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Design and Modification of Anticancer Peptides

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Abstract

Cancer is a great concern in the public health and development of novel therapeutic agent or strategy become urgently. Anticancer peptides (ACPs) have become promising anticancer drug candidate molecules due to their several extraordinary properties, such as small size, high activity, low immunogenicity, good biocompatibility, diversity of sequence and more modification sites for the functional molecules. However, the low stability and short half-life of peptides are the major barriers for the application. In this review, we focus on the methods of peptide design and modification to improve the stability, half-life time and specificity of ACPs, which including amino acid substitution, cyclization, hybridization, fragmentization, modification of C- and N-terminal of peptide by polymer or targeting molecules, etc.

Keywords: Anticancer peptide; Design and modification; Tumor homing peptide; Polymer

Introduction

Cancer is a great concern in the public health and development of novel therapeutic agent or strategy become urgently. Compared to other small organic molecules and protein, anticancer peptides (ACPs) have several extraordinary properties, such as small size, high activity, low immunogenicity, good biocompatibility, diversity of sequence and more modification sites for the functional molecules. Thus, ACPs have become promising anticancer drug candidate molecules.

According to the biological effects of ACPs, they can be classified into three groups: inhibitory activity, necrosis activity and apoptosis activity. For the inhibitor activity, most peptides are cellular adhesion molecules such as RGD or YIGSR, etc. and come from the common conservative sequence of basement membrane glypeotein of laminin and fibronectin, etc. [1,2]. They can target on the integrin receptors on the surface of cancer cells and inhibit the migration and metastasis. Thus, they can also be used as targeting molecules for drug designing. For the necrosis activity, most peptides are membrane activity peptides, such as lytic peptide or derived from the antimicrobial peptides (AMPs). In the first, they can quickly bind to the highly negative charged cancer cell membrane by the electrostatic interactions, then destabilize and disrupt the integrity of cell membrane through the hydrophobic interactions leading to necrosis of cancer cells [3-5]. For the apoptosis activity, as we know, most tumor cells resist apoptosis due to a deregulation of pro- and anti-apoptotic proteins, however, partly ACPs can result to the releasing of cytochrome c (Cyt c) and lead to the apoptosis of cancer cells by the permeation and swelling of mitochondria membrane if ACPs internalized inside eukaryotic cells. Briefly, the releasing of Cyt c from damaged mitochondria induces Apaf-1 oligomerization, caspase 9 activation and subsequently the conversion of pro-caspase 3 to caspase 3, which is responsible for many of the hallmarks of apoptotic symptoms [6-8].

The peptide therapeutics is a promising field against different diseases. However, the low stability and short half-life of peptides are the major barriers for the application because most peptides are usually prone to degradation by various proteases *in vivo*. In this review, we focus on the methods of peptide designing and modification to improve the stability, half-life time and specificity of ACPs, which including amino acid substitution, cyclization, hybridization, fragementization, modification of C- and N-terminal of peptide by polymer or targeting molecules, etc. The schematic diagram of major design and modification strategies of ACPs are shown in Figure 1.

Design of ACPs

Up to now, more than 2750 AMPs have been found and 199 peptides exhibited anticancer activities (named ACPs), see the website: http://aps.unmc.edu/AP/main.php. Some ACPs come from the AMPs and exhibit similar properties, such as net positive charge, amphipathic conformation, mechanism, etc. Thus, the method for the design of AMP also can be used for the design of ACP, including amino acid substitution, cyclization, hybridization, fragementization, etc. Based on these methods, the activity, stability of ACP can be improved.

Amino acid substitution

As we know, most anticancer peptides are membrane activity peptides, particularly α -helical cationic anticancer peptides and the net charge, hydrophobicity and helicity usually affect their activities [9,10]. Several researches have been reported that amino acid substitution is a useful tool to improve the activity and specificity of ACPs. Amino acid substitution cannot only change the biophysical parameter of ACP, but also influence the activity and stability of ACP, even alter the mechanism of action of ACP. Temproin-1CEa, a naturally occured α-helical cationic antimicrobial peptide, exhibits a potent anticancer activity and a moderate hemolytic activity [11,12]. Yang et al. designed and synthesized six analogs of temporin-1CEa by reserving the amphipathicity levels and α-helical structural patterns, while changing their cationic property and hydrophobicity. The results indicated that

