Medical Journal of Ahl al-Bayt University, Vol.4, No.1, Aug.2025

A Study of Therapeutic Peptides and Their Application in Cancer Treatment and Recovery with an Emphasis on Anti-Cancer and Cell-Penetrating Peptides

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ABSTRACT
Cancer is known as one of the main causes of death in today's world. Given the fact that there are limitations to the use of common methods for cancer treatment, e.g. chemotherapy, due to drug resistance and lack of specificity for tumors, discovering new methods to overcome these challenges is necessary. Peptides have attracted scientists' attention due to their properties, including easy synthesis, small size, biological diversity, and high activity and specificity. In this regard, cationic anticancer peptides (ACPs) and cell-permeable peptides (CPPs) have been considered for cancer treatment in recent years. The present study makes an attempt to review a number of available studies on ACPs and CPPs. The results show that antimicrobial peptides with anticancer properties act against cancer cells and tumors through membrane and non-membrane mechanisms. Moreover, CPPs conjugated to therapeutic agents are considered as an effective mechanism in cancer treatment by overcoming drug resistance. In addition, ACPs and CPPs can be proposed as a candidate for cancer treatment due to their properties, including low toxicity, mode of action, and ability to penetrate the cell membrane. Nevertheless, to understand the mechanism of action of these peptides with therapeutic potential, further studies should be conducted.

Keywords Cancer, anticancer peptides, cell permeable peptides (CPPs), antimicrobial peptides.

NOMENCLATURE

Antibacterial Peptides (ABP)
Anticancer peptides (ACP)
Antifungal peptides (AFP)
Antiparasitic peptides (APP)
Antiviral peptides (AVP).

INTRODUCTION

After cardiovascular diseases, cancer has been identified as the second most common deadliest diseases in the world, so that according to the statistics, it has led to the death of more than 9.2 million people throughout the world in 2018 (Bray et al, 2018). In the past few years, chemotherapy, surgery, radiotherapy, and other treatment methods have been used for cancer treatment and recovery; however, they have some disadvantages, including non-specificity for tumors, heavy expenses, and complications and consequences (Mahassni and Al-Reemi, 2013). For example, today doxorubicin, as a chemotherapy compound used to treat many tumors, has side effects such as oxidative damage in human organs (Marqus et al, 2017). Moreover, as there are also reports confirming the development of secondary malignancies caused by chemotherapy agents, e.g. cyclophosphamide, knowledge of and familiarity with new treatment methods seems necessary.

It can be said that peptides are short biologically diverse amino acid sequences. Although peptides and proteins are very similar, their size and structure are the main factors that distinguish peptides from proteins. Peptides are molecules with 2-50 amino acids, while proteins have more than 50 amino acids. Peptides, which are responsible for a large part of vital factors, exist in all human tissues and cells, and their classification is based on their function and source; for example, they can be grouped into bacterial, plant, endocrine, and fungal peptides. Currently, peptides are synthesized by various methods such as peptide coupling reagents, green peptide synthesis, solid-phase synthesis, protecting groups schemes, microwave-assisted peptide, and solid supports (Petrou et al, 2018).

Peptides are proposed as a suitable therapeutic candidate for cancer treatment due to their easy synthesis, biochemical and biological diversity, high activity and specificity, and their ability to cross the cell membrane. Moreover, due to their small size these compounds can be rapidly removed from the blood circulation through renal filtration. In addition, their side effects are low due to their non-accumulation in organs such as the liver (Marqus et al, 2017). Today, peptides can be considered effective agents in cancer treatment through carrying cytotoxic drugs, vaccines and hormones. Despite their many advantages, there are limitations to their use due to defects such as low resistance to destruction by proteinases and short half-life. Most anticancer peptides have a short amino acid sequence, as studies show that peptides with shorter amino acid sequences interact more effectively with the phospholipids of cancer cell membranes due to their diffusion and greater molecular mobility (Chalamaiah and Wu, 2018). Ren et al. (2013) found that the truncated FK-16 peptide derived from LL-37 has a stronger effect on colon cancer cells compared to the LL-37 peptide. Another study showed that citropin, maculatin, caerin, and aurein peptides affect the integrity of membrane layers through different mechanisms despite having the same sequence. Therefore, shorter tersaurein and citropin peptides indicate a surface interaction mechanism, while longer caerin and maculatin peptides can form pores in membranes (Fernandez et al. 2009). Reports and statistics show that out of 214 antimicrobial peptides with anticancer properties (ACPs) in the database, 34.11% have a sequence of 21-30 amino acids and 28.04% have a sequence of 11-20 amino acids. Figure 1 shows that the range of 21-30 amino acids is the most optimal length for ACP peptides because the number of peptides with anticancer activity decreases as amino acid length increases (Shoombuatong et al., 2018). The results of another study showed that 44% of the examined plant ACPs have a sequence of 25-30 amino acids, among which the most common amino acids are cysteine and serine. In recent years, a large number of studies

have been conducted on the use of peptides in the treatment of various diseases, especially types of cancer (Kharazmi-Khorassani and Asoodeh, 2019). In fact, the use of therapeutic peptides is proposed as a new scientific and promising approach to develop anticancer agents. Currently, therapeutic peptides for cancer treatment are divided into different groups, e.g. cell-penetrating and antimicrobial peptides. Accordingly, the current study makes an attempt to investigate the effectiveness of therapeutic peptides as anticancer agents.

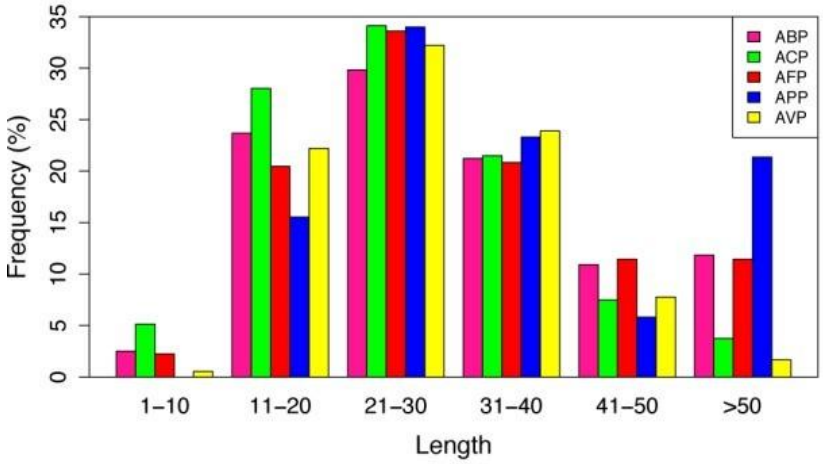


Figure 1. The distribution percentage of peptides based on their amino acid length (Shoombuatong et al, 2018).

Antibacterial Peptides (ABP), Anticancer peptides (ACP), Antifungal peptides (AFP), Antiparasitic peptides (APP) and Antiviral peptides (AVP).

Materials and Methods

Antimicrobial peptides with anticancer properties (ACPs): ACPs have short cationic sequences and are naturally present in most living organisms (positive charge 2-9). Considering their broad and specific activity against a wide range of pathogens, including viruses and bacteria, these peptides are critical for the innate immunity of organisms (Hancock et al., 2016; Asoodeh et al., 2014). Although gram-positive and negative bacteria are the main targets of ACPs, they also act against fungi and viruses (Yang et al., 2019). A wide range of ACPs of synthetic and natural origin are known today. ACPs attack the cell wall of bacteria and cause their loss of function and death through electrostatic interaction (Felício et al., 2017). The high density of negatively charged compounds, e.g. cardiolipin, phosphatidylserine, and phosphatidylcholine, on the surface of the bacterial membrane strengthens the connection of these peptides with the membrane. In fact, antimicrobial peptides lead to membrane permeability and disintegration in different ways, for example by thinning of the two membrane layers and formation of pores in the lipid membrane by a model of carpet-like, barrel-stave, or toroidal mechanism (Oren and Shai, 1998). The property of degradation and entry of these antimicrobial peptides into cells depends on various factors such as amino acid sequence, peptide secondary structure, overall net charge, and hydrophobicity. The ability to neutralize lipopolysaccharide (LPS) and interfere in the regulation of the immune system, for example by stimulating the production of cytokines, are other activities of ACPs (Rosenfeld and Shay, 2006). Currently, several antimicrobial peptides have entered the clinical phase to treat diseases such as cystic fibrosis and acne (Giuliani et al, 2007). In recent years, anticancer activities have been reported for

antimicrobial peptides, in which case they are known as anticancer peptides (ACPs). Many anticancer activities are known for antimicrobial peptides (Table 1).

Table 1. Examples of ACPs and their mechanism of action in cancer therapy.

Peptide	Sequence	Target tissue	Mechanism	Reference
BMAP-28	GGLRSLGRKILRAWKKYGPIIVPIIRI	leukemia	Membrane permeability/calcium influx	Risso et al, 1998
Cecropin B-LHRH	KWKVFKKIEKMGRNIRNGIVKAG PA-IAVLGEAKALSYGLRPG	Ovarian and endometrial cancer	Apoptosis	Li et al, 2016
Pardaxin	GFFALIPKIISSPLFKTLLSAVGSAL SSSG-GQE	Hepatocellular carcinoma	Apoptosis induction through caspase-3 signaling pathway	Han et al, 2016
BPC96	LKLKKFKKLQ	servicex	Apoptosis	Feliu et al, 2017
MG2A	GIGKFLHSAKKFGKAFVGEIMNS GG-QRLGNQWAVGHLM	Lung, cervix and melanoma	Association with gangliosides and apoptosis induction	Liu et al, 2013
D-K6L9	LKLLKKLLKKLLKLL	prostate	Necrosis through membrane depolarization	Oren and Shai/ 1998
K4R2-Nal2-S1	Ac-KKKKRR- β -naphthylalanine- β -naphthylalanine-KKWRKWLAKKNH2	Oral squamous cell cancer	Apoptosis	Chu et al, 2015
FK-16	FKRIVQRIKDFLRNLV	colon	Caspase independent of apoptosis and autophagy	Ren et al, 2013
Temporin-1CEa	FVDLKKIANIINSIF	cervix	Damage to the cell membrane	Wang et al, 2013
KT2 , RT2	NGVQPKYKWWKWWKKWWNH2, NGVQPKYRWWRWWRRWW-NH2	lung	Calcium release and production of reactive oxygen species (ROS)	Theansungnoen et al, 2016
human β-defensin-3	GIINTLQKYYCRVRGGRCAVLSC LPKEE	breast	Necrosis induction by interaction with phosphatidylserine	Hanaoka et al, 2016
Brevinin-2R	GRFKRFRKKLKRLWHKVGPFVGP ILHY	Kidney, breast, melanoma, leukemia	Reduction of mitochondrial membrane potential and activation of mitochondrial lysosomal death pathway	Emelianova et al, 2018
	KLKNFAKGVAQSLLNKASCKLSG QC	breast		Ghavami et al, 2008
ChMAP-28	VVGQAATI-NH2		necrosis	Li et al, 2018
myristoyl-CM4	GRWKIFKKIEKVGQNIRDGIVKA GPAVA -		Mitochondrial disorder, induction of apoptosis	Baindara et al, 2016

Temporin-Ra	FLKPLFNAALKLLP	Cervix and breast Breast	Increased expression of IL-1β and IL-8 in cancer cells	Lu et al, 2016
Laterosporulin10	ACVNQCPDAIDRFIVKDKGCHGV EKKYYKQVYVACMNGQHLYCRTEW GGPCQL	Leukemia and lung	Apoptosis induction, membrane disintegration with dihydrogen lactate release	Asadi et al, 2013
Melittin	GIGAVLKVLTTGLPALISWIKRKR QQ	Leukemia and cervix	Apoptosis induction	Wang et al, 2009

Aurein 1.2 is one of the ACPs that acts on the activity against bacteria with anticancer properties for different types of cancer cells (Rozek et al, 2000). Based on the structure, it can be divided into two main categories: β-sheet and α-helical (Figure 2). These structures, which usually consist of a predominantly cationic side, are completely amphipathic in nature (Hilchie et al, 2019). Mixed, extended helical, and cyclic structures are other structures of ACPs. Among α-helical structures, Cecropins and BMAP can be mentioned, and β-sheet peptides include Lactoferricin, Tachyplesin I, and Defensins.

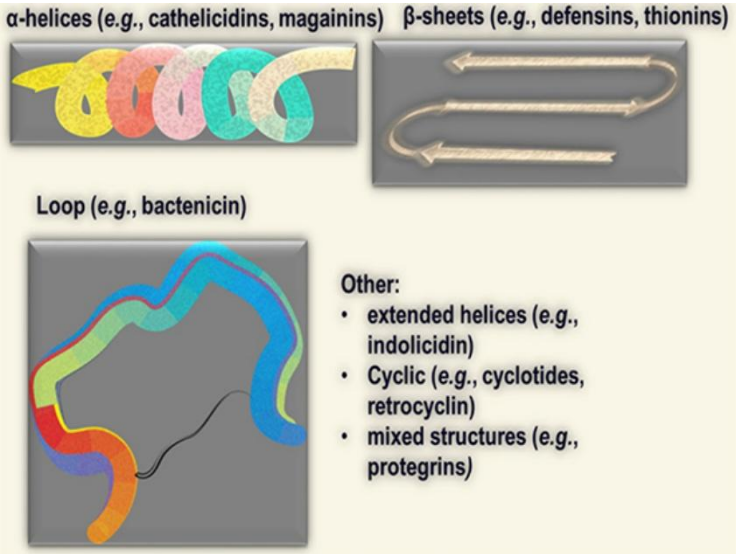


Figure 2. Different structures of ACP peptides (Deslouches and Di, 2017).

In another category and based on the target, ACPs can be divided into two main groups: the first group, with no effect on healthy cells, include anticancer peptides that target cancer cells and microbes (Magainins); the second group includes peptides that target cancer cells, microbes, and normal cells (HNP-1: Human Neutrophil Defensins). In addition to the intended structure and target, ACPs are also divided into two main groups, membrane and non-membrane, based on the mechanism of action. Similar to the barrel-stave and carpet models, which are defined for the interaction of antimicrobial peptides with the bacteria membrane and membrane destruction, these activities also take place in connection with ACPs (Schweizer, 2009). Antimicrobial peptides interact with the membrane of cancer cells in the membrane mechanism and cause cell death by developing necrosis or apoptosis. In the case of necrosis, the aforementioned peptides interact with negatively charged molecules on the surface of the cancer cell membrane and cause cell destruction. In fact, ACPs penetrate

into the interior of the cell by creating a connection with the back membrane of cancer cells and lead to cell membrane disruption along with the creation of a hole, while in the membrane mechanism of other antimicrobial peptides, they destroy the mitochondrial membrane, release cytochrome C, and lead to apoptosis (Gaspar et al., 2013) (Figure 3). Table 1 has reviewed a number of ACPs and their function in cancer treatment. The Tilapia piscidin (TP) 4 peptide showed a cytotoxic effect on A549 lung cells through disrupting the structure of microtubules. It seems that the mechanism of this peptide is related to the interaction between α-Tubulin and Tilapia piscidin (TP) 4 (Ting et al., 2018). Li et al showed that increasing hydrophobic activity through peptide myristoylation can be suggested as an option for using ACPs in cancer treatment. They showed that the mitochondria can be destroyed through mechanisms such as the release of cytochrome C, changes in the mitochondrial membrane potential, and an increase in the production of reactive oxygen species by the myristoylated CM4 peptide. Moreover, this peptide can lead to the activation of caspase 9 and 3, resulting in the stimulation of apoptosis (Li et al, 2018).

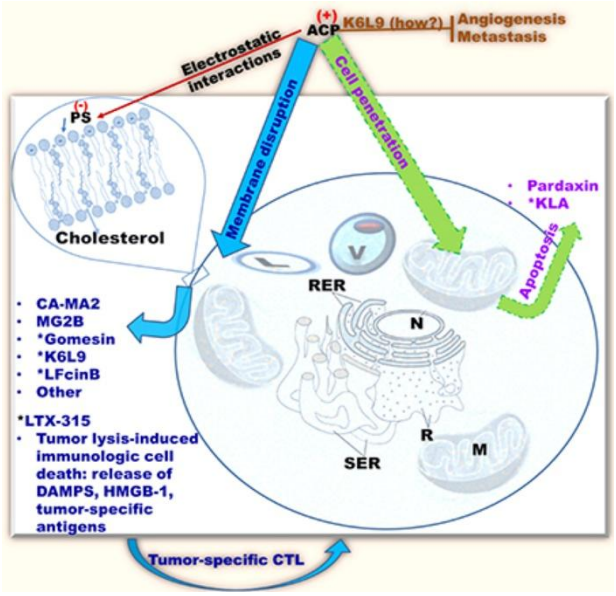


Figure 3. Membrane mechanisms of ACP peptides including apoptosis and necrosis (Deslouches and Di, 2017).

Moreover, Paradaxin peptide identified from marine fish works through caspase 3 activation and cell cycle arrest in G2/M phase and thus inhibition of cell proliferation in 4-SCC cells (Han et al., 2016). In 2008, a study showed that Brevinin-2R peptide leads to a decreased amount of cellular ATP and mitochondrial membrane potential, as well as an increased production of reactive oxygen species. In addition, Brevinin-2R-induced cell death is independent of caspase activation and may be modulated by the Bcl2 family (Ghavami et al, 2008). Melittin peptide leads to the activation of apoptosis through activating protein kinase Ca2+/calmodulin, transforming growth factor β-activated kinase and JNK/p38 MAPK pathway. The results showed that in the presence of calcium chelator, due to factors such as inhibition of protein kinase Ca2+/calmodulin, JNK and P38, this peptide leads to the inhibition of the apoptotic effect of Melittin (Wang et al., 2009). A number of anticancer peptides act through necrotic cell death. Lu et al. concluded that treatment of leukemia cells with LF11-322 peptide leads to increased calcium concentration and necrosis through

membrane disruption. However, after the treatment, signs of apoptosis, including the increased pro-apoptotic proteins and chromatin condensation, were not observed (Lu et al., 2016). Also, the results showed that the toxicity effect of 28-ChMAP peptide is due to the occurrence of necrosis as well as the permeability of the cytoplasmic membrane, and in fact, it has no effect on apoptosis (Emelianova et al., 2018).

Today, various biophysical methods have been proposed to understand the interaction of peptides with the cell membrane. Some these methods and an example of the peptides identified by these methods are shown in Figure 4. For example, in the Fluorescence Spectroscopy method can be used to evaluate the information related to the membrane stability due to the peptide-membrane interaction, the peptide depth, and the affinity of the membrane with the desired peptide. The Atomic Force Microscopy method is used to derive the information regarding the presence of peptides and its relationship with structural changes and membrane destabilization. The Circular Dichroism Spectroscopy method can be used to study the changes in the secondary structure and the secondary structure of peptides due to contact with the membrane in different environmental conditions (Avci et al, 2018); this method has been used for peptides such as Cecropin and Indolicidin.

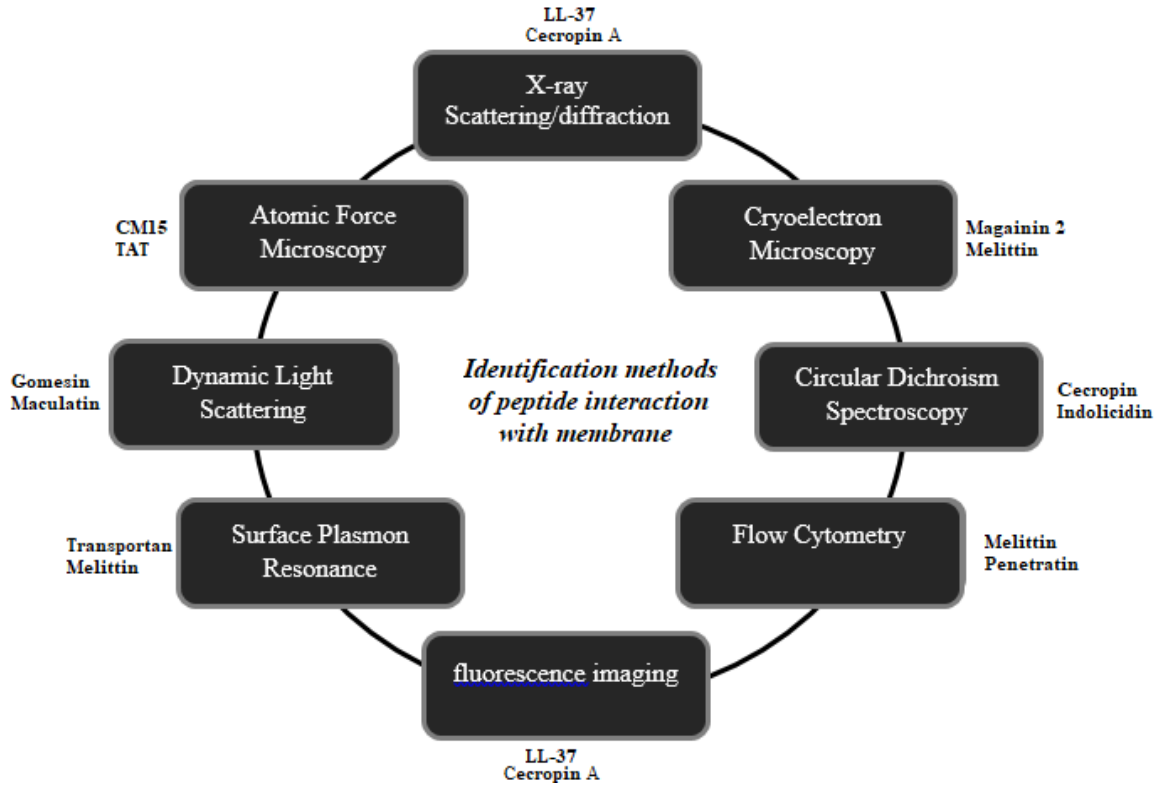


Figure 4. Methods of investigating the peptide-membrane interaction and an example of peptides identified by these methods.

Non-membrane activities of ACPs: Membrane disruption and mitochondrial destruction are not the only activities of ACPs, and in addition to membrane mechanisms, they have several non-membrane activities, including involvement in the regulation of the immune system, inhibition/stimulation of proteins, and inhibition of angiogenesis (Figure 5) (Wu et al., 2014).

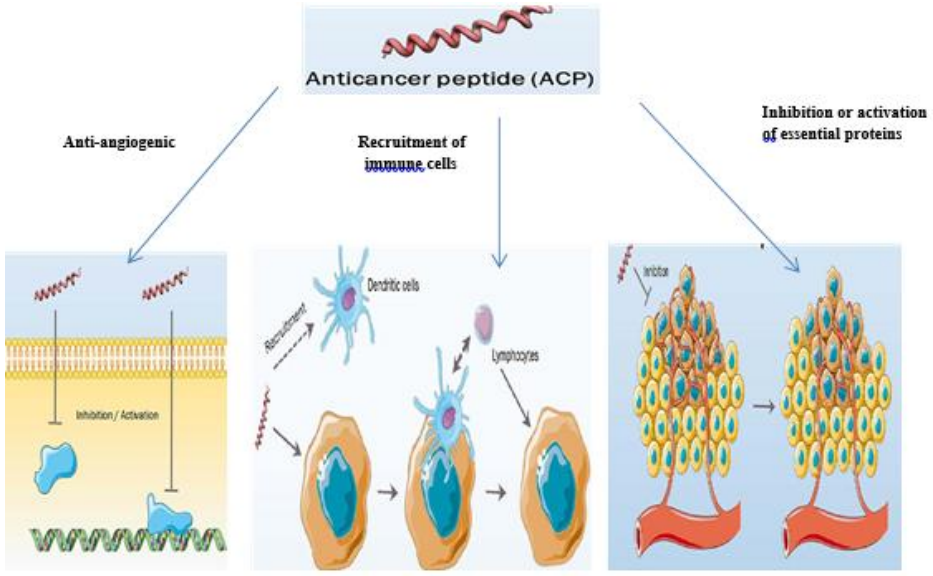


Figure 5. Different non-membrane mechanisms of ACPs (Felício et al., 2017).

Today, the immune system activation is known as a promising method in the cancer treatment. Recent studies have investigated the use of vaccines to create immunity against cancer. On the surface of tumor cells, antigens called tumor-associated antigens (TAA) are expressed, which are recognized by the host immune system. The these TAAs are injected in order is to induce a systemic immune response in cancer patients, which may destroy the growing cancer in different tissues of the body (Thundimathil, 2012). After vaccine injection, antigenic products are endocytosed through antigen-presenting cells (APC) and begin to migrate to lymph nodes, which lead to the activation of CD4 + T and CD8 + T cells (cytotoxic T lymphocytes (CTLs)). T cell antigen receptor recognize the small antigen located in the antigen binding groove of the MHC molecule. In fact, APC leads to presentation of MHC bound antigen to T cells and causes T cell activation. Finally, the production of tumor-specific CTLs causes the lysis of tumor cells (Tardón et al., 2019). As helper cells, CD4+T cells recognize antigens attached to MHC class II molecules and lead to cytokine secretion to attract more CTLs.

Adjuvant supplements are a group of compounds that can enhance the immune response through various mechanisms. As studies have confirmed (Bartnik et al, 2013), Antimicrobial peptides with anticancer properties (ACPs) can be considered as vaccine supplements. Huang et al, studied a vaccine using shrimp anti-lipopolysaccharide factor (SALF) peptide and mouse bladder carcinoma cell inactive extract (2-MBT). They found that this vaccine increases inflammatory factors such as 12-IL, IL-6 and 1β-IL and leads to further stimulation of the creation of 2-MBT specific tumor antigens and the expression of cytotoxic T cells in mouse model [43]. By evaluating the effectness of anticancer peptide pardaxin in combination with 2-MBT as a cancer vaccine, another study showed that using pardaxin with 2-MBT reduces tumor growth in mice. In addition, the expressions of T cell receptors, natural killer (NK) cells, and T toxic cells increase (Huang et al, 2013).
Another study (2009) showed that the anticancer peptide 1-HNP can stimulate an immune response to the tumor of breast and colon cancer mouse models through the activation of dendritic cells (DCs). Camilio et al. found that some ACPs lead to immune response against

tumor antigens through the release of Danger-Associated Molecular Patterns (DAMPS) molecules, e.g. ATP and HMGB1 protein, from cancer cells. Intratumoral injection of anticancer peptide 315-LTX induces cell lysis through the release of DAMPs and membrane destabilization. This release leads to stimulating the absorption of tumor antigens by DC cells and subsequently the maturation of these cells, followed by the presentation of tumor antigens to T cells. Finally, tumor-specific cytotoxic T lymphocytes (CTLs) are produced, leading to the destruction of tumor cells (Figure 6) (Hilchie et al., 2019).

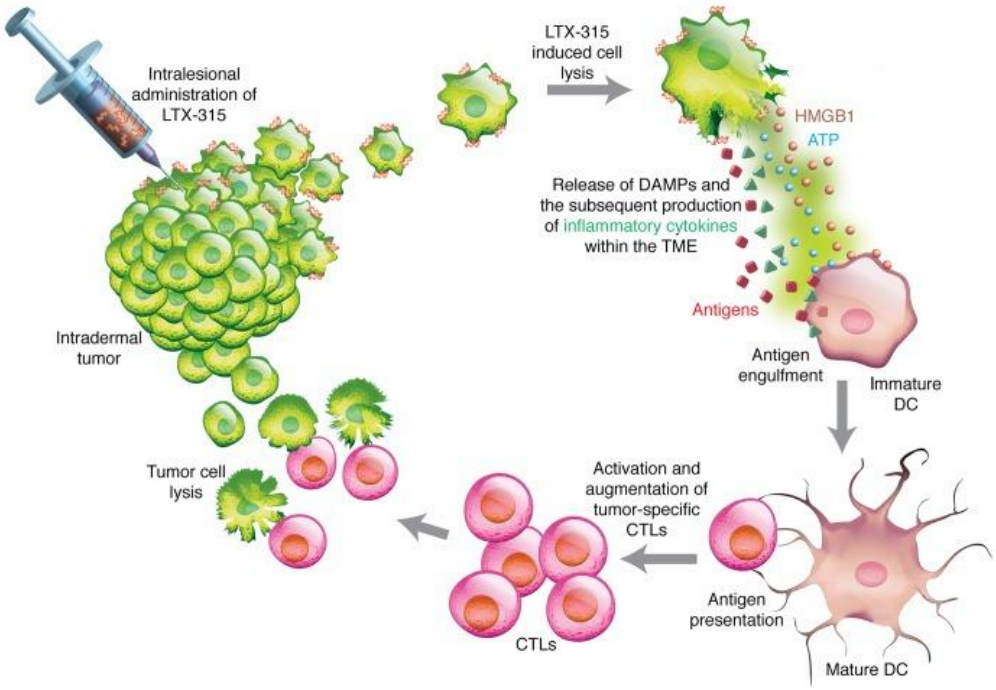


Figure 6. Immune regulatory activity of anticancer peptide LTX-315 through activation of CTLs (Camilio et al., 2014).

Yamazaki et al. (2016) showed that in tumor beds, the anticancer peptide 315-LTX leads to a significant increase in the level of CD3+ leukocytes, including CD4+, CD8+ and T lymphocytes, while the level of regulatory CD4+ T cells with OX40+ CTLA4 or CD25+ FoxP3 decrease due to contact with 315-LTX. Also, ACPs can stimulate a systemic immune response that leads to the destruction of all neoplastic cells; this immune response is activated through the release of DAMPs induced by ACPs. In their research, Mader et al. found that LL-37 peptide from the Cathelicidin class (Chen et al., 2018) lead to the destruction of regulatory CD4+CD25+FoxP3+ (Treg) T cells through apoptosis and creating an anti-tumor immune response. In addition, it has been shown that Brevinin-2R antimicrobial peptide stimulates the expression of IL-1β, IL-b, IL-8, and IL-6 factors in HepG2 and A549 cancer cells, which play an effective role in regulating the immune system (Homayouni-Tabrizi et al., 2015).

Another non-membrane activity of ACPs is inhibition of angiogenesis. Koskimaki (2009) found that intraperitoneal administration of 1-Chemokinostatin, Properdistatin, and Pentastatin-1 peptides in the 231-MDA-MB breast cancer model leads to adequate suppression of tumor growth and inhibition of angiogenesis. Furthermore, Wang et al. (2009) showed that 1-HNP peptide increases apoptosis and inhibits angiogenesis in mice model. A research in 2011 showed that the structural N-myristoylated peptide shows its non-membrane

anticancer function by inhibiting DNA replication and synthesis on several types of cancer cells including breast, lung, and colon. In general, the results showed that ACPs exhibit their anticancer effect through non-membrane activities in addition to membrane mechanisms including apoptosis and necrosis.

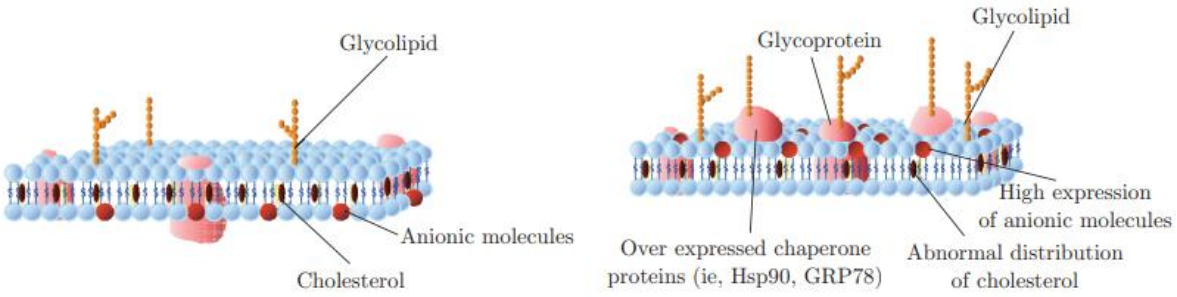


Figure 7. The difference between the membrane of cancer and healthy cells (Liu et al., 2015).

The difference between the membrane of cancer and healthy cells: evidence shows that there are many differences between the membrane of cancer and normal cells, which causes the identification and interaction of ACPs with malignant cells (Figure 7). It seems that electrostatic interactions between ACPs and negatively charged compounds on the cell membrane surface are considered as a main mechanism in the selective killing of cancer cells by anticancer peptides. For example, in healthy cells, there is a compound of phosphatidylserine in the inner layer of the plasma membrane, while this symmetry between the inner and outer membrane does not exist in cancer cells; therefore, phosphatidylserine is expressed in the outer layer and creates a negative charge on the membrane surface.
Moreover, the existence of other compounds such as chaperone proteins GRP78 and HSP90, sialic acid, and O-glycosylated mucins leads to the creation of a negative charge on the surface of cancer cells (Schweizer, 2009). For example, cationic peptides such as CopA3 and D-K6L9 interact with the phosphatidylserine compound in the outer layer of the cancer cell membrane and cause necrosis in the cells (Lee et al., 2015). In 28-BMAP peptide, negatively charged sialic acid chains on the surface of U937 cell line membrane are considered as sites for initial interaction with the peptide. The results of another research indicated that compounds such as phosphatidylserine and glycosylated mucins lead to the electrostatic interaction of temporin-1CEA peptide with the membrane of 7-MCF cells (Wang et al., 2013). Also, cationic peptide Buforin IIb derived from histone H2A exerts its cytotoxic effect through interaction with gangliosides containing sialic acid on the surface of cancer cell membranes. The low level of cholesterol in cancer cells is another notable feature that leads to increased fluidity. In general, the membrane of cancer cells has more fluidity compared to normal cells, resulting in increased lytic activity of ACPs by facilitating the membrane destabilization. However, it has been reported that in a number of cancer cell lines, including prostate, the presence of cholesterol reduces the effect of ACPs on cancer cells (Li et al., 2006). In addition, one of the other features is an increased surface area due to an increase in the number of microvilli, which leads to an increased contact of ACPs with malignant cells. The presence of negatively charged compounds in combination with increased surface and fluidity of the membrane leads to the induction of ACPs activity in cancer cells.

Limitation of the use of ACPs: In the past few years, ACPs have attracted researchers' attention due to their ability to destroy tumors and cancer cells. Despite the identification of a wide range of anticancer peptides, few of them are in the clinical phase, and the higher cost of their production, compared to the synthesis of antibiotic molecules, is one of the reasons for limiting the use of these peptides in the clinical phase. Also, their toxicity against normal cells in high peptide concentrations is another disadvantage of anticancer peptide-based treatment methods. One of the ways to overcome this problem is to use the target sequences attached to the desired selected peptide (Hilchie et al., 2019). These short target sequences that interact with specific cell surface molecules on cancer cells are usually added to the target peptide through a glycine-glycine binder. An example of target sequences is Bombesin peptide, which interacts with many receptors on the surface of cancer cells. The use of Bombesin attached to Magainin 2 led to a 10-fold decrease in IC_{50} on cancer cells, which was significantly lower than the IC_{50} on normal cells (Liu et al., 2011).
Amino acid replacement to reduce toxicity against normal cells is one of the other methods that involves making simple changes in the properties of peptides, including changing their charge. One of the characteristics of a solid tumor is the acidic environment around it compared to normal cells. In a research, the lysine amino acids of the peptide [D]-K6L9 with a pKa of 10.5 were replaced with three and six histidine amino acids, leading to a decrease in the pKa of the peptide to 1.6. In fact, histidine amino acids in the peptide [D]-H6L9 are protonated in acidic pH and become active, while they are inactive in neutral pH. Although the peptide [D]-K6L9] has cytotoxicity against the prostate cancer model, despite its therapeutic potential, this peptide has significant systemic toxicity in slightly higher concentrations compared to the treatment method. In this study, peptides [D]-H6L9] and [D]-K3H3L] caused a decrease in prostate tumor growth, and compared to the peptide [D]-K6L9, it showed a much lower systemic toxicity effect (Makovitzki et al., 2009).
Another disadvantage of ACPs is their lack of stability and sensitivity to proteolysis. ACPs, with a half-life of approximately 2 minutes in the blood, are rapidly distributed to all body tissues. This limitation is not considered for ACPs that operate at high speed. To overcome this issue, some methods, e.g. the use of nanoparticles, have been proposed for packaging these peptides in order to reach the tumor environment. In order to transfer the drug to the right place, stable and non-toxic nanoparticles are used. Among these nanoparticles, Perfluorocarbon can be mentioned, which has the ability to transfer a wide range of drugs. Due to their small size, ACPs are easily incorporated into Perfluorocarbon nanoparticles to increase their delivery to the tumor site (Winter, 2014). According to the results, Melittin peptide mounted in Perfluorocarbon nanoparticle leads to a decrease in B16 melanoma tumor volume and size.

Cell-penetrating peptides (CPPs): CPPs are a group of peptides with the ability to pass through the cell membrane and transfer molecules such as siRNA, DNA, plasmid, and protein (Regberg et al., 2012). The ability of PPCs to pass molecules has made this group of peptides to be used as a promising candidate for drug delivery. CPPs are usually considered sequences containing 5 to 30 amino acids and are hydrophobic. Various factors such as cell type, temperature, peptide concentration, and carrier size play an important role in the entry of CPPs into the cell (Marqus et al., 2017). Most of these peptides contain 5 positive cationic charges. Endocytosis and direct permeation are considered as two main mechanisms for the entry of CPP peptides into cells, both of which differ in how energy is used. In the direct penetration

model, CPPs pass through the lipid bilayer without the interference of receptors and independently of energy. While in the process of endocytosis, CPPs enter the lysosome or endosome along with their therapeutic molecules with energy consumption. Based on their origin, CPPs are divided into three main categories: synthetic, natural, and spherical (Mostafavi and Asoodeh, 2019); and based on their structural characteristics, they are divided into two main categories: arginine-rich and amphipathic CPPs. Frankel and Pabo (1988) found that the transcripting protein (TAT) from the HIV virus has the potential to penetrate the cell membrane, and this discovery can be considered as an introduction to the identification and description of different CPPs. TAT peptide has the ability to carry molecules with different molecular weight, including antisense oligonucleotides, siRNA, and therapeutic agents (Marqus et al., 2017).

CPPs with anticancer properties: Currently, it has been shown that CPPs can be considered as a candidate for cancer treatment. For example, in 2013, Lim et al. introduced a new CPP, called BR2, which showed the ability to interact with tumor cell membrane gangliosides and had a cytotoxic effect on B16-F10, HCT116, and HeLa cancer cells. One of the important applications of CPPs is their use as carriers for the transfer of anticancer drugs. Although chemotherapy is considered as a treatment method for most cancers, drug resistance is one of the main challenges of this treatment method. One of the important mechanisms of drug resistance is a decrease in membrane permeability and drug metabolism (Bolhassani et al., 2017). The evidence shows that this drug resistance can be mitigated through addition of anticancer drugs to CPPs. In recent years, drug delivery using CPPs has been considered for many diseases, including cancer. The available evidence indicates that cytotoxic drugs are easily transferred in tumor cells by CPPs, which leads to the apoptosis induction. It has been also shown that the use of CPPs in combination with silver nanoparticles has stronger effects in killing 7-MCF cancer cells by increasing the penetration of silver nanoparticles into cancer cells compared to silver nanoparticles alone (Farkhani et al., 2017). Considering the interaction between drug and CPP, CPPs can be classified into two main categories: the first category requires chemical bonding with the drug; and the second one involves the formation of stable non-covalent complexes with the drug. In the past few years, a large number of studies have investigated CPPs conjugated to small molecules and macromolecules, in order to treat cancer (see Table 2).

Table 2. Examples of conjugated CPPs and their application in cancer treatment.

CPP name	CPP sequence	Cargo	Application	Reference
SCPP-PS	RLWMRWYSPRTRAYGC	MTX	A549 lung cancer cells	Yang et al., 2018
LDP12	LKHLLHLR$_8$KHLLKLS$_5$G	siVEGF	HeLa cervical cancer cells	Hyun et al., 2018; Kwon et al., 2013
	DSLKSYWYLQKFSWR	SiRNA	Colorectal cancer cells	
	RAGLQFPVGRLLRRLLR	EGFP	HeLa cervical cancer cells	
	TAPKRKRTKTKK		HeLa cervical cancer cells	
YTA4	IAWVKAFIRKLRKGPLG	Fluorescein and MTX	MDA-MB-231 breast cancer cells	Mäe et al., 2012
CPP6	RLWMRWYSPRTRAYGC	MTX	MCF-7 breast cancer cells	Yang et al., 2019

R8	CKIKKVKKKGRKKIKKVKKKGRK	DOX	HeLa cervical cancer cells	Xiang et al., 2018
dNP2	KLKLALALALAVQRKRQKL-MP	DOX	U87 glioblastoma cancer cells	Zhang et al., 2016
R9	Octa-arginine	Taxol	OVCA-429T ovarian cancer cells	Dubikovskaya et al., 2008
CB5005	R9PLGLAGDGGDGGDGGDG	DOX	Tumors with high expression of MMP-2/9	Shi et al., 2012
LKH-stEK	RQIKIWFQNRRMKWKK	p16MIS	Animal models of pancreatic tumor	Wang et al., 2016

Using CPPs to deliver small molecules: despite effective distribution of small molecule anticancer drugs on the tumor due to their small size, the development of tumor resistance to the drug is one of their main problems. To overcome this challenge, scientists have investigated the effect of drugs conjugated to CPPs. For example, it has been reported that the DOX molecule conjugated to CPPs is a more effective therapeutic method in the treatment of tumors compared to the DOX alone. Aroui et al. (2009) showed that the CCP peptide Maurocalcine conjugated to DOX enhances the entry of this drug into MDA-MB-231 and MCF-7 cells, overcoming the DOX resistance in MDA-MB-231 cells. It has been reported that the different chemical sensitivity of MCF7 and MDA-MB-231 cell lines is due to the different expressions of Rad51 protein, which is highly expressed in MDA-MB-231 cells and is less expressed in MCF7 cells. Investigating the effect of penetratin and TAT peptides conjugated to DOX on different cell lines, Aroui et al. showed that penetratin peptide has a stronger effect on DOX entry than TAT does. Also, DOX-penetratin increases the toxicity of DOX about 11.53, 4.87, and 7.19 times in HUVEC, 231-MDA-MB, and CHO cells, respectively, compared to DOX alone (Aroui et al., 2010). Conjugating TAT peptide to Chitosan/DOX resulted in increased entry into CT-26 cells compared to free DOX. In fact, a small amount of free DOX compound enters the cell and only a part of it enters the nucleus of the cells, while Chitosan/DOX/TAT concentrates in a significant amount in the cytoplasm. In addition, Chitosan/DOX/TAT has a stronger role in killing 26-T cells.

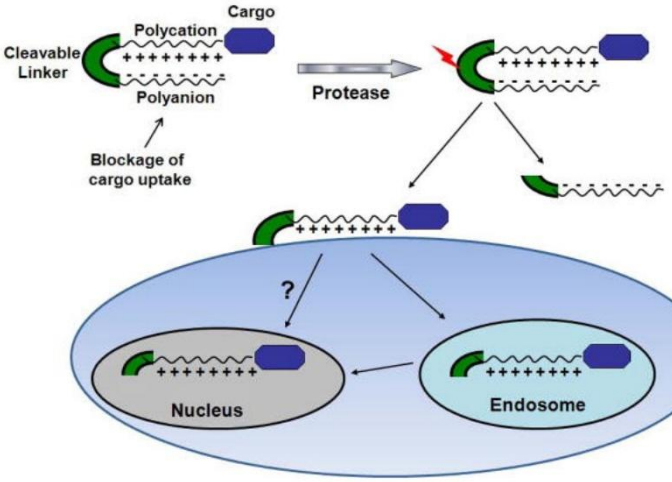
Morshed et al. (2016) found that modified TAT peptide with gold nanoparticles conjugated to DOX leads to increased toxicity of DOX in breast cancer brain metastasis cells. In addition, treatment with 200 nM TAT-Au-Dox increases DOX absorption (91.5%) compared to treatment with DOX (18.4%) (Morshed et al., 2016). Methotrexate (MTX), an anticancer agent with limited use due to resistance issues, inhibits tumor proliferation by disrupting purine nucleotides through inhibition of the dihydrofolate reductase enzyme in the cytoplasm (Regberg et al., 2012). In a study (2006), the authors evaluated the effect of YTA2 peptide conjugated to Methotrexate (MTX) on resistant breast cancer cells. Their results showed that the EC_{50} of MTX-YTA2 is about 5 times lower than that of MTX drug alone. In addition, MTX-YTA2 mitigates cancer cell resistance against MIX (Lindgren et al., 2006). It has been reported that disruption of polyglutamation, which is a major step in the mechanism of action of methotrexate, is often one of the main reasons for resistance to this drug. Szabo et al., in their study on the effect of MTX along with pentaghutamylated analogs attached to Octa-arginine, CPP, and penetratin peptides on breast cancer cells, showed that the use of MTX-Glu5-GFLG-Penetratin led to a decrease in IC_{50} in breast cancer cells compared to free MTX (Szabó et al., 2016).

Using CPPs to transfer macromolecules: evidence shows that the covalent binding of CPPs to peptides can interfere with the function of active biological molecules, leading to steric hindrance in the drug reaching the target (Regberg et al., 2012). Therefore, the formation of non-covalent CPP complexes is considered as a more effective method in drug delivery for carrying macromolecules. In recent years, a large number of studies have investigated the anticancer properties of CPPs binding to macromolecules. Apoptosis process is induced during different cell stresses and this process is controlled by tumor suppressor proteins such as p16 and p53. Evidence shows that mutations in these tumor suppressor genes are observed in 50% of human cancers. Various studies have investigated the effect of p53 protein and its derivatives conjugated with CPPs in order to improve the function of p53.
In a study (2006), researchers have shown that adding the N-terminal end of p53 protein to TAT peptide leads to apoptosis induction. Snyder et al. (2004) showed that intraperitoneal injection of TAT peptide fused to all-D retro-inverso (ri)-p53 into a mouse model of peritoneal carcinomatosis leads to stimulation of apoptosis in cancer cells and increased survival in the mouse model. The use of FHV CPP in combination with the penetration accelerating sequence and the C-terminal end of p53 in a concentration-dependent manner leads to the inhibition of tumor growth and the induction of autophagic cell death in glioma-initiating cells (Ueda et al., 2012). Another example of CPPs is p28, which prevents the degradation of p53 protein in tumor cells. p28 also facilitates the entry of exogenous proteins GFP and GST into cultured cells (Bolhassani et al., 2017). Intraperitoneal injection of Antp-p16 non-covalent complex inhibits the growth of pancreatic cancer cells in mouse models. In another study using mitochondrial apoptosis regulator protein called SMAC, it was shown showed that SMAC-TATp induces apoptosis stimuli including TRAIL. Combined transfer of SMAC-TATP along with 0.6 and 2 μg of TRAIL resulted in complete tumor eradication in a mouse model (Shin et al., 2014). In general, conjugating CPPs with small molecules and macromolecules is considered as a suitable mechanism in cancer treatment.
Limitations of using CPPs in cancer treatment: Evidence shows that CPPs can be used as a drug delivery method. The main problem of using these peptides is the lack of selectivity and specificity against cancer cells and tumors. Researchers are looking for ways to overcome this problem (Figure 8) (Bolhassani et al., 2017).

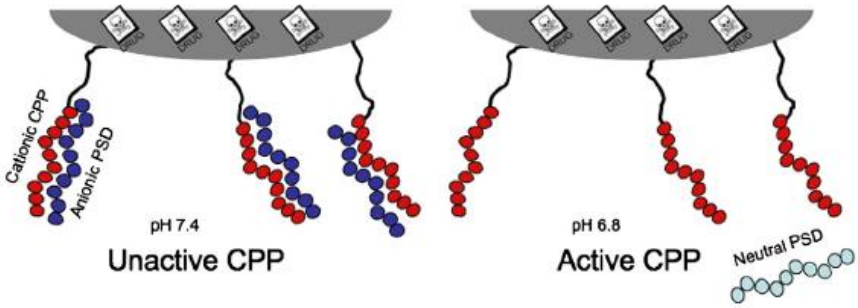
Active CPP (ACPP) production strategy (using matrix metalloproteases): In this method, a polycationic CPP is used in the form of arginine homopolymer (r9; nine D-form arginine residues) that contains a carrier molecule (drug). r9 is coupled with a polyanionic sequence (e8; eight D-form glutamate residues) using ionic interactions, which temporarily disables CPP positive charges. Also, in this method, two ionic parts are bound through a binder that has a cutting sequence of matrix metalloprotease (MMP-2 or 9) in tumor cells. When this drug system enters the blood stream, the location of the cut sequence is not recognized by these enzymes on the CPP due to the low amount of MMP in the blood circulation; as a result, the unwanted interactions of the CPP with the negatively charged surfaces of the inner cells of the vessels and subsequently the transfer of the drug to healthy cells are prevented. While in cancer cells and tumors, there is a large amount of MMP around the tumors, this enzyme identifies the site of the MMP cleavage sequence and separates the two ionic parts, which activates the CPP containing the drug transporter molecule and thus causes the interaction of the CPP with the surface of negatively charged cancer cells. Subsequently, drug molecules covalently bound to the CPP enter the target cell. The researchers have found that r9 in doses

higher than 5 µmol/kg can cause severe systemic toxicity, eventually leading to the death of mice due to respiratory failure. However, injection of ACPP even at 4 times the tolerated dose caused very mild toxicity (Figure 8.A) (Shin et al., 2014).

A:



B:



C:

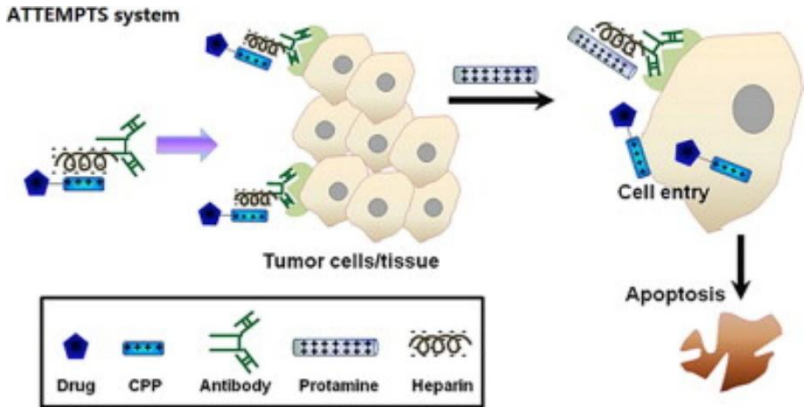


Figure 8. Methods to overcome the limitation of using CPPs. A) (active CPP production strategy (ACPP) (Shin et al., 2014); B) use of acidic pH around tumors (Vivès et al., 2008); and C) using modified ATTEMPTS CPPs (Ye et al., 2015).

Use of acidic pH: presence of some pathological conditions, e.g. cancer, leads to development of an acidic environment. In general, the pH around tumors is around 6.8 and acidic, while in normal conditions it is around 7.20. It has been reported that the target drug molecule can be specifically transferred to the tumor cells by using the acidic pH around the tumors. In this method, the positively charged CPP is covered by a polyanion called PSD and its charge is neutralized, as a result, the CPP remains inactive until it reaches the tumor site. The

sulfonamide group in the PSD composition is highly sensitive to acidic pH. Due to the acidity of the environment around tumors, when this system reaches the tumor site, the sulfonamide group is protonated and separated from the cationic part. As a result, the active CPP interacts with negatively charged cancer cells, which leads to the selective transfer of drugs to cancer cells (Figure 8.B) (Vivès et al., 2008).

Using ATTEMPTS modified CPPs: Antibody Targeted Triggered Electrically Modified Prodrug Type Strategy (ATTEMPTS) is used for the specific delivery of drugs to the tumor site, which consists of 2 main parts: the antibody conjugated to heparin and the anionic effector part formed from the CPP bound to the drug. In fact, this drug transfer system takes place through the formation of an electrostatic complex between the antibody conjugated to heparin and CPP-drug, and the positive charge of CPP is neutralized through the negative charge of heparin. In the first stage, the drug delivery system enters and accumulates at the tumor site by targeting the antibodies. In the next step, protamine is injected. In fact, protamine leads to the release of the CPP-drug from the drug transferring system due to the stronger interaction of heparin with protamine compared to that of heparin with CPP, subsequently, making the CPP-drug able to penetrate the tumor cell membrane (Ye et al., 2015). Currently, a new ATTEMPTS strategy for the treatment of colorectal cancer has been reported. In this study, the anionic targeting part includes T84.66 antibody conjugated to heparin and the CPP-drug part includes TAT and CPP fused to the drug gelonin. The results showed that the created TAT-gelonin/T84.66-Hep complex is able to bind to LS174T colorectal cancer cells with CEA overexpression. In addition, the transfer of TAT-gelonin to the target tumor using this system is enhanced about 58 times compared to TAT-gelonin alone (Shin et al., 2014) (Figure 8.C).

Results and Discussion

The present study investigated the effectiveness of the use of therapeutic peptides, including cell-permeable peptides (CPPs) and cationic antimicrobial peptides with anticancer properties (ACPs), for the cancer treatment. Considering their properties including easy synthesis, ability to penetrate the membrane, and small size, peptides are considered suitable candidates for treatment of infectious diseases and cancer. ACPs can be mentioned as therapeutic peptides, which interact with negatively charged compounds on the surface of the membrane. This group of peptides leads to the membrane disintegration through cell lysis or the mitochondria destruction through apoptosis. ACPs also exhibit their anticancer properties through several non-membrane activities. CPPs are another group of therapeutic peptides, which are used in cancer treatment through covalent or non-covalent binding with small and macromolecules and entering cells. Despite many advantages of ACPs and CPPs, they have limitations such as high cost and lack of specificity. However, various methods have been proposed to overcome these challenges. Considering the fact that the use of ACPs and CPPs is suggested as a promising method for cancer treatment, more extensive studies are needed in order to use these therapeutic peptides in the clinical phase and to understand their mechanism.

Conclusions

ACPs and CPPs can be proposed as a candidate for cancer treatment due to their properties, including low toxicity, mode of action, and ability to penetrate the cell membrane

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