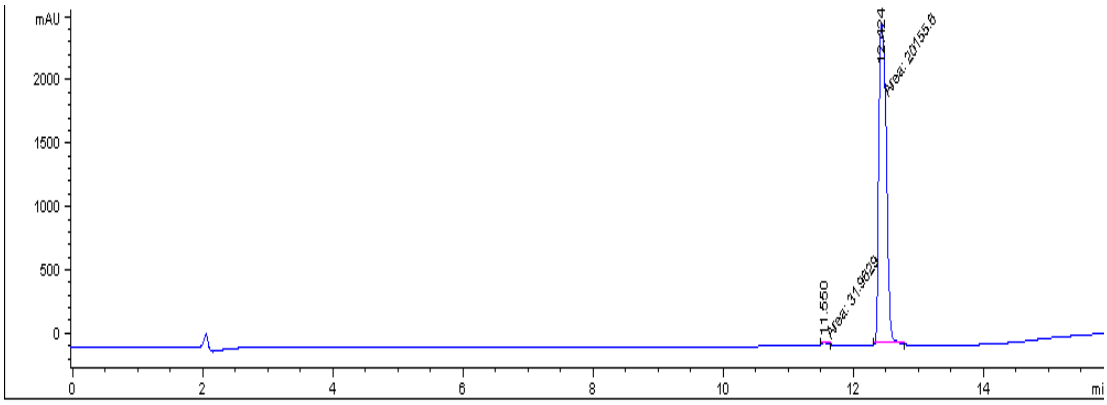

Figure 2.22 The HPLC chromatogram of FITC-labeled peptide ER2C

CHAPTER 3: BINDING SPECIFICITY OF HER2-TARGETING PEPTIDE

3.1. Introduction

The signaling pathway in cells starts from the binding of ligands and receptor. Ligand and receptor come in closely matched pairs, with receptors recognizing specific ligands. Another similar signaling pathway is antibody-antigen interaction. The antibody specifically binds to the antigen expressed on the surface of cells. After binding with an antigen, the antibody-antigen complex is transported into cells. The design of antibody-drug conjugates utilizes this characteristic of antibody and antigen complex. By linking the drug to an antibody, the conjugate can deliver the drug to the specific target cancer cells. The specificity of antibody leads to high potency and low cytotoxicity of antibody-drug conjugate, the same concept can be applied to the peptide-drug conjugates.

Immunofluorescence (IF) is a conventional laboratory technique used to identify microbiological samples with a fluorescent microscope. This technique is used for studying specificity of antibodies binding to a specific antigen by monitoring fluorescent dyes in specific bio-molecular targets or cells. Immunofluorescence labeled antibody can recognize and stain particular cellular protein. The specificity of the antibody-fluorescence conjugation can be verified by using cell line with known expression target or level. The specificity of peptides can be determined by similar method with the use of fluorophore-conjugated targeting peptides. HER2 is

overexpressed in breast cancer cell lines, and it undergoes receptor-mediated endocytosis when trastuzumab binds to HER2. To evaluate the peptides' binding specificity to HER2 receptor, confocal and fluorescent microscopy was used with 5-FITC conjugated HER2-targeting peptides. Fluorescent microscopy showed the binding specificity, while confocal microscopy confirmed the internalization of the samples.

The purpose of this chapter is using immunofluorescence to verify the specificity of HER2-targeting peptide.

3.2. Materials

The Alexa Fluor 594 wheat germ agglutinin SlowFade were obtained from Invitrogen (Carlsbad, CA, USA). Formaldehyde (4%) in PBS was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All chemicals and solvents were used without purification. PBS was ordered from Gibco (Invitrogen Corporation, Japan).

The MDA-MB 361, ZR 75, and HEK 293 cells were purchased from ATCC (Manassas, VA, USA). Dulbecco's modified eagle medium (DMEM), 4.5 g/L glucose, L-glutamine and 1% penicillin-streptomycin (5000 I.U./mL) were obtained from Mediatech (Manassas, VA, USA). Sodium pyruvate containing 10% heat-inactivated fetal bovine serum was ordered from Gemini (Sacramento, CA, USA)

3.3. Methods

The MDA-MB 361, ZR 75, and HEK 293 cells were cultured in 75-cm^2 culture flasks in DMEM supplements with 10% FBS and 1% penicillin-streptomycin. Cells (1×10^5) were placed on cover slips in 6-well plates and cultured at 37 °C for 24 h. The mediums were removed from 6-well plates. The cells were then washed with PBS and serum-free media. FITC-labeled peptides were used to treat cells in the serum-free media for 20 min. Then, the medium was removed, and the cells were washed with PBS twice in order to remove the unbound peptides. Alexa Fluor 594 wheat agglutinin (2.5 µg/mL in PBS) was then used to treat cells for 10 min to stain the cell membranes. The cells were washed two times with PBS and fixed with 4% paraformaldehyde for 15 min. The cover slips were attached to the slides with a drop of *SlowFade* antifade reagent. The cells were then imaged under brand BZ-X710 All-in-One Fluorescence Microscope (Keyence, Itasca, USA) using green and red filters with 40 X objective. The samples prepared for fluorescence microscope were also used for visualization by an inverted Leica DMIRE2 confocal microscope (Leica Microsystems GmbH, Germany) with Yokogawa CSUX1 63X magnification (Yokogawa Electric Corporation, Japan) to verify internalization. All the images were analyzed and transformed to the bmp format by Image J software.

3.4. Result and Discussion

Ligand binding assays (LBA) is an analytical assay used to measure the strength

of ligand binding to receptors. Numerous techniques are used to measure the ligand binding including fluorescence resonance energy transfer (FRET), fluorescence polarization (FP), and surface plasmon resonance (SPR). In this study, the specificity of HER2-targeting peptides has been confirmed. HER2 specific - peptides should preferentially bind to tumor cancer cells that over express HER2 rather than to normal cells. Binding specificity studies are used to determine the specific binding and uptake of different molecules in the target cells. Confocal and fluorescent images provide the visual information about the peptides binding to target cells. To determine the influence of cysteine added in peptide sequence, four specific peptides ER1 (MARAKE), ER1C (MARAKEC), ER2 (MARSGL), ER2C (MARSGLC), and one random peptide RA (MAKRAE) were chosen to study the binding specificity.

Three different cell lines were used to study the binding specificity of peptides. MDA-MB-361 cells and ZR-75-1 cells are cell lines that overexpress HER2, while HEK293 cells are the human normal kidney cells that do not express HER2 [84]. All four FITC-labeled peptides and cysteine-modified peptides were found to be specifically bound to the cell membrane and internalized into the cells that overexpressed HER2 within the first 20 min at 37˚C. As shown in Figure 3.1- Figure 3.4, significant higher fluorescence was observed in MDA-MB 361 and ZR 75-1 cells as compared with the negligible fluorescence found in HEK 293 cells, which demonstrated the binding specificity of the four specific peptides towards HER2. In the case of FITC-labeled control peptide RA1, no binding was observed in all of three

cell lines. The binding specificity of the ER1 and ER2 peptides confirmed the claim in the literature [83], and the binding specificities of cysteine-modified peptides were also proved. The result of the confocal microscopy studies suggested that cysteine-modified peptides retain their binding capability to HER2-positive cell lines.

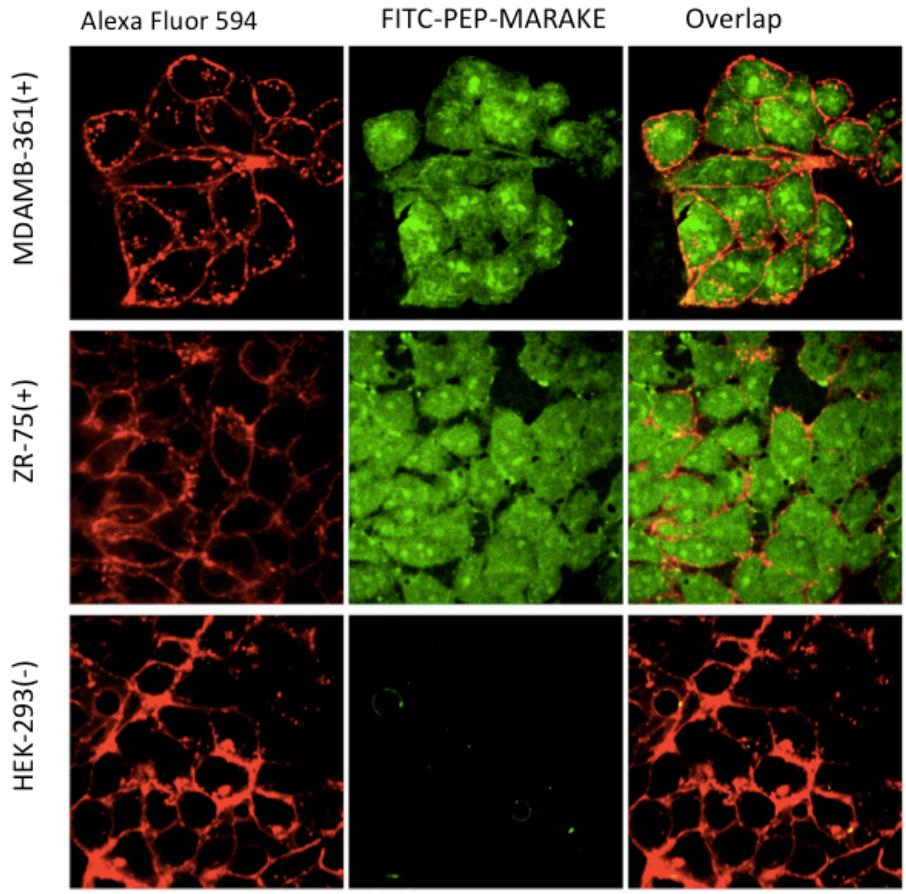

Figure 3.1 Confocal images of peptide ER1. Alexa Fluor594 column represents the cell treated with membrane dye, FITC-PEP-MARAKE displays the cells treated with FITC conjugated peptide and overlap column represent the merge of the two images.

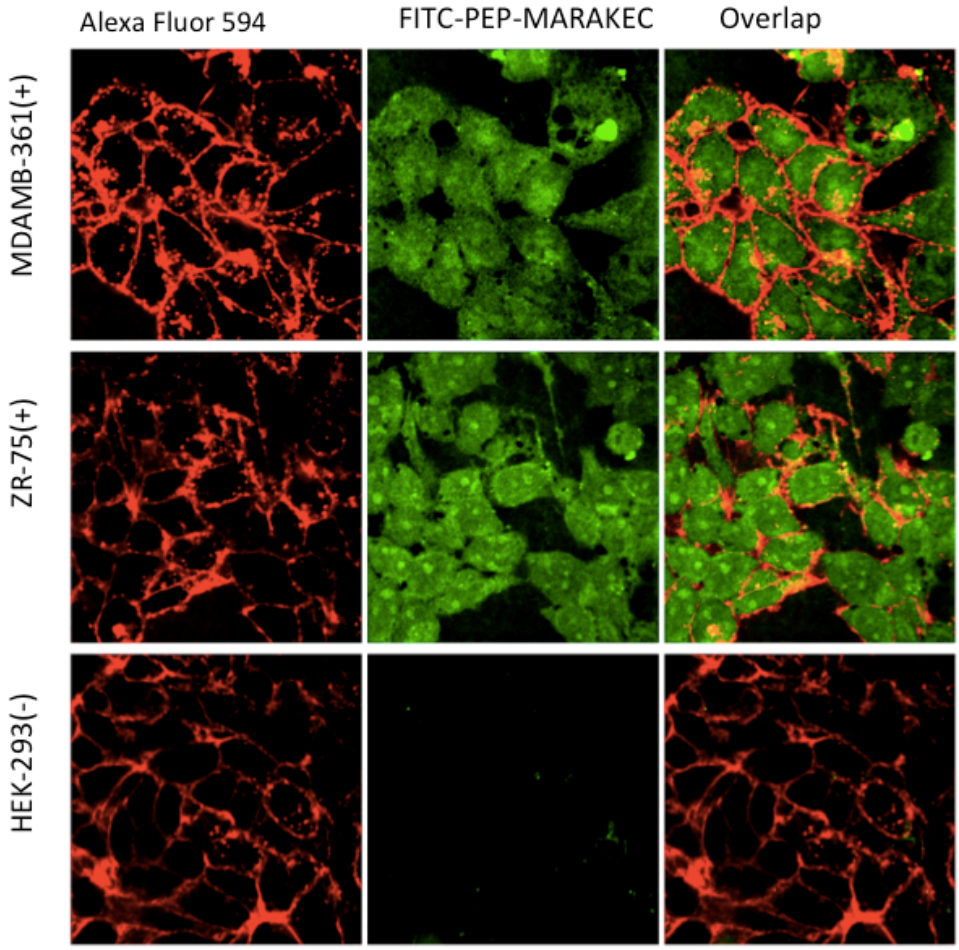

Figure 3.2 Confocal images of peptide ER1C. Alexa Fluor594 column represents the cell treated with membrane dye, FITC-PEP-MARAKEC displays the cells treated with FITC conjugated peptide and overlap column represent the merge of the two images.

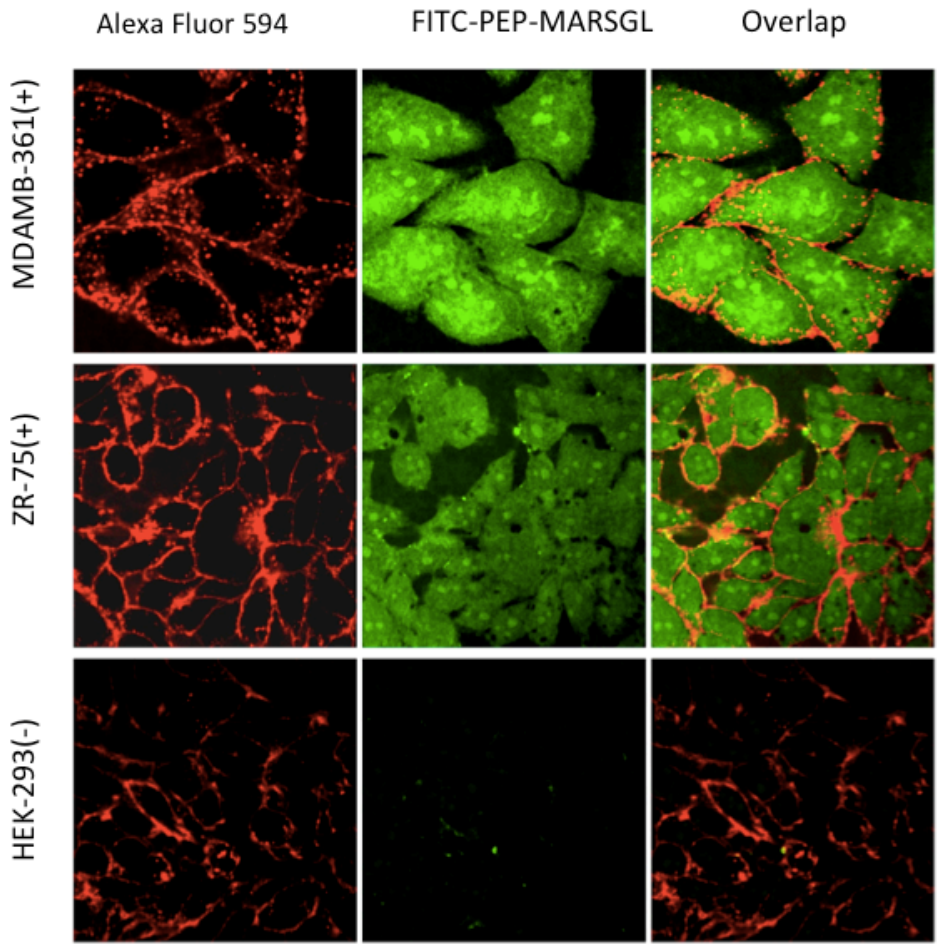

Figure 3.3 Confocal images of peptide ER2. Alexa Fluor594 column represents the cell treated with membrane dye, FITC-PEP-MARSGL displays the cells treated with FITC conjugated peptide and overlap column represent the merge of the two images.

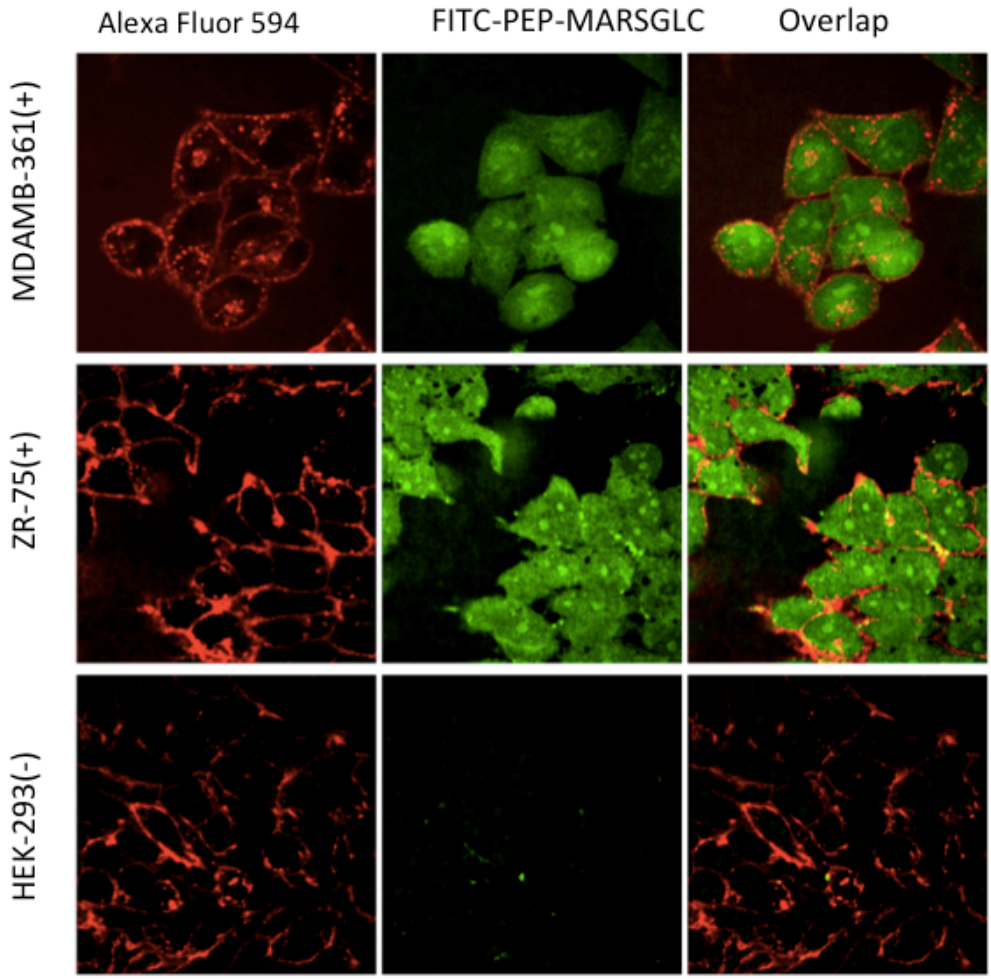

Figure 3.4 Confocal images of peptide ER2C. Alexa Fluor column represents the cell treated with membrane dye, FITC-PEP-MARSGLC displays the cells treated with FITC conjugated peptide and overlap column represent the merge of the two images.

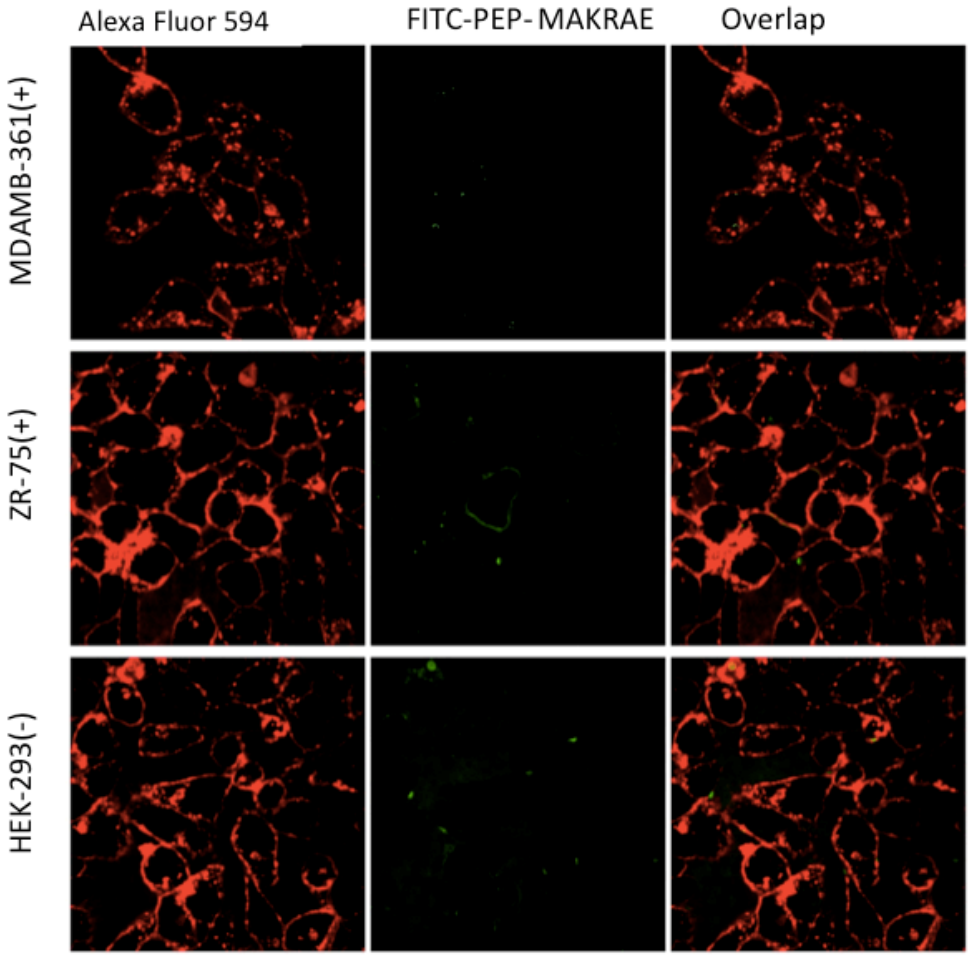

Figure 3.5 Confocal images of peptide RA. Alexa Fluor594 column represents the cell treated with membrane dye, FITC-PEP-MAKRAE displays the cells treated with FITC conjugated peptide and overlap column represent the merge of the two images.

CHAPTER 4: SYNTHESIS AND CHARACTERIZATION OF PEPTIDE-DRUG CONJUGATES AND HYBRID PEPTIDES

4.1. Introduction

As stated in Chapter 1, peptide–drug conjugates share similar concepts with antibody–drug conjugates; the flexibility of PDCs in structure and low molecular weight lead to enhanced drug loading and higher penetration into the solid tumor tissues. The peptides used in PDCs can be divided into two categories: cell penetration peptides (CPPs) and cell-targeting peptides (CTPs). The linker used in PDCs can be categorized into two types: non-cleavable linkers and cleavable linkers. Carbon chain, amide bond, and ether bond are commonly used in non-cleavable linker designs. They are stable in biological fluids and can reduce the premature drug release in the blood circulation. PDCs with the cleavable linkers can release the drug after reaching the tumor site. The cleavable linkers can be further divided into three different types: pH-sensitive, redox-sensitive, and enzyme-sensitive. Hydrazone bond, vinyl ether bond, and acetal bond are pH-sensitive linkers that release drug in an acidic environment in the endosomes. Redox-sensitive linkers release the drugs in endosomes mediated by GSH. The cleavage of the enzyme-sensitive linkers are mediated released drug by Cathepsin B. For the objective of this study, HER2-targeting peptides MARAKE and MARSGL were chosen as targeting moiety

to design the peptide–drug conjugates. Two type linkers: non-cleavable Maleimidocaproyl (MC) linker and cleavable valine-citrulline (VC) linker were used to link the cytotoxic drug MMAE and targeting peptides to form the peptide–drug conjugates.

Cytolytic peptides, which emerge from eukaryotic cells, were found to have anticancer function. In this study, a design based on the fusion of cytolytic peptide as anticancer agent and HER2 binding peptide was carried out to generate a "peptide-peptide conjugate" for targeted cancer delivery of cytolytic peptide [84]. In our study, a lytic peptide, melittin [67] that was developed from nanobees was chosen to fuse with HER2-targeting peptide in order to form hybrid peptides. Table 4.1 indicates the design of hybrid lytic peptides. When cytolytic peptides are used in the design, non-cleavable amid linkage is used.

The purpose of this chapter is to synthesize, characterize and purify peptide-drug conjugates and hybrid peptides for in vitro studies.

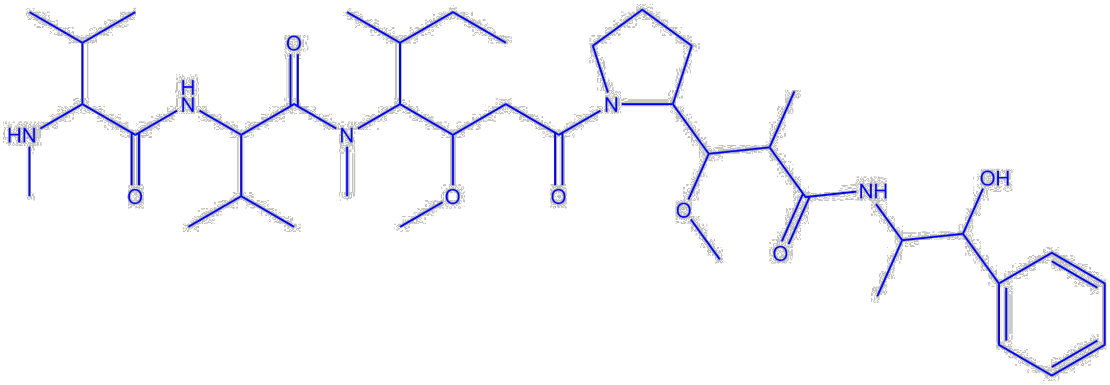

Figure 4.1 Structure of MMAE（1）

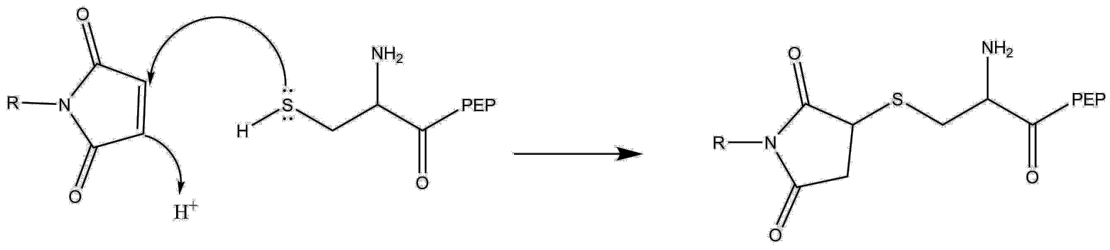

Figure 4.2 Mechanism of peptide–drug conjugates reaction

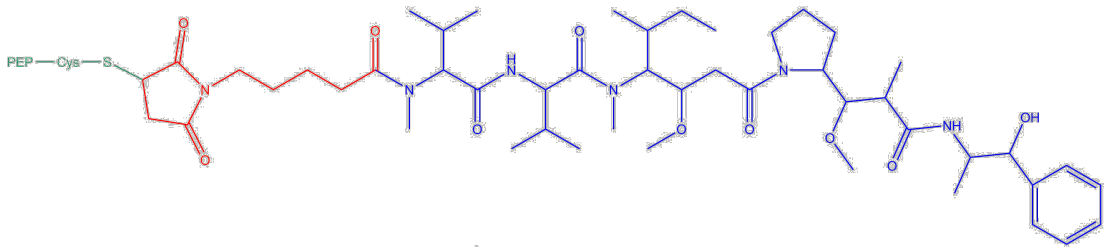

Figure 4.3 Structure of PDC based on MC-MMAE (2). The linker consisting of attachment group maleimidocaproyl is marked by red. The drug MMAE is blue while the peptide is green.

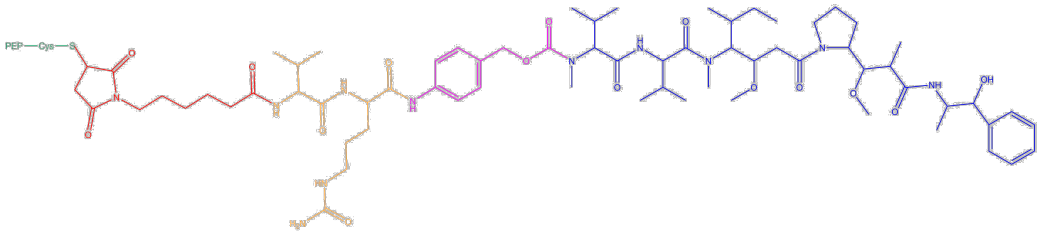

Figure 4.4 Structure of PDC based on VC-MMAE (3). The linker consisting of cleavable dipeptide valine (Val)-citrulline(Cit), the spacer (p-aminobenzylcarbamate) and the attachment group maleimidocaproyl. The dipeptide is marked by yellow; the spacer is pink; the attachment group is red; the color of drug MMAE and the peptide are the same as Figure 4.3.

Table 4.1 Summary designing of peptide-drug conjugates

Name of PDCs	Peptide	Linker
YW1 (4)	MARAKEC	MC-MMAE (2)
YW2 (5)	MARSGLC	MC-MMAE (2)
YW3 (6)	MARAKEC	VC-MMAE (3)
YW4 (7)	MARSGLC	VC-MMAE (3)

Table 4.2 Sequence of lytic peptide and hybrid peptides

Peptides	Structure	MW
LY1 (8)	GIGAVLKVLTTGLPALISWIKRKRQQ	2847.46
HY1 (9)	MARAKE-GGG-GIGAVLKVLTTGLPALISWIKRKRQQ	3705.44
HY2 (10)	MARSGL-GGG-GIGAVLKVLTTGLPALISWIKRKRQQ	3634.36

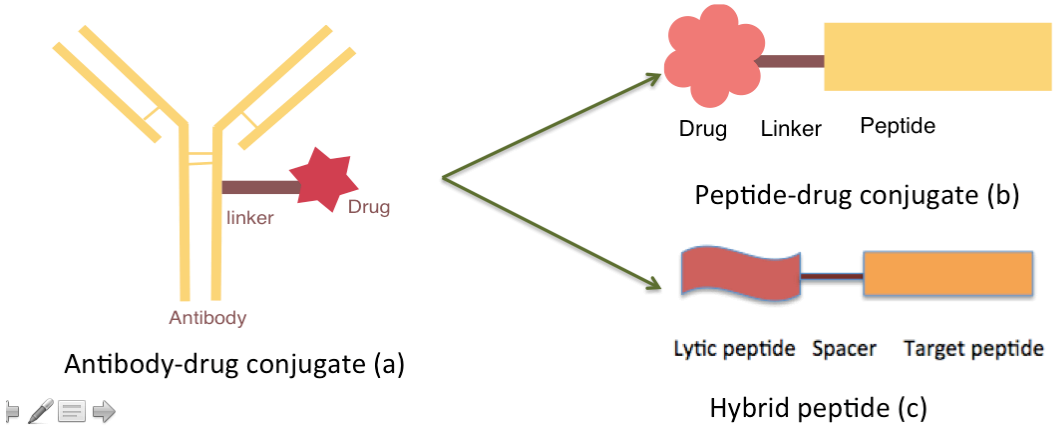

Figure 4.5 Model of Antibody-drug conjugate (a), peptide-drug conjugate (b), and hybrid peptide (c).

4.2. Materials

MC-MMAE and VC-MMAE were obtained from Boc Science (Shirley, NY, USA). MMAE was ordered from Medchem Express (New Jersey, USA), and N,N'-dimethylformamide (DMF) and Triethylamine (TEA) were purchased from Axros Organics (New Jersey, USA).

4.3. Methods

4.3.1. Synthesis of MC-MMAE Peptide–drug Conjugate

Peptide (1.1 eq) with a free thiol group and MC-MMAE (1 eq) were dissolved separately in DMF. The peptide was added to the MC-MMAE DMF solution drop-wise, to which 10 µL of TEA was added into the reaction mixture under nitrogen protection. The reaction mixture was stirred for 2.5 h at the room temperature and then evaporated under reduced pressure.

4.3.2. Synthesis of VC-MMAE Peptide–drug Conjugate

VC-MMAE was used in the place of MC-MMAE and other procedures and conditions were the same as that described in 4.3.1.

4.3.3. Purification of Peptide–drug Conjugate

All peptide–drug conjugates were dissolved in methanol and then purified on RP-HPLC with the Agilent 1100 System (Agilent Zorbax SB-C18; 4.6 x 150 mm, 3.5-µm column) at 230 and 280 nm. The mobile phase solvents were water (solvent A) and methanol (solvent B), and both solvents were used with 0.1% TFA. Elution was performed with a linear gradient for 30 min from 5% to 95% solvent B at the flow rate of 1 mL/min at 23.8°C.

4.3.4. Characterization of Peptide–drug Conjugate

The products were characterized by using Electro Spray Ionization Mass Spectrometry (ESI-MS; AB Sciex PI 3000 Ion Trap Mass Spectrometer). The samples were prepared in methanol and water mixtures and infused at the speed of 10 µL/min. Ion source parameters are listed as follows: Nebulizer Gas (NEB) 8 Psi, Ion Spray Voltage 5500 V; TEM （Temperature） 300°C; CUR （Curtain Gas） 8 Psi.

4.4. Results and Discussion

In this study, HER2-targeting peptide ER1 and ER2 were selected to synthesize peptide-drug conjugates. Cysteine was used to modify HER2-targeting peptides to generate a functional group for the linkage to the drug. The specificity of cysteine-modified peptide ER1C and ER2C were retained after modification as

confirmed in Chapter 3.

These modified targeting peptides were conjugated with MMAE, which is a non-selective but potent tubulin polymerization inhibitor that develops high toxicity in tumor cells. Two type linkers: non-cleavable Maleimidocaproyl (MC) linker and cleavable valine-citrulline (VC) linker were used to prepare the peptide–MMAE conjugates. Both MC linker and VC linker have the maleimide group, which can react with the thiol group and form the thiol-ether bond between the peptide and the drug. The reaction mechanism of thiol group and maleimide group was illustrated in Figure 4.2.

All the intermediate and final products were characterized by ESI-MS as shown in Table 4.2. The mass spectra for MMAE, MC-MMAE, VC-MMAE, YW1, YW2, YW3, and YW4 were observed as both single- and double-charge species (Figures 4.9–4.15). RP-HPLC was performed to check the purity and to purify the PDCs. The purity of all compounds was found to be >95% (Figure 4.5-4.8; Table 4.2). All PDCs were purified through RP-HPLC on the C18 column and used for further experiments.

The lytic peptide and hybrid peptides were successfully synthesized and were characterized by ESI-MS as shown in Table 4.3. The mass spectra for LY1, HY1, HY2 were observed from quadruple to octuple charge (Figure 4.16-4.18).

In this chapter, four peptide-drug conjugates were successfully synthesized and purified. ESI mass spectrum was used to characterize the molecular weight of conjugates, while HPLC was used to purify the conjugates. The purities of

peptide-drug conjugates were confirmed more than 95%. Peptide with more than 90% purity also named high-level of purity peptide, which is beneficial for in-vitro cytotoxicity studies.

The lytic and hybrid peptides were also successfully synthesized and characterized. However, the purities of lytic and hybrid peptides were range from 69% to 73%, which was considered in mid-range levels of purity. This level of purity is appropriate for screenings peptides in the preliminary studies. Lytic and hybrid peptides cytotoxicity studies were performed in Chapter 5.

Table 4.2 MMAE, intermediates and PDC MS and HPLC data

Molecule	HPLC Purity (%)	MW (g/mol)
MMAE (1)	99%	718
MC-MMAE (2)	99%	911.2
VC-MMAE (3)	99%	1316.65
YW1 (4)	98%	1720
YW2 (5)	98%	1649
YW3 (6)	99%	2124
YW4 (7)	98%	2053

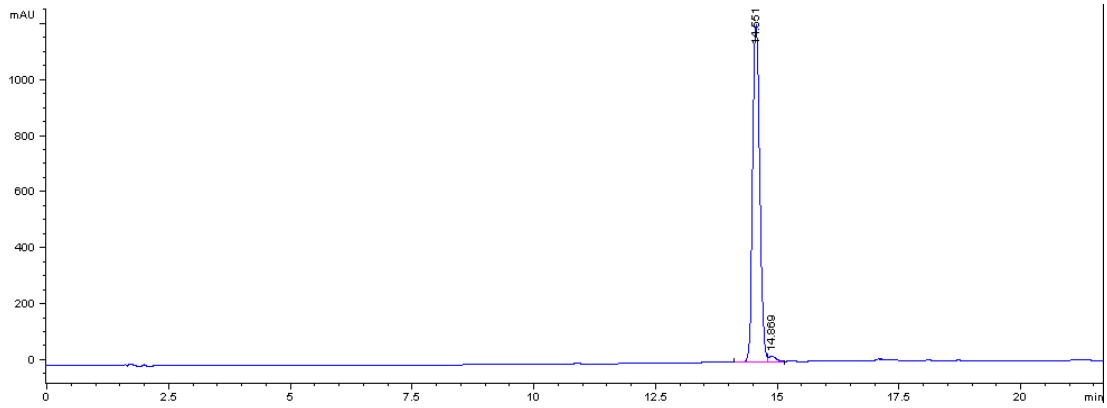

Figure 4.5 HPLC chromatogram of YW1

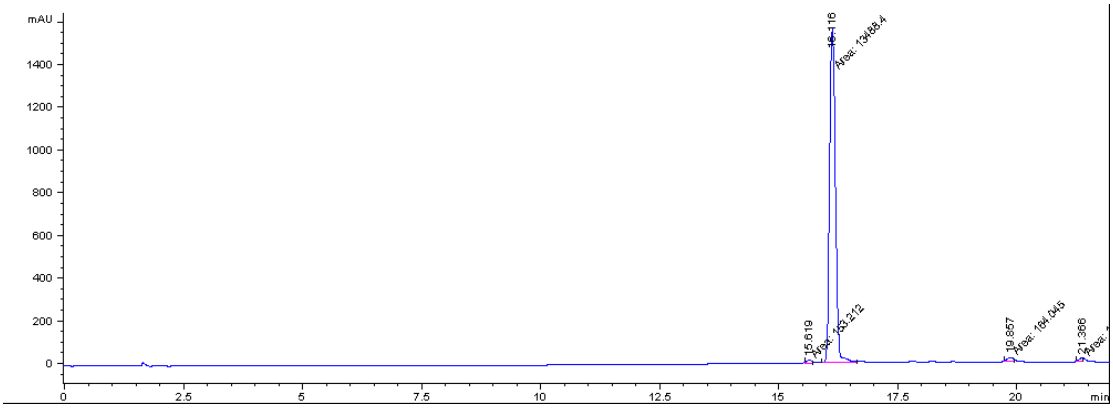

Figure 4.6 HPLC chromatogram of YW2

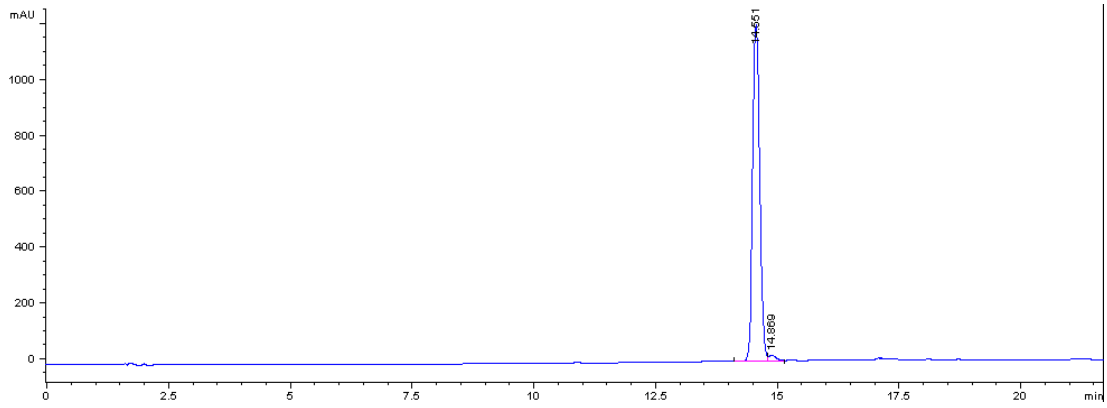

Figure 4.7 HPLC chromatogram of YW3

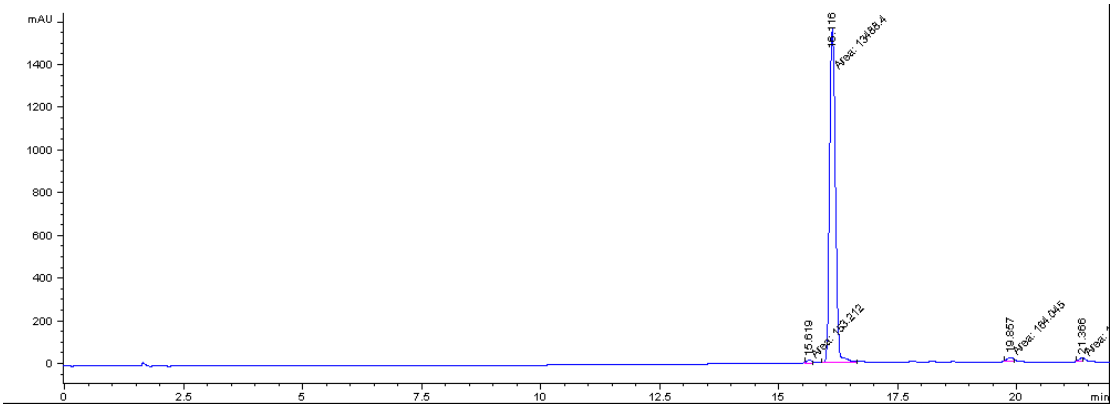

Figure 4.8 HPLC chromatogram of YW4

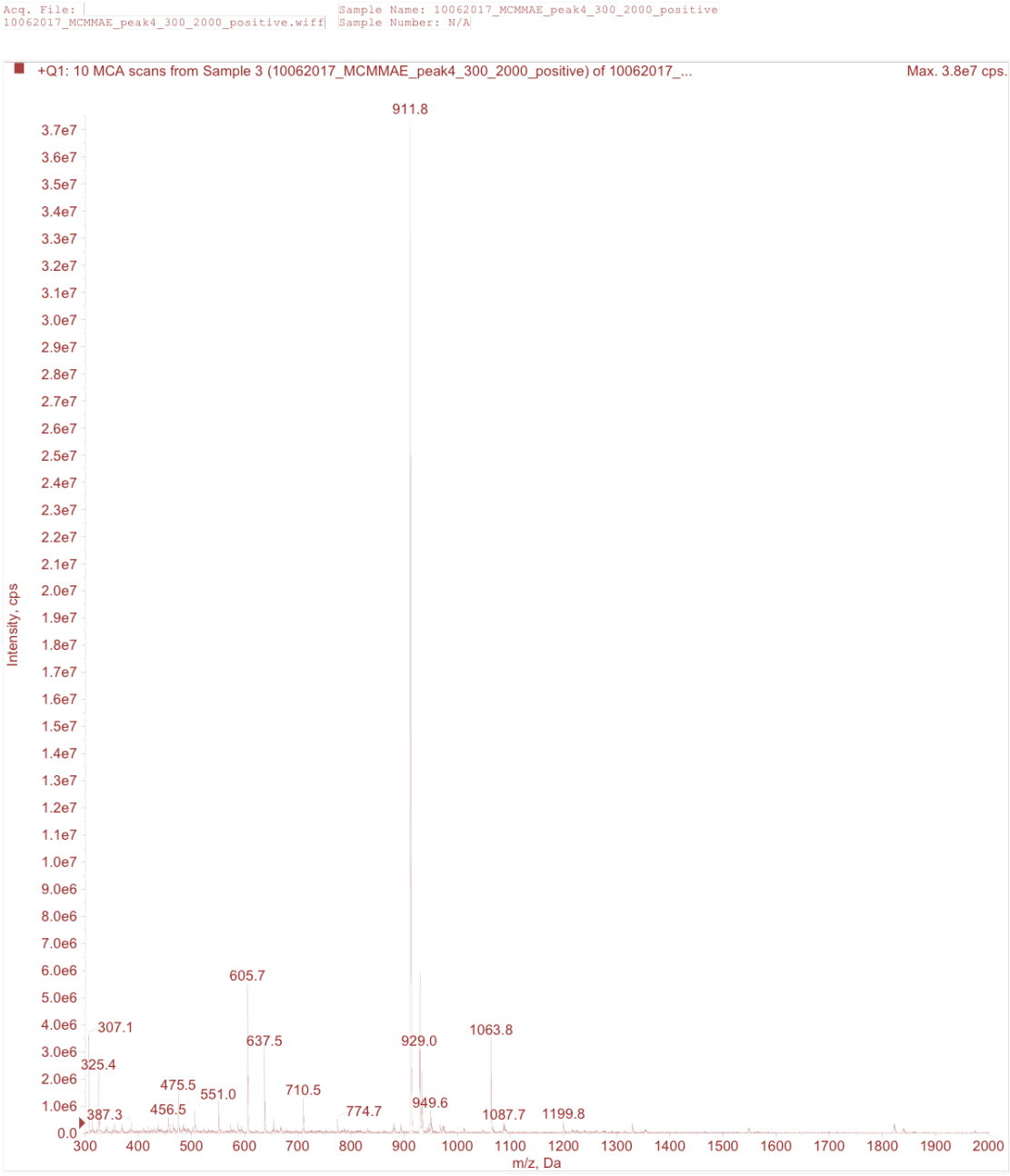

Figure 4.10 ESI Mass Spectrum of MC-MMAE

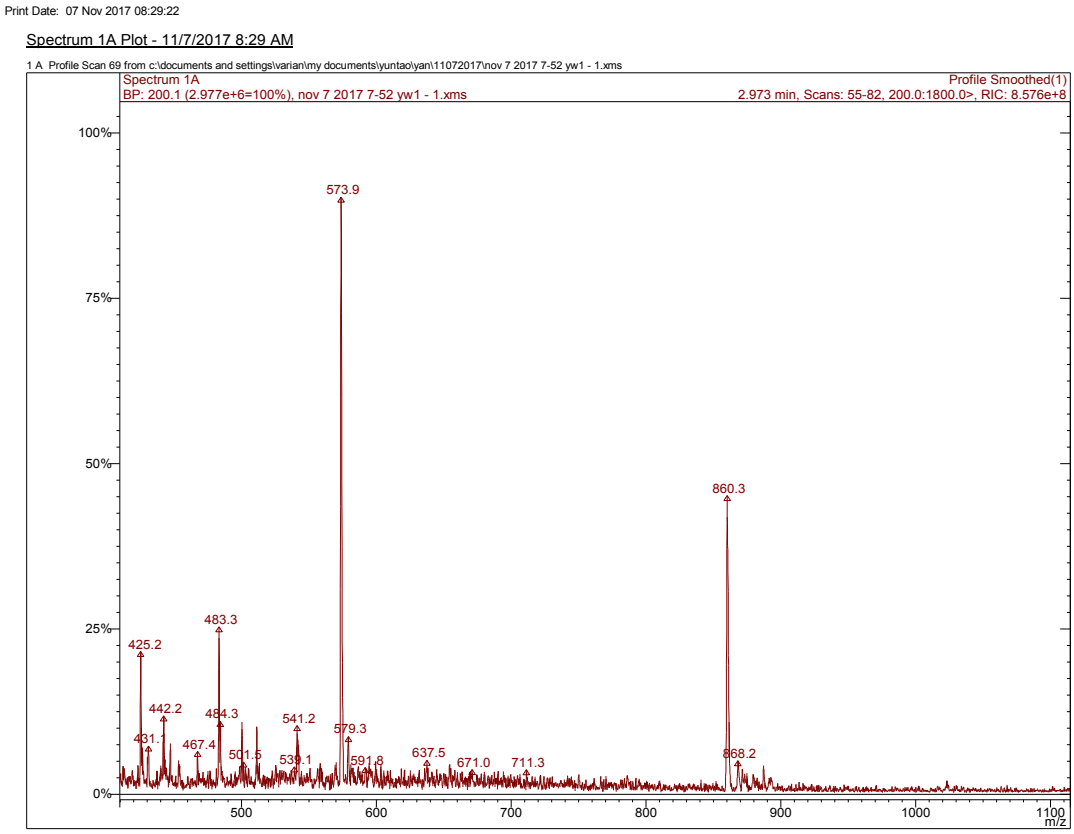

Figure 4.12 ESI Mass Spectrum of YW1

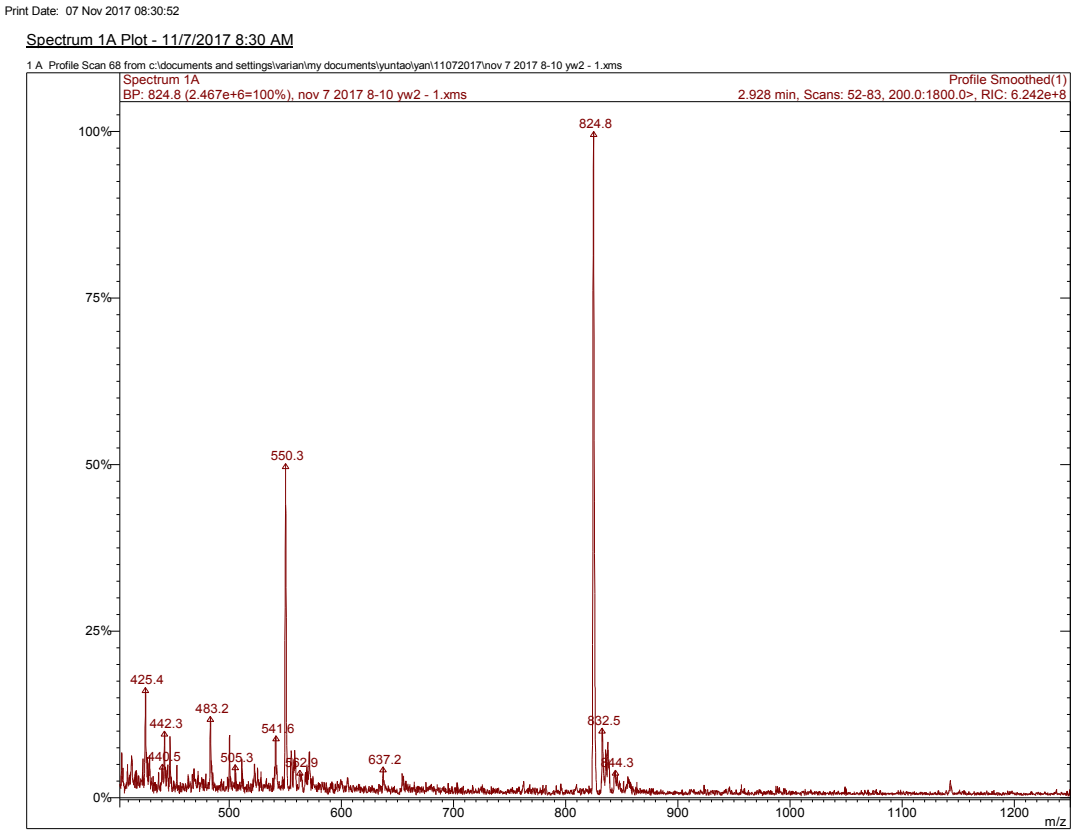

Figure 4.13 ESI Mass Spectrum of YW2

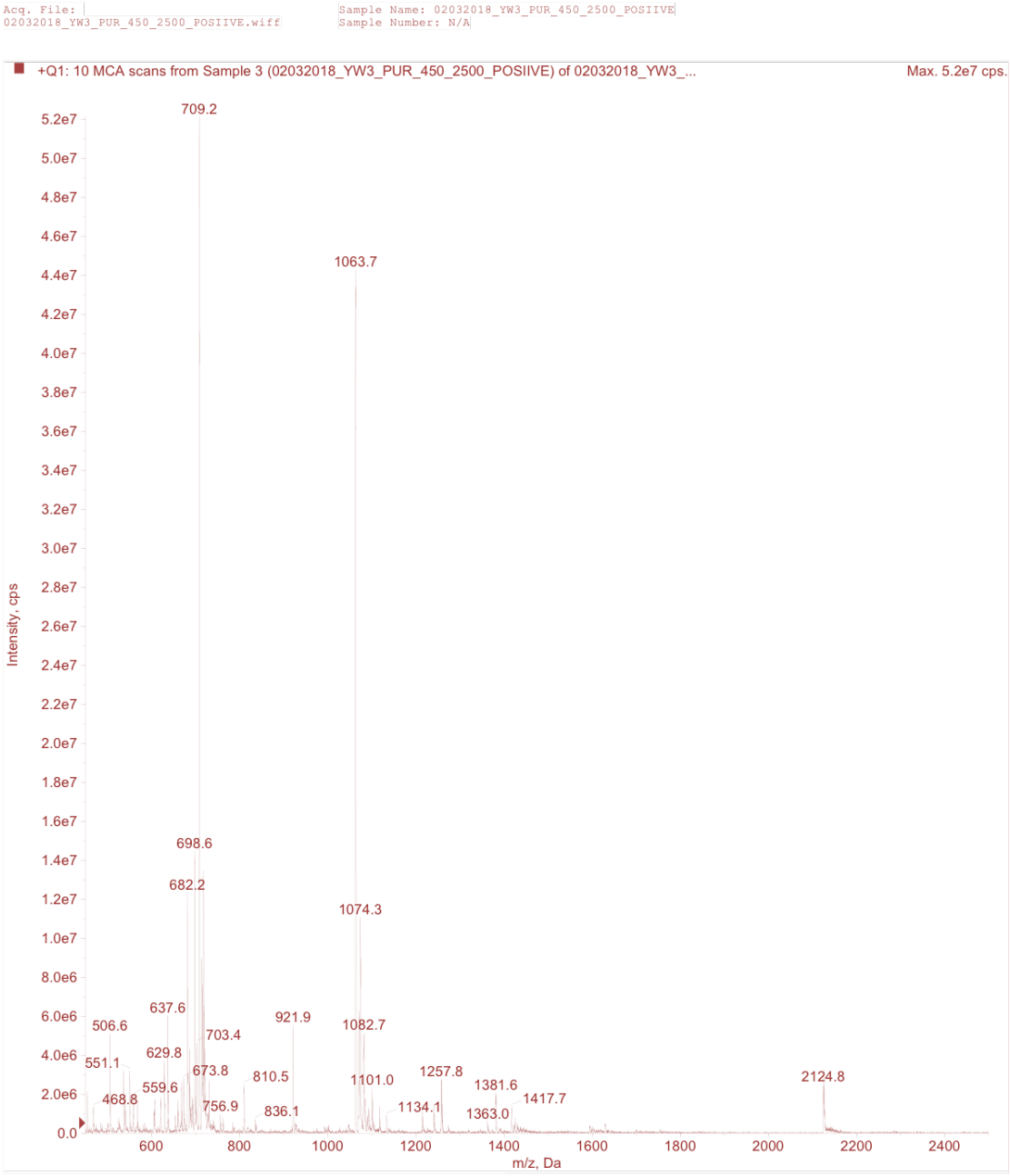

Figure 4.14 ESI Mass Spectrum of YW3

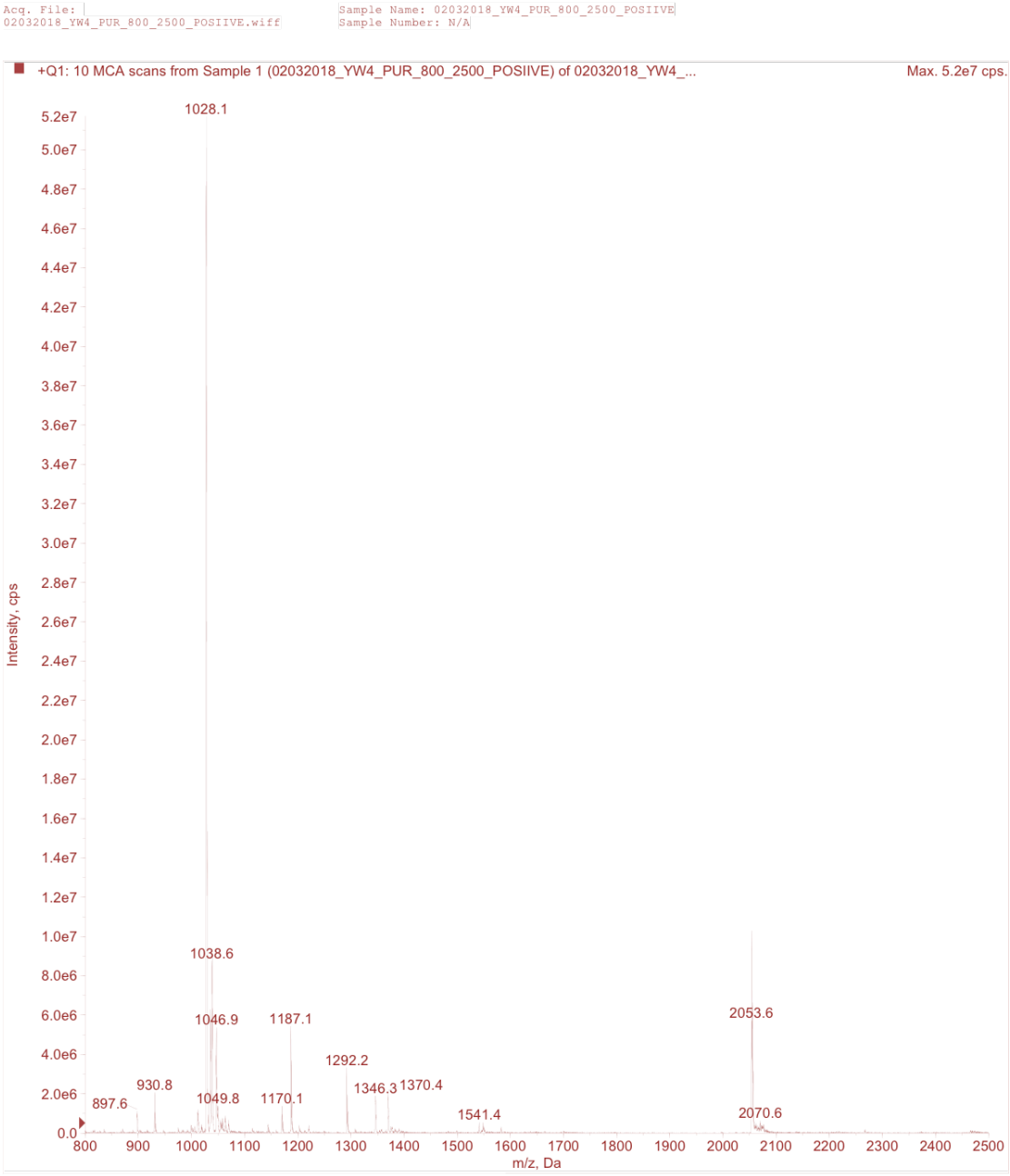

Figure 4.15 ESI Mass Spectrum of YW4

Table 4.3 Lytic peptide and hybrid peptides MS and HPLC data

Peptides	Purity	MW
LY1 (8)	73%	2847.46
HY1 (9)	69%	3705.44
HY2 (10)	70%	3634.36

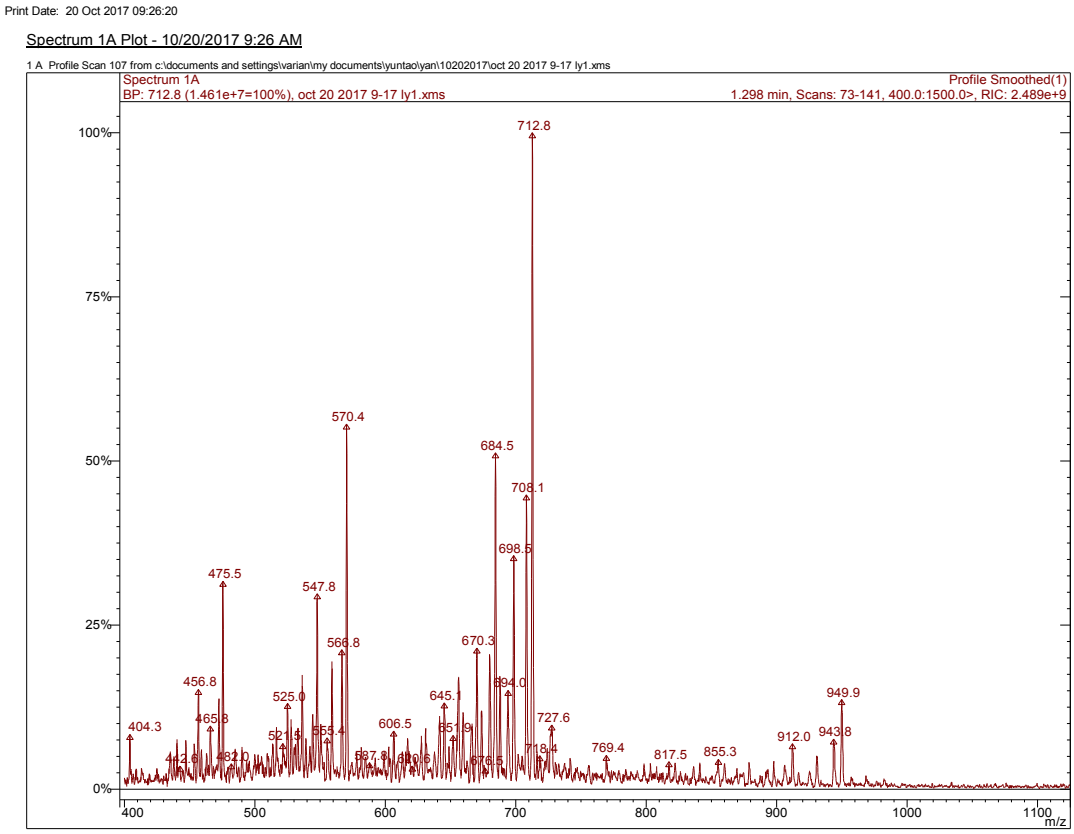

Figure 4.16 ESI Mass Spectrum of LY1

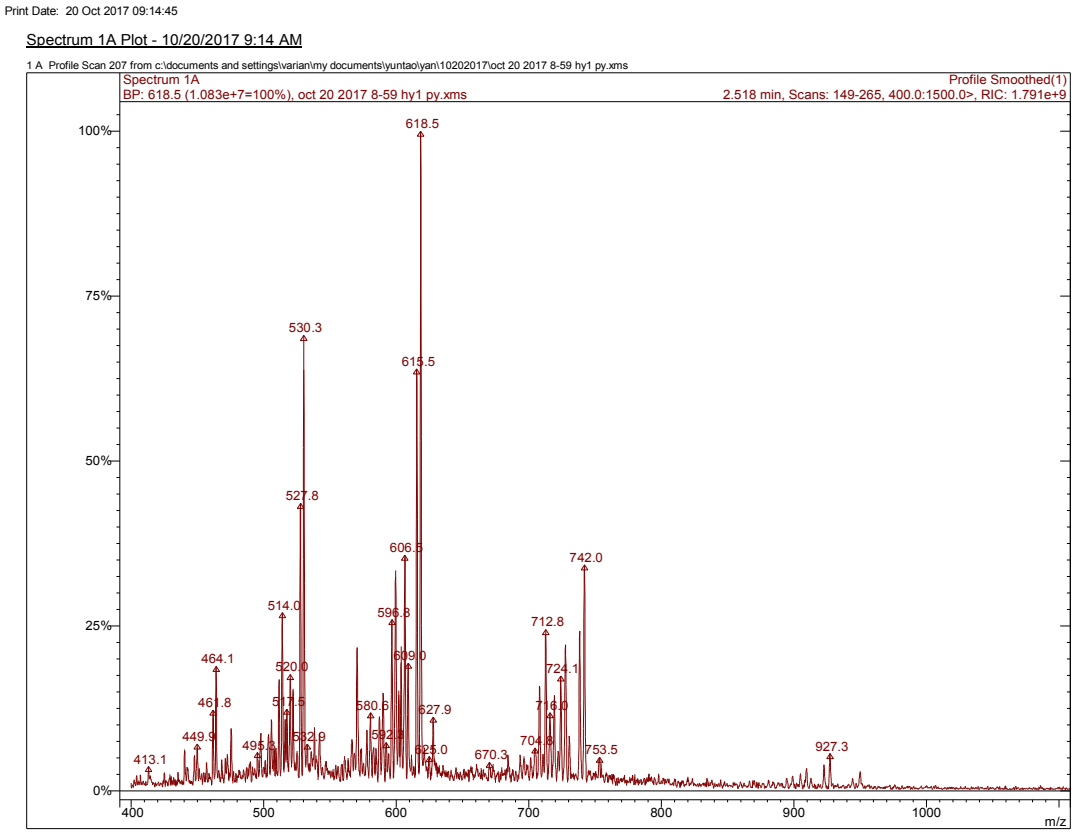

Figure 4.17 ESI Mass Spectrum of HY1

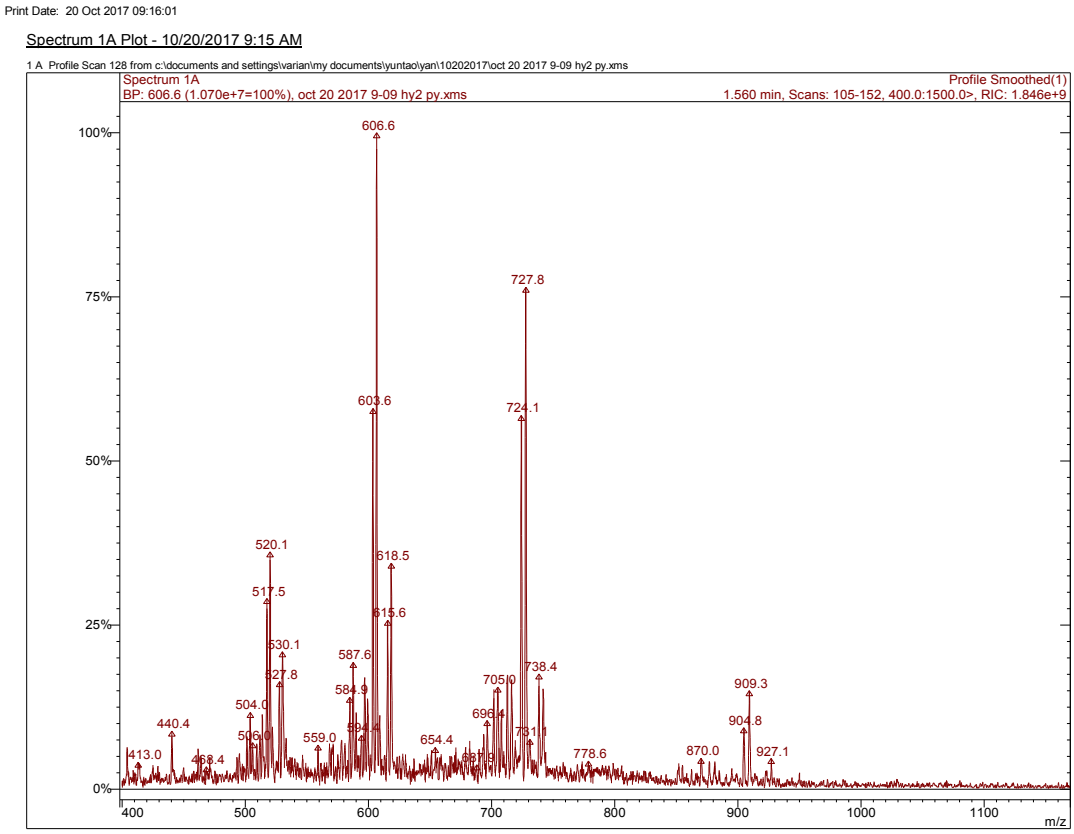

Figure 4.18 ESI Mass Spectrum of HY2

CHAPTER 5: IN VITRO CYTOTOXICITY OF PEPTIDE-DRUG CONJUGATES AND HYBRID PEPTIDES

5.1. Introduction

Cell viability and cytotoxicity study have been used for drug biological evaluation, screening, and cytotoxicity on the cells *in vitro* to observe the response on cell growth, reproduction, and morphology. Cell cytotoxicity test is an important indicator for toxicity evaluation of compound as it is simple, fast, and highly sensitive. Various cytotoxicity assays are based on the cell viability at different stages of the cell cycles. Lactate dehydrogenase (LDH) assay and trypan blue exclusion assay are membrane integrity assay used to evaluate the extent of cell damage by cytotoxicity agents. To analyze the cellular metabolic activity, MTT [3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] assay is a sensitive and reliable indicator as compared to ATP and 3H-thymidine incorporation assay, which is used to measure the end-point of viable cells. Despite the fact that MTT assay is a sensitive and reliable method, drug-efflux pump inhibitor or anti-oxidants may interfere with the MTT assay resulting in a false test result.

To overcome the drawback of MTT assay, Sulforhodamine B (SRB) assay was proposed by Skehan in 1990 and applied to large-scale drug screening by Vichal and Kirtikara in 2006 [86][87]. SRB assay determines cell viability by measuring the cellular protein content. The death of cells can be observed by staining the cellular

DNA with propidium iodide staining method, followed by flow cytometry [88]. SRB is a protein dye that binds to protein in a pH-dependent condition of cell protein amino acid residues fixed by trichloroacetic acid. SRB binds to protein in mild acidic condition, while it can be extracted and solubilized for quantitative measurement under basic conditions [86]. Linear relation between the cell number and cellular protein measured at cellular densities can reach 1–200‰ of confluence. As compared to MTT and other cell cytotoxicity assay, the SRB assay was performed with similar results and a stable end-point makes it an appropriate and sensitive assay for measuring the cytotoxicity of peptide–drug conjugates.

The purpose of this chapter is to utilize SRB assay to measure the cytotoxicity of synthesized peptide-drug conjugates and hybrid peptides.

5.2. Materials

MDA-MB 361, ZR75, and HEK293 cells were purchased from ATCC (Manassas, VA, USA). Dulbecco's modified eagle medium (DMEM), 4.5 g/L glucose, L-glutamine and 1% penicillin-streptomycin (5000 I.U./mL) were obtained from Mediatech (Manassas, VA, USA). Sodium pyruvate containing 10% heat-inactivated fetal bovine serum was ordered from Gemini (Sacramento, CA, USA). Trichloroacetic acid, acetic acid, and tris base were obtained from Chem-Impex International Inc. (Wood Dale, IL, USA). SRB was obtained from MP Biomedical, Inc. (Santa Ana, CA, USA). The 96-well plates were purchased from Santa Cruz Biotechnology, Inc..

5.3. Methods

The cytotoxicity study of MMAE, peptide–drug conjugates, lytic peptides, and hybrid peptides was performed using MDA-MB 361, ZR75 cells, and HEK293 cells. The cells were cultured in 75-cm^2 culture flasks in DMEM medium supplements with 10% FBS and 1% penicillin-streptomycin. The cells were seeded into 96-well plates at a density of 1.25 x 10^4/mL and grown for 24 h until they were attached to the wells. The cells were then incubated with MMAE, peptides, and peptide–drug conjugates at the following concentrations: 0.0001, 0.001, 0.01, 0.1, 1, 10, 100, 1000, and 10000 nM. After 72 hours, the 96-well plates were removed from incubator and 50-µL of pre-chilled 50% trichloroacetic acid was added to each well and then the plates were stored at 4°C refrigerator for 1 hour to fix the cells. Finally, the cells were washed with deionized water 5 times. After drying at the room temperature, the cellular proteins were stained by adding 60 µL of 0.4% SRB prepared in 1% acetic acid. After 20-min staining, the unbound dye was removed by washing with 1% acetic acid for 5 times. After drying in the air overnight, the cell-bounded SRB was dissolved in 150 µL of 10 mM non-buffered Tris-base solution at pH 10.5. The SRB absorbance was measured at 490 nm wavelength by using a microplate reader (BioTek Instruments, Inc., VT, USA).

The percent of cell viability was calculated by using the following equation:

Percent of cell cytotoxicity = (At-Ab)/Ab

At = Absorbance value of test compound

Ab = Absorbance value of blank

Percent of cell viability = 1-(Percent of cell cytotoxicity)

The percent of cell viability was analyzed using the Graph Pad Prism Version 7 software (GraphPad Software Inc, CA, USA) with a nonlinear regression dose-response curve fit (variable slope three parameter equation).

5.4. Results and Discussion

Cytotoxicity studies were performed to determine the potency of peptide, drug, and peptide–drug conjugates. As shown in Table 5.1, the tubulin inhibitor MMAE demonstrated similar potent cytotoxicity and IC50 in both HER2-positive cell line and HER2-negative normal cell line, which indicated the lack of specificity of the drug. Peptides showed low toxicity in both HER2-positive cell lines and normal cell line as compared with MMAE.

As for the toxicity of MMAE intermediate, the MC-MMAE exhibited a low toxicity similar to that of the peptides. Furthermore, when conjugating MC-MMAE with HER2-targeting peptide, the peptide–drug conjugates YW1 and YW2 presented an even lower potency than MMAE intermediate in both HER2-positive cell lines and normal cell line. There is the minor difference of toxicity of MC-MMAE PDC between the HER2-positive cell line and HER2-negative cell line as shown in Table 5.1. This result indicated that PDCs with MC linker selectively bind to HER2 overexpressed cell lines due to the lower toxicity in the normal cell line. MC-MMAE

PDCs demonstrated lower toxicity in HER2-negative cell line as compared to the toxicity in the HER2-positive cell line. This may be attributed to the non-cleavable linker maleimidocaproyl, when conjugated with MMAE, the structure of MMAE was modified and the toxicity of MMAE was altered. Moreover, the amide bond between 6-maleimidocaproic acid and MMAE is stable and difficult to be cleaved by a protease enzyme.

In case of a cleavable MMAE intermediate, the MC-VC-MMAE exhibited similar high potency as MMAE, when MC-VC-MMAE was conjugated with HER2-targeting peptide, the peptide–drug conjugates YW3 and YW4 presented with higher potency in the HER2-positive cell lines MDA-MB 361 and ZR75 and a distinct lower toxicity in the normal cell line HEK 293 in comparison to MMAE. Both peptide-drug conjugates displayed higher toxicity in HER2-positive cell lines MDA-MB 361 and ZR 75 as compared to the normal cell line HEK293 as shown in Table 5.1. This result indicated that the peptide–drug conjugates based on cleavable linker selectively binds to and internalizes via the receptor-mediated endocytosis in HER2 overexpressed cell lines.

The proposed cell-killing mechanism of PDCs based on the MC-VC-PABC linker may start upon binding of targeting peptides to HER2, which is overexpressed on the surface of the cancer cells. After binding, the PDC-HER2 complex undergoes internalization through receptor-mediated endocytosis. Following the internalization, the complex releases cytotoxic agents after protease (Cathepsin B) cleavage. Then,

after spontaneous elimination of p-aminobenzoyloxycarbonyl (PABC), the cytotoxic agent MMAE was released and it exerted its cytotoxic effect by binding to tubulin and inhibiting polymerization, leading to apoptosis of the target cells. In comparing to the MC-MMAE PDC results, it can conclude that the linker or derivation of MMAE to link to targeting peptides play a crucial role in cytotoxicity for HER2 overexpress cells. Although the data from MC MMAE PDC would not provide indication of specificity of binding and internalization, the MC MMAE PDC could bind and internalized into HER 2 overexpress cells. However, the low potency of MC MMAE may not exert its cytotoxicity. The significant low toxicity of both MC-MMAE PDCs and VC-MMAE PDCs in HER2 overexpress cells compared to normal cells indicated the specificity of PDCs.

As shown in Table 5.2, the lytic peptide demonstrated similar cytotoxicity and IC50 in both HER2-positive cell line and normal cell line, which indicated the lack of specificity of the lytic peptides. 10-folds higher toxicity of hybrid peptides than lytic peptides was observed on HER2-positive cell line ZR 75 and MDA-MB 361. Approximately 5-folds lower toxicity of hybrid peptide than lytic peptides was detected on the normal cell line. Furthermore, the significant higher cytotoxicity of hybrid peptides on HER2 positive cell line compared to the cytotoxicity on the normal cell line exhibited the enhanced specificity of hybrid peptides. By conjugating the lytic peptide with HER2-targeting peptide, the hybrid peptides demonstrated selectivity on killing HER2 positive cell lines.

Table 5.1 *In-vitro* cytotoxicity of PDCs and free MMAE, MMAE intermediate on HER2 positive cell lines and normal cell lines**

Cell line	ZR75	MDAMB 361	HEK293
	IC50 (nM, Avg±SD)		
MMAE*	7.59±0.39	$(1.35\pm0.02)\times10^{-1}$	6.68±0.15
MC-MMAE	$(1.33\pm0.09)\times10^{4}$	$(7.07\pm0.14)\times10^{3}$	$(1.04\pm0.13)\times10^{3}$
YW1	$(3.95\pm0.07)\times10^{4}$	$(1.46\pm0.02)\times10^{4}$	$(3.29\pm0.07)\times10^{4}$
YW2	$(7.40\pm0.91)\times10^{4}$	$(3.89\pm0.04)\times10^{4}$	$(3.91\pm0.04)\times10^{4}$
VCMMAE	4.43±0.13	$(4.16\pm0.12)\times10^{-1}$	5.45±0.37
YW3	$(7.00\pm0.32)\times10^{-3}$	$(3.90\pm0.16)\times10^{-2}$	$(1.22\pm0.17)\times10^{2}$
YW4	$(1.30\pm0.02)\times10^{-2}$	$(1.50\pm0.05)\times10^{-2}$	$(1.55\pm0.28)\times10^{2}$
MARAKE	$(5.52\pm0.08)\times10^{4}$	$(1.80\pm0.06)\times10^{4}$	$(6.11\pm0.17)\times10^{4}$

Abbreviations: Avg=Average (n=6); SD= Standard deviation

* P<0.001 P-values were determined by analysis of variance (ANOVA).

** P<0.0001 P-values were determined by analysis of variance (ANOVA).

Table 5.2 *In-vitro* cytotoxicity of lytic peptide and hybrid peptides on HER2-positive cell lines and normal cell lines*

Cell line	ZR75	MDAMB361	HEK293
	IC50 (nM, Avg±SD)		
LY1	83±1.5	301±12	$(1.30\pm0.01)\times10^2$
HY1	6.58±0.61	21.1±1.4	$(5.01\pm0.09)\times10^2$
HY2	2.88±0.27	32.3±0.27	$(5.45\pm0.13)\times10^3$
MARAKE	$(5.52\pm0.03)\times10^4$	$(1.80\pm0.12)\times10^4$	$(6.11\pm0.27)\times10^4$

Abbreviations: Avg=Average (n=6); SD= Standard deviation

* $P<0.001$ P-values were determined by analysis of variance (ANOVA).

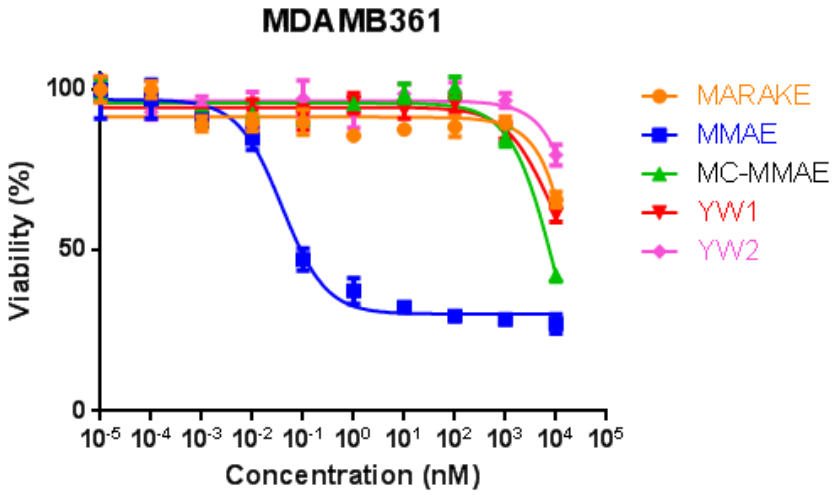

Figure 5.1 IC50 value of MMAE, MC-MMAE, peptide and PDC on MDA-MB 361 cell line.

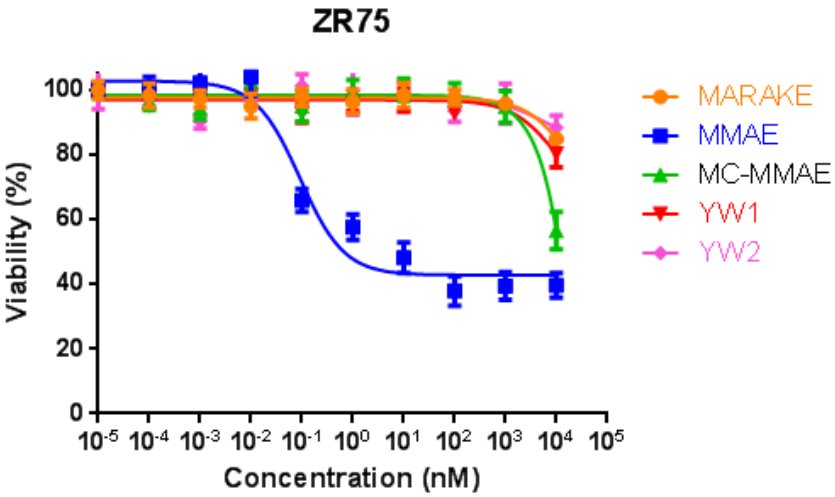

Figure 5.2 IC50 value of MMAE, MC-MMAE, peptide and PDC on ZR75 cell line.

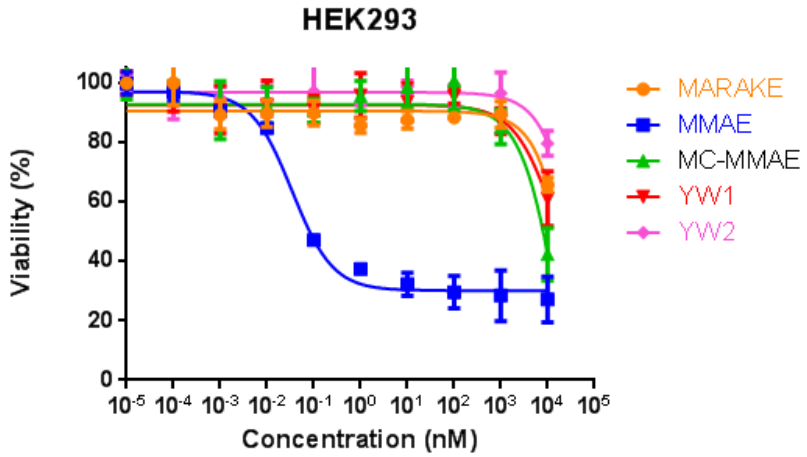

Figure 5.3 IC50 value of MMAE, MC-MMAE, peptide and PDC on HEK293 cell line.

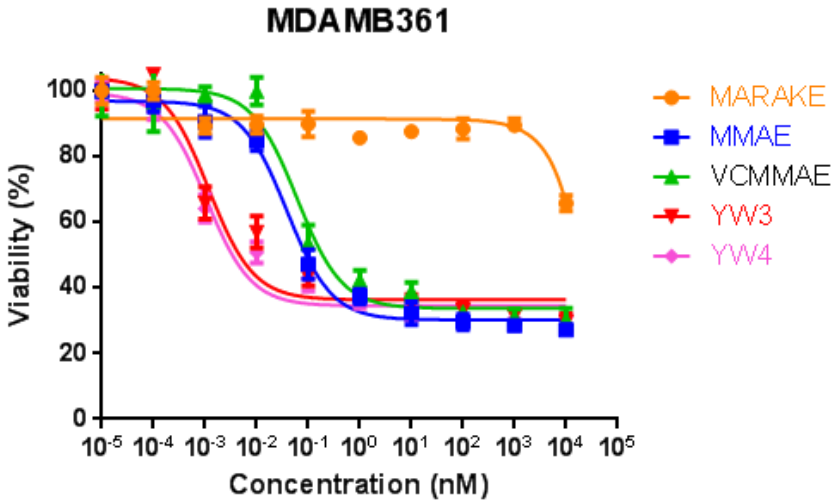

Figure 5.4 IC50 value of MMAE, VC-MMAE, peptide and PDC on MDA-MB 361 cell line.

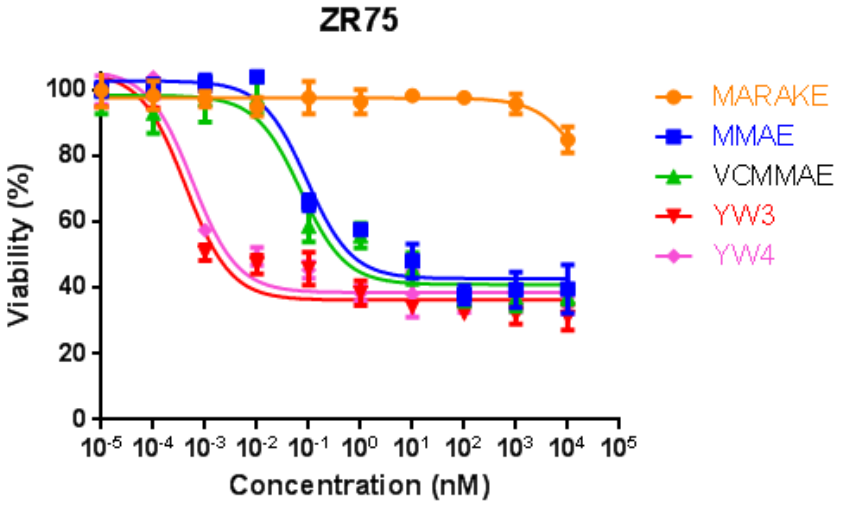

Figure 5.5 IC50 value of MMAE, VC-MMAE, peptide and PDC on ZR75 cell line.

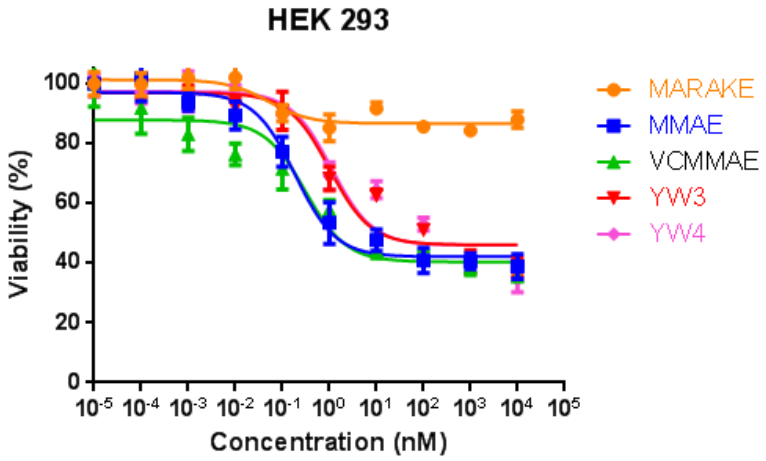

Figure 5.6 IC50 value of MMAE, VC-MMAE, peptide and PDC on HEK293 cell line.

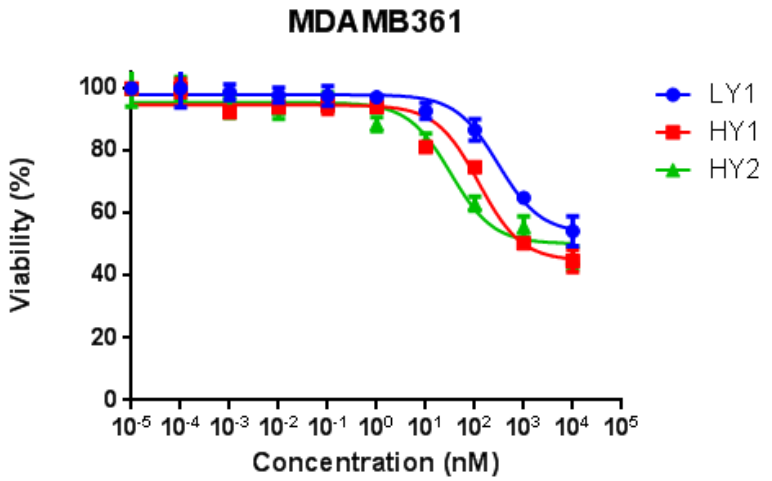

Figure 5.7 IC50 value of lytic peptide and hybrid peptides on MDA-MB 361 cell line.

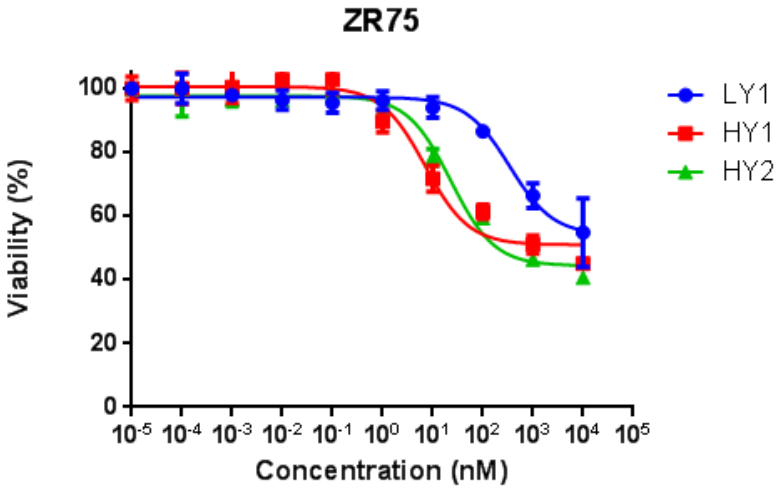

Figure 5.8 IC50 value of lytic peptide and hybrid peptides on ZR75 cell line.

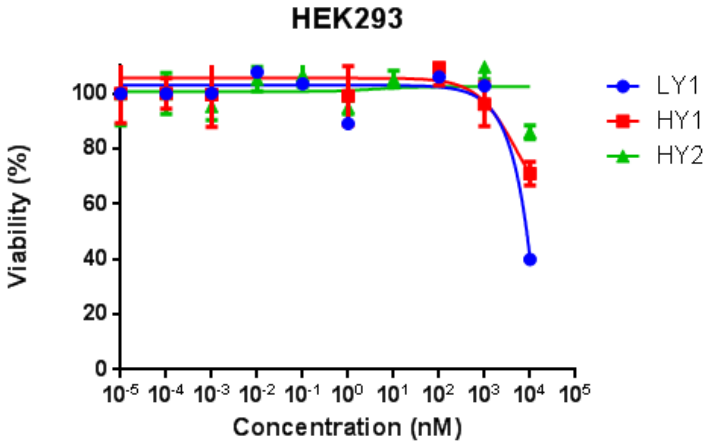

Figure 5.9 IC50 value of lytic peptide and hybrid peptides on HEK293 cell line

CHAPTER 6: SUMMARY AND CONCLUSIONS

In this study, HER2-targeting peptides MARAKE and MARSGL were used as a target moiety to design peptide–drug conjugates and hybrid peptides. Both non-cleavable and cleavable linkers were used for developing the peptide–drug conjugates. Melittin, a cytolytic peptide was used as a cytotoxic agent to conjugate with same HER-targeting peptides to form the hybrid peptides.

To verify the binding specificity of targeting peptides and their derivative, two cell lines that overexpress HER2 and one control cell line without HER2 expression were used to prove the specificity of the HER2-targeting peptides. Confocal microscopy study was performed to verify the uptake of peptides on these cell lines. The result indicated that the peptides and cysteine-modified peptides were bond and internalized selectively into the HER2-overexpressed cell lines. The random peptide as control showed negligible uptake as compared to HER2-targeting peptides.

Two designs using peptide-drug conjugate were studied in this thesis. Both designs used HER2 binding peptides as targeting moieties, but the drug portion of peptide-drug conjugate was either cytolytic peptide or cytotoxic small molecule.

Peptide–drug conjugates were designed by linking HER2-targeting peptides and MMAE, a highly potent and non-specific anti-cancer drug, via MC or VC linker. The results showed that the MC linker-based peptide–drug conjugates did not exhibit

any toxicity in both HER2-overpressed cell lines and normal cell lines. To the cleavable VC linker, the peptide–drug conjugates were 10-folds more cytotoxic to HER2-overpressed cancer cells as compared to the drug itself and were 100-fold less cytotoxic to cells with no HER2 expression. These results suggest that the use of peptide–drug conjugate approach could be a potential approach in cancer therapy.

Hybrid peptides with cytolytic peptide and HER2-targeting peptide MARAKE and MARSGL were also designed by using a GGG as a spacer to link the two peptides. The sequence of the designed hybrid peptides was 35 amino acids. Due to the high molecular weight, high purity of hybrid peptides was not obtained. The purity of peptides was 70 %. However, the purity of peptide was sufficient for in vitro toxicity study. The preliminary cytotoxicity study demonstrated limited potential of hybrid peptides. Both hybrid peptides showed slightly enhanced toxicity in the HER2-overpressed cell lines as compared to the lytic peptide while reducing toxicity to non-HER2 overexpress cells. These results may lead to the further studies in designing hybrid peptides with shorter and more potent lytic peptides.

In conclusion, HER2-targeted delivery peptide–drug conjugates were successfully synthesized by using an enzyme-sensitive cleavable linker. Peptide–drug conjugates using non-cleavable linker did not enhance the toxicity to the cancer cell lines. Hybrid peptide is a potential approach for cancer therapy but further research with different lytic peptides will be needed.

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