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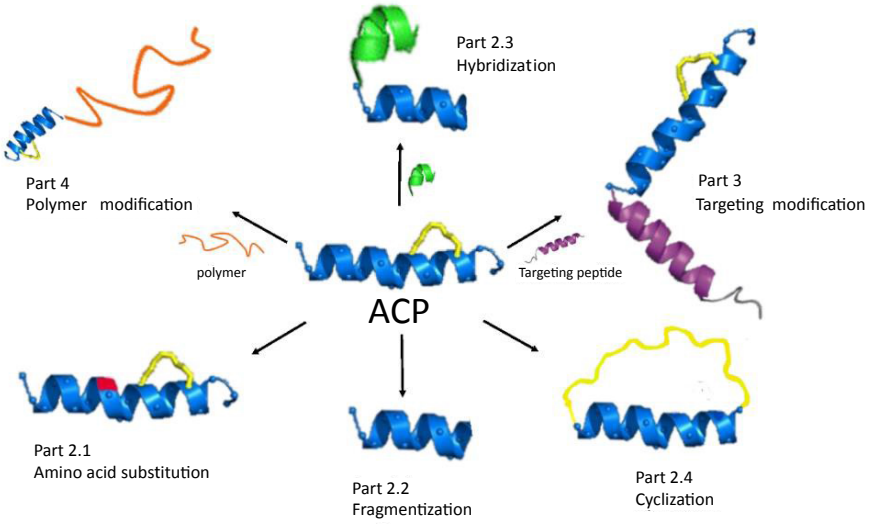

Figure 1: The schematic diagram of design and modification strategies of ACPs.

increasing the cationicity while reserving the moderate hydrophobicity may be a strategy to improve the cytotoxicity against tumor cells and decrease the hemolytic activity [13]. Similarly, Alyteserin-2a, is an amphipathic α-helical cationic cell-penetrating peptide, which displays relatively low cytotoxic potency against A549 human non-small cell lung adenocarcinoma cells and low hemolytic activity against human erythrocytes [14]. Several alyteserin-2a analogs were designed, in which amino acids on the hydrophobic face of the helix were replaced by L-tryptophan and amino acids on the hydrophilic face were replaced by one or more L-lysine or D-lysine residues. The Trp-containing peptides display an increasing of cytotoxicity against tumor cells, but the hemolytic activity against human erythrocytes also increase in parallel [15].

A 26-residues amphipathic peptide of V13K can adapt to α-helical conformation in the hydrophobic environment [16]. In order to increase the hydrophobicity of V13K, we designed single Leu-substituted peptides A12L, A20L, double Leu-substituted peptides A12L/A20L and triple ones A12L/A20L/A23L by replacing alanine to leucine at corresponding position. Among of them, peptide of A12L/A20L showed the strongest activity against HeLa cell line. Our data's suggested that the hydrophobicity of peptide plays a crucial role in the action against cancer cells due to the necrotic-like membrane disruption mechanism [3]. In addition, helicity was systematically modulated by introducing D-amino acids to replace the original L-amino acids on the non-polar face or the polar face of the helix and the therapeutic index of A12L/A20L against HeLa cells was improved by 9-fold and 22-fold, respectively [17]. These data's noted us needing to consider the balance of anticancer activity and hemolytic activity by modulation the hydrophobicity and helicity for the designing and modification of ACPs.

Due to the proteolytic degradation of nature peptide, D-amino acids substitution is a useful method to overcome this limitation and enhance peptide stability. NRC-03, acationic anticancer peptide from winter flounder, can cause the lysis of breast cancer and multiple myeloma cells and NRC-03-mediated cell death correlated with peptide binding to anionic molecules of cellular surface [18-20]. However, the effect may be limited because of its susceptibility to proteases. Hilchie et al. [19] designed a analog of NRC-03 by replacing all the original amino acids with the corresponding D-amino acids, named [D]-NRC-03, which exhibited higher anticancer activity than NRC-03 due to the stability against human serum or trypsin [21]. Except D-amino acid, other unnatural amino acids also have been used to modify the anticancer peptide to increase their stability or bioactivity [22-24]. Anticancer peptide of $_{D}(KLAKLAK)_{2}$ is a well-known pro-apoptotic peptide that locate to mitochondria and disrupt this organelle, also named $_{D}(KLA)_{2}$ or kla [25]. Horton designed a serious of analogues by exchanging the leucine residue with three different amino acids: phenylalanine (F), cyclohexyl-alanine (FX) or 6-carbon alkyl chain residue (Hex). The data showed that engineered peptides exhibit more toxicity than the parent compound against a wide variety of cancer cell lines, while their cell-type specificity are remained [26-28].

Besides affecting the activity and stability, amino acid substitutions may even alter the mechanism of action of anticancer peptides. Zhou et al. designed a mutant ZXR-2 (FKIGGFIKKLWRSLLA) based on ZXR-1(FKIGGFIKKLWRSKLA) by replacing Lys to Leu at the 14th position. ZXR-1 is an anticancer peptide derived from a known anticancer peptide mauriporin [29]. It is interesting that ZXR-2 exhibits anticancer activity, but the mechanism is completely different. Briefly, ZXR-1 is a pro-apoptotic peptide, it can translocate into cells and target on the mitochondria, then induce cell apoptosis, while ZXR-2 is a lytic peptide that directly targets on the cell membranes and causes membrane lysis.

Fragmentization

Fragmentization is a strategy to obtain short bioactivity peptide from the bioactivity proteins or the long and complex bioactive peptides. Short peptide has several advantages such as low immunity, easy synthesis, low cost, etc. Here, we divide the fragmentization method into two different types.

The first type is the bioactivity peptide coming from a bioactive protein. For example HPRP-A1 is derived from the N-terminus of ribosomal protein L1 of *Helicobacter pylori* and exhibited a broad spectrum anticancer activity [30]. AFP is a safe, naturally occurs human protein produced during pregnancy, which has anti-estrogenic and anti-breast cancer activity [31]. The oncostatic activity is localized to an eight amino acid sequence (amino acids 472–479) in domain IIIB. Based on this, a linear peptide of EMTPVNPG was synthesized

[32] and then a series of analogues were designed. Among them, the activity of cyclo[EMTOVNOGQ] had been proved [33]. BH3 proteins promote apoptosis through anti-sequestration or direct activation of apoptosis effectors [34,35]. Liu et al. designed an artificial 18-mer BCL-2 homology 3 peptide (ABH3) via charge tuning and conformation constraining based on original BH3 sequence. The data indicated that it induces cell death in an apoptosis-independent manner through the lytic properties of the peptide that causes disruption of cell membrane [36].

The second type is truncation from the long and complex bioactive peptides [37]. Buforin IIb (RAGLQFPVG[RLLR]3), a histone H2A-derived peptide, has strong cell penetrating ability and anticancer activity against various cancer cell lines but also showed cytotoxicity against normal cells at high concentrations [38]. By stepwise elimination of the C-terminal regular α-helical motif RLLR repeats motif of buforin IIb, Lim et al. designed several peptides consisted of different numbers of motif RLLR and named BR1 (RAGLQFPVGRLLR) and BR2 (RAGLQFPVGRLLRRLLR). The data indicated that BR2 can efficiently internalize into various cancer cell lines without cytotoxicity against normal cells [39]. Cecropin B is an amphipathic polycationic peptide; the signal sequence is located at N-terminus [40]. CB1a was constructed by repeating the N-terminal ten amino acids of CB three times and including a hinge near C-terminus. Compared to CB, CB1a has been demonstrated promising activity against several cancer cells but low toxicity against non-cancer cells and has become a promising anticancer agent [41].

Hybridization

Peptide-peptide hybrid is an important strategy for the modification of ACPs. In the design of AMPs, hybridization is a very common method to improve the activity, reduce the toxicity, etc. [42,43]. In the design of ACPs, most hybrid peptides usually combine cell penetrating peptide or targeting peptide with a lytic peptide or pro-apoptotic peptide, to improve the activity, stability or selectivity of ACP. Here we focus on the modification of cell penetrating peptide, the modification of targeting peptide will be reviewed in next part of targeting modification.

In our previously study, we constructed of a new hybrid peptide, HPRP-A1-TAT(FKKLKKLFSKLWNWKRKKRRQRRR), comprising the cell-permeating peptide TAT(RKKRRQRRR) [44] linked to the C-terminus of HPRP-A1(FKKLKKLFSKLWNWK). Compared to HPRP-A1, HPRP-A1-TAT exhibited stronger anticancer activity and higher therapeutic index [30]. It is interesting that the cellular concentration of HPRP-A1-TAT was higher than that of HPRP-A1 after 24 h incubation with HeLa cells. We suggested that TAT protects HPRP-A1 against degradation, maybe attribute to its high number of positively charged amino acids or the further release of peptides into cancer cells from endocytotic vesicles [30]. Another example of hybrid peptide is r7-kla (D forms). Benedict Law et al. incorporated the mitochondrial membrane disrupting peptide of kla with a cell-penetrating domain of r7. As we know, kla cannot easily penetrate through the cell membrane, r7 as a delivery vector can increase the membrane-crossing ability of kla. Thus, the hybrid peptide of r7-kla showed stronger cellular uptake rate and stability due to the resistant ability to protease digestion and resulted to more cells apoptosis [45].

In addition, the hybrid peptide has also been applied in intravital imaging. Ts (SKKPVPIIY CNRRSGKCQRM) is a mammalian free cell membrane-penetrating peptide [46] and Pc1 (CIRTPKISKPIKFELSG) is a αvβ3-binding peptide [47]. Yan et al. linked Ts to Pc1, and created a hybrid peptide, PTS. Then the hybrid was labeled with an FITC or Cy5.5 as an imaging indicator to evaluate its *in vitro* and *in vivo* bioactivity [48].

Except the cell penetrating peptides modification, the hybrid of different AMPs also has been reported. Cecropin A-magainin 2 and cecropin A-melittin hybrid peptides also have been designed and synthesized by Shin and examined the relationships between structure and biological activity [42]. The results suggested that hybridization is a useful method for the design of ACP and the activity of ACP is closed to the structure.

Cyclization

Besides enhancing the biological stability of peptides, cyclization can also stabilize the conformation suitable for better binding to other sites and improved biological activity of ACPs [49,50]. There are two main forms of peptide cyclization, cyclization by the formation of the amide bond between the N-terminal and the C-terminal amino acid residues and cyclizations involving the side chains of individual amino acids [51,52].

Cyclization by the formation of amid bond between the N-terminal and C-terminal amino acids can be also called head-to-tail cyclization [51] or backbone to backbone cyclization [52]. Tørfoss et al. discovered a series of synthetic anticancer heptapeptides (H-KKW$\beta_{2,2}$WKK-NH_2) containing a central achiral and lipophilic $\beta_{2,2}$-amino acid, which showed high proteolytic stability but low toxicity against normal cells [53]. They further prepared a series of seven to five residue cyclic peptides containing the two most promising $\beta_{2,2}$-amino acid derivatives as part of the central lipophilic core and proved that a considerable increase in anticancer potency following head-to-tail peptide cyclization [54].

Conotoxins are disulfide-rich peptides from the venoms of marine cone snail. Some kind of conotoxins can specifically target different subtypes of nicotinic acetylcholine receptors [55] and have very promising anticancer potential [56]. However, they are susceptibility to degradation by proteases. Clark et al. used (Native chemical ligation) NCL to synthesize a range of cyclic conotoxin analogues for evaluation as potential drug leads [57].

TAT is a well-known cell-penetrating peptide. Oh et al. [58] compared the activities and conformations between cyclic TAT and linear TAT. They found that cyclic TAT transduces with higher efficiency than linear TAT and the guanidinium groups are more distant in cyclic TAT meanwhile guanidinium group separation enhances uptake kinetics [59]. Conibear et al. examined three different cyclization approaches using a tumor homing peptide epitope of LyP1 by replacing the disulfide bond with a stable triazole or fluorobenzene ring or grafting it into a h-defensin or cyclotide scaffold. Although these analogs are not as active as expected, but their study highlights the potential of the cyclic cystine ladder and cyclic cystine knotmotifs as stable and versatile peptide scaffolds [60].

Modification Strategy

Targeting modification

Based on the combine peptide library and phage display technology, hundreds of targeting peptides have been identified to specifically distinguish many types of cancer cells and tumor angiogenesis, named Tumor Homing Peptide (THP). These THPs can also be used to delivery chemical agents to the cancer site while with low affinity against the normal cells [61]. Here, we divide the THPs into three different types. The first one, the THPs are tumor specific tumor homing peptides, such as TCP-1, it can specifically target towards the

vasculature of orthotropic colorectal cancer [62]. The secondly one, the THPs are tumor independent tumor homing peptides, such as RGD-4C, NGR, Lyp-1, TMTP1, etc., they can target to the common antigenic markers with broad spectrum [63]. The thirdly one, the THPs not only have homing capability, but also have cell penetrating ability, therefore they can deliver the drug into the depth of tumor tissue, for example, iRGD [64]. As shown above, anticancer peptides (ACPs) are potential candidate drug for cancer treatment, but the drawback of less selectivity toward tumor cells is still the major challenge for clinical use [65]. Based on this problem, many researches have been done by conjugating a THP on the N-terminal or C-terminal of peptide to increase the specificity of ACPs [63], including pro-apoptotic peptide $_{D}(KLA)_{2}$, Tachyplesin and ATAP, etc. and membrane activity peptide, such as Magainin 2(MG2). In this way, the THP mainly can improve the specificity of ACPs against cancer cell lines and reduce the toxicity of ACPs against normal cells.

RGD

The first isolated THP was RGD (Arg-Gly-Asp) peptide, which was identified by *in vivo* phage display in tumor bearing mice in 1997. The RGD peptide has high affinity to the alpha v integrin's when intravenously injected into tumor-bearing mice [66]. Based on the peptide of RGD, a targeting hybrid peptide was synthesized which compose a RGD and an anticancer peptide of Tachyplesin, which is present in leukocytes of the horseshoe crab. The results *in vitro* showed that RGD-tachyplesin can inhibite the proliferation of both cultured tumor and endothelial cells and reduce the colony formation of TSU prostate cancer cells. Meanwhile, RGD-tachyplesin can induce apoptosis through both mitochondrial and Fas-dependent pathways. The studies *in vivo* also indicated that the RGD-tachyplesin can inhibit the growth of tumors on the chorioallantoic membranes of chicken embryos and in syngenic mice [67].

NGR

Ellerby et al. [68] also synthesized both CNGRC and ACDCRGDCFC (RGD-4C) conjugated with pro-apoptotic peptide of $_{D}(KLA)_{2}$. The two homing peptide CNGRC and RGD-4C guided the peptide targeting to cancer cell and internalization. Then the peptide of $_{D}(KLA)_{2}$, can induce apoptosis by disruption of mitochondrial membranes. Smolarczyk et al. [69] also examined the therapeutic effect of peptide RGD-4C-GG-$_{D}(KLA)_{2}$. The results indicated that the peptide can induce apoptosis in B16 (F10) cell line *in vitro* and inhibit the tumor growth *in vivo* by intratumoral administration. It is interesting that the tumor growth is faster and the animals die off after the administration termination.

iRGD

Sugahara et al. identified a novel tumor-penetrating peptide of iRGD (CRGDK/RGPD/EC) [64]. iRGD contains an internalizing RGD sequence, which can bind to αv integrins that are specifically expressed on the endothelium cells of tumor vessels. Then it can be proteolytically cleaved to expose the CRGDK/R sequence on the C-terminal, named C-end Rule (CendR) motif (R/KXXR/K), which has the high affinity to the receptor of neuropilin-1 (NRP-1) and triggers tissue penetration [70]. iRGD has been extensively used as a targeting delivery and penetration tools for nanoparticles [71], peptides [72], monoclonal antibody [73], etc., by chemical conjugated or co-administration [70]. iRGD is one of the most widely used THPs to modify many kind of ACPs, like apoptotic peptide $_{D}(KLA)_{2}$, ATAP, CDD, Thymopentin, TP5, etc.

ATAP derived from Bfl-1, is an amphipathic α-helix peptide. It can target on mitochondria and induce caspase-dependent apoptosis [74]. De [72] conjugated the iRGD sequence of CRGDKGPDC to the carboxyl-terminal of ATAP and the results indicated that ATAP-iRGD can penetrate into cancer cells and distribute the mitochondria network. Meanwhile, the peptide of ATAP-iRGD can also induce apoptosis through release of Cyt c on DU145 cells. In addition, ATAP-iRGD-M8 also suppressed tumor growth on DU 145 and PC-3 prostatic cancer xenograft model by intravenous administration.

Wang et al. also modified a variant of $_{D}(KLAKLA)_{2}$ to $_{D}(KLAKLAKKLAKLA)_{L}K$, named m(KLA) by iRGD. As we know, the -$_{D}$(A)K- amino acid bond is a unique substrate of Cathepsin B (CTSB), which is overexpressed in the cytoplasm of tumors and in human tumor-associated cells [76]. The confocal microscope results indicated that iRGD can guide m(KLA) enter into the αvβ3 and NRP1 positive cells, such as MDA-MB-231, SKBR3 and 4T1 after administrated for 2 h and induce apoptosis through both mitochondrial pathway and the death receptor pathway, whereas it cannot enter into the NRP1-negative B16 cells. Furthermore, m(KLA)-iRGD spread extensively within the tumor tissue when it was injected into 4T1 tumor bearing mice. The m(KLA)-iRGD peptide reduction in tumor volume ($P<0.05$) and the total inhibition of metastasis at the end of the treatment [75].

Thymopoietin pentapeptide (Thymopentin, TP5), is an active fragment of thymopoietin (residues 32-36, Arg-Lys-Asp-Val-Tyr) [77] and has been widely used as immunomodulatory for treating immune deficiency, cancer and infectious diseases. However, poor penetration into tumors limits the clinical use of TP5. In order to overcome this drawback, a homing peptide iRGD was introduced with the C-terminal of TP5 to form TP5-iRGD by Lao et al. [78]. The MTT result showed that TP5-iRGD exhibited high inhibition of cell growth activity against B16F10, MCF-7 and H460 cell line. Meanwhile, the test *in vivo* on B16 xenograft model also indicated that the TP5-iRGD can significantly inhibit the tumor growth than TP5.

Bit1is a 179-amino acid residues protein. Its C-terminus constitutes the catalytic domain and the N-terminus serves as a mitochondrial localization signal [79,80] and the N-terminal domain has been reported with apoptotic activity and defined cell death domain (CDD). A recombinant protein of iRGD-CDD has been also indicated to trigger the tumor cell death both in cultured tumor cell and the xenograft breast tumor in mice. Repeated treatment with iRGD-CDD strongly inhibited tumor growth, resulting in an average reduction of 77% in tumor volume [81].

T3

Lactaptin, which is a proteolytic fragment of human kappa-casein (residues 57–134) was found in human breast milk. It has been reported that lactaptin has the ability of reducing cell viability and inducing apoptosis in cultured tumor cells [82]. RL2 is the recombinant analogue of lactaptin that has the activity of inducing different of human cancer cells, such as MCF-7, MDA-MB-231, A549, HEP-2 and HA1 [83]. More efforts have been made to obtain the targeting properties of lactaptin. Recently, Nemudraya et al. [84] reported that two targeting peptides of T3(YTYDPWLIFPAN) and iRGD had the specificity for cancer cell and penetration to targeted cancer cell and tumor tissue. They were used to construct the fusion proteins T3-RL2, RL-iRGD-His and RL2-iRGD and evaluate the activity both *in vitro* and *in vivo*. The results *in vitro* showed that the fusion proteins exhibited higher activity than RL2 in MDA-MB-231 and MCF-7 cell lines. The data *in vivo* also indicated that T3-RL2 protein significantly inhibit tumor growth in a MDA-MB-231 xenograft model compared with RL2.

IL-4Rα ligand

High-affinity interleukin-4 receptor α(IL-4Rα) is highly expressed on the cell surface of various human solid tumors, for example renal cell carcinoma, melanoma, breast carcinoma, ovarian carcinoma, glioblastoma, AIDS-related Kaposi's sarcoma and head and neck squamous cell carcinoma [85-90]. Recently, a hybrid peptide of IL-4Rα-lytic peptide containing a target peptide targeted to IL-4Rα(KQLIRFLKRLDRNG) and a lytic peptide of KL**L**LK**LL**K**K**LLK**L**LKKK-OH (bold letters are D-amino acids) has been designed by Yang et al. [91]. The data indicated that the expression levels of IL4Ra in cells were correlated well with IC50 ratio of lytic peptide to IL-4Rα–lytic peptide.

The authors suggested that the increasing of cytotoxic activity can be attributed to the targeting (IL-4Rα) moiety of hybrid peptide. The results *in vivo* also indicate that the IL-4Rα–lytic hybrid peptide can selectively target to cancer cells and inhibit the tumor growth in the BXPC-3 and MDA-MB-231 xenograft model.

z13

An endometriosis targeting peptide of z13, which displayed the sequence VRRADNRPG, was from T7 phage-based library. z13 can strongly bind with the targeted protein CNGB3 in endometriosis. Sugihara et al. [92] linked z13 to an 18-mer pro-apoptotic peptide, KLAKLAKKLAKLAKKLAK, abbreviated dKLAK (D forms) or a variant peptide of HLAH by replacing lysine with histidine to form hybrid peptide of dKLAK-z13 or HLAH-z13, respectively. The effects of mixture of dKLAK-z13 and HLAH-z13 peptides on baboon endometriosis models *in vivo* by co-administered were investigated. The results indicated that cells in lesions selectively underwent apoptosis with no effect on neighboring organs and presented a strategy that could be useful to treat peritoneal endometriosis in humans.

EGFR ligand

Oesophageal squamous cell carcinoma (OSCC) is a major histologic type of oesophageal cancer [93] and the key therapy against advanced or metastatic OSCC is chemotherapy [94,95]. Epidermal growth factor receptor (EGFR) is over expressed in OSCC tissues about 71%-88% [96]. Recently, a hybrid peptide as a new agent of EGFR-targeting therapy was designed and synthesized, which contains an EGFR-targeting peptide of YHWYGYTPQNVI and a lytic peptide of KL**L**LK**LL**K**K**LLK**L**LKKK-OH (bold letters are D-amino acids) [97]. The hybrid peptide could kill EGFR-expressing cells through the combined process of specific binding to EGFR on the cell surface and subsequently disintegrate cell membranes.

TCP-1

TCP-1 (CTPSPFSHC) is a peptide that could target the vasculature of orthotropic colorectal cancer identified by the *in vivo* phage display technology [62]. The data demonstrated that TCP-1 also recognizes the blood vessels of human colorectal cancer and can be used to deliver fluorescein and the pro-apoptotic peptide. Li et al. [62] conjugated TCP-1 to the N-terminal of the pro-apoptosis of $_{D}(KLA)_{2}$ with the double glycine as linker. The results showed that the TCP-1 peptide can guide $_{D}(KLA)_{2}$ entering into the cancer cell and inducing apoptosis through caspase 3 pathway both by chemical conjugate and uncoupled mixture.

TMTP-1

A 5-amino acid residues peptide of TMTP1 has been reported that it can bind to a series of highly metastatic cancer cell lines both *in vitro* and *in vivo*, particularly those from atypical liver micrometasteses that contained small number of neoplastic cells [98]. Ma et al. [99] coupled TMTP1 to the pro-apoptosis peptide $_{D}(KLA)_{2}$ and named as TMTP-1-DKK. The data *in vitro* indicated that TMTP1-DKK could trigger rapid apoptosis in human prostate and gastric cancer cells through both the mitochondrial- induced apoptosis pathway and the death receptor pathway. Furthermore, direct injection of TMTP1-DKK into mice with prostate and gastric xenograft cancers also resulted in decreasing of tumor volumes and a significant delay in tumor progression and metastasis *in vivo* [99].

BRBP1

A linear dodecapeptide peptide of BRBP1 (MYPWTEPSYLSN) was identified through random peptide phage display bio-panning and can against preferentially bind to the human "brain-seeking" breast carcinoma cells (231-BR cells) in a concentration- dependent and energy dependent manner *in vitro* [100]. Fu et al. [101] designed and synthesized a new hybrid peptide of BRBP1-TAT-KLA, which containing three elements: a brain metastatic breast carcinoma cell (231-BR)-binding peptide BRBP1, a cell penetrating peptide TAT and a pro-apoptotic peptide KLA. In their study, the antitumor activity of BRBP1-TAT-KLA on brain metastatic breast cancer both *in vitro* and *in vivo* was evaluated. And the results *in vitro* showed that the peptide of BRBP1- TAT-KLA efficiently internalized in 231-BR cells and consequently induced the membrane damage of mitochondrial and cellular apoptosis, significantly decreased cell viability and increased apoptotic ability. The results *in vivo* also demonstrated that BRBP1-TAT-KLA selectively homed to the tumors and induced cellular apoptosis, significantly delayed tumor growth and enhanced antitumor selectivity while without significant toxicity on non-tumor tissues.

Bld-1

A Bld-1 peptide of CSNRDARRC, which binds to bladder tumor cell, has been identified by phage display [102]. Jung et al. combined the tumor targeting peptide Bld-1 with a pro-apoptosis $_{D}(KLA)_{2}$ and formed a hybrid peptide of Bld-1-kla. The hybrid peptide of Bld-1-kla can selectively bind to HT1376 bladder tumor cells and efficiently internalize into cells but not to other tumors or normal cells, exhibiting higher apoptotic ability than Bld-1 or kla alone. It is interesting that the binding and cytotoxicity of Bld-1-kla was inhibited when pretreated HT1376 cells with Bld-1. The data of confocal microscope showed the peptide localized in the mitochondrial. The test *in vivo* on tumor-bearing mice got the same results that the peptide of Bld-1-kla induced apoptosis of tumor cells and inhibited tumor growth more efficiently than the peptide of kla [103].

LTV, GR and Bombesin

Based on phage peptide libraries, many other cancer-cell targeting peptides have been identified and used for the diagnosis and treatment of tumors. The LTVSPWY peptide (LTV peptide) is a targeting peptide. It can deliver antisense oligonucleotides and small molecules to cancer cells *in vitro* [104,105]. Furthermore, various fusion proteins carrying the LTV peptide have been designed to target cancer cells *in vitro* and *in vivo* [106]. A gastrin-releasing peptide (GNHWAVGHLM, GR peptide) also is a targeting moiety, whose receptor is expressed by most cancer types, including breast, ovarian, prostate and lung cancer [107,108]. Bombesin is a 14-amino acid tumor homing peptide isolated from frog skin [109]. Its receptors are overexpressed in a variety of common human cancers, such as neuroblastoma and small cell lung cancer, as well as cancers of the prostate, kidney, uterus, ovary, breast, pancreas, gastrointestinal tract, head

and neck, and esophagus [110]. Liu et al. [111] reported that by attaching magainin 2(MG2) to the N-terminus of Bombesin, the anticancer effect of the hybrid peptide of MG2B increase 10 times over than unconjugated MG2 *in vitro*. While MG2B also exhibited higher antitumor effects in mice bearing MCF-7 tumor grafts [111].

As shown above, THPs can increase the anticancer activity and selectivity of the ACPs while without change the secondary structure or charges, some of them can help more ACPs enter into the cancer cells and induce cell apoptosis through interrupting mitochondrial membranes. These results suggested that chemically coupled of targeting peptide with the pro-apoptosis peptide or others anticancer drugs is a promising therapeutic strategy for targeted therapy of cancers. We believe that the targeting modification of ACPs will be having a very beautiful future in clinical use. The targeting modifications of ACPs are listed in Table 1.

Polymer modification

Polymer is consisting of one or several units, having high molecular weight connected by covalent bond. Polymer generally possesses some special characteristics, such as negligible toxicity, high biocompatibility and strong degradability. Many polymers have been used in drug delivery system to improve the stability and solubility of the drug, for example, PEG, carboxymethyl dextran (CMD), PLLA, etc. In addition, polymer modification also can used to design prodrug system.

Among all the kinds of polymers used for the modification of anticancer peptide, PEG was studied most frequently and thoroughly. It has been approved safety by FDA for food, drug delivery vehicle [112-115]. PEGylated protein and peptide have many advantages, such as the hydrophilic characteristic of PEG moiety enabling to increase the ability of ACPs to escape from degradation of blood serum protein and making the peptides obtained larger molecular weight. In this part, several strategies for the modification of ACPs have been reviewed as follow:

The PEGylation of ACPs

PEGylated ACPs was first reported by Kawasaki [116]. In this study, a laminin related peptides of YIGSR was conjugated to PEG and formed YIGSRG-[amino-poly(ethylene glycol)] hybrid molecule. It showed a better stability and resistance to blood serum *in vivo* and exhibited the most potent inhibitory effect on the metastasis of B16 melanoma BL6. Then, Kawasaki [117] developed two novel different methods to conjugate PEG to ACPs which the NH_2-PEG and COOH-PEG were successfully synthesized and attached to the amino or carboxyl groups of peptide, respectively. Then, NH_2-PEG-COOH was also synthesized by Maeda's [118-120] and introduced into SPPS, just like an ordinary amino acids. A bifunctional PEG polymer of PDSGR-aaPEG-YIGSR was built combining PDSGR and YIGSR together and exhibited higher inhibiting effect of tumor metastasis.

Similarly, Brinckerhoff et al. [121] also reported the PEGlytion of anticancer peptide MART - 1. It is predicted that immunogenic tumor peptides would have short half life time *in vivo* due to the degradation of peptidases in plasma. However, when an amino-PEG was attached to the C terminal of MART - 1 to form a MART-1 PEG hybrid, the stability of MART-1 was markedly prolonged; the immunogenicity of these peptides might also be enhanced by creating modifications that enhance stability.

Nisin is an amphiphylic peptide with 34 amino acid residues belonging to the family of lantibiotics and exhibites anticancer activity [122,123]. Guiotto et al. [124] reported that nisin with several shortcomings, including its low solubility in neutral aqueous solutions, instability at physiological pH and rapid breakdown by proteolytic enzymes. The PEG-nisin conjugate could protect nisin from degradation of enzymes and improve its solubility.

It must be noted that high molecular weight PEG moiety leads to a decrease of anticancer activity of peptide-PEG block polymer as the stability increase. For this reason, ACPs modified by short PEG chains was synthesized by Zhang et al. [125]. The studies *in vivo* and *in vitro* indicated that PEGylated peptide by short PEG chains exhibited a prolonged circulating life times in plasma without losing of bioactivity. Except the linear PEG, the branched PEG was also employed for the PEGylation of ACPs. Alpha-momorcharin (α-MMC), from a

Table 1: The list of targeting modification of ACPs.

Homing peptide	Anticancer Peptide	Cancer Type	Reference
RGD	Tachyplesin	TSU prostatic cancer B16 melanoma tumor	[66,67]
RGD	D(KLA)2	MDA-MB-435 tumor	[68]
RGD-4C	D(KLA)2	B16F10 melanoma tumor	[69]
iRGD	ATAP	DU145, PC-3	[72,74]
iRGD	m(KLA)	MDA-MB-231, SKBR3, and 4T1 breast tumor	[75,76]
iRGD	TP5	B16F10 melanoma tumor	[77,78]
iRGD	CDD	MCF- 10CA1a, 4T1 tumor	[79-81]
T3	RL2	MDA-MB-231, MCF-7	[82-84]
IL-4Rα ligand KQLIRFLKRLDRNG	D(KLA)2	BXPC-3 and MDA-MB-231	[91]
z13	dKLAK	baboon endometriosis models	[92]
EGFR ligand YHWYGYTPQNVI	KL**L**LK**LL**K**K**LLK **L**LKKK-OH	human K-ras mutation negative and positive cancers	[94,95]
TCP-1	D(KLA)2	Orthotopic Colorectal cancer	[62]
TMTP-1	D(KLA)2	PC-3M-1E8 prostate and MKN-45sci gastric	[98,99]
BRBP-1	D(KLA)2	231-BR brain metastatic breast cancer	[100,101]
Bld-1	D(KLA)2	HT1376 bladder tumor	[102,103]
LTV&GR	D(KLA)2	MCF-7 and MDA-MB-231	[104-106]
Bombesin	magainin II	MCF-7	[111]

ribosome-inactivating protein (RIP) exhibited excellent cytotoxicity against tumor cells and strong immunogenicity and short half-life time in plasma. Bian et al. [126] synthesized PEGylated-α-MMC using a branched mPEG. The results indicated that the complex preserved moderate anticancer activity and a longer circulation time with 36% acute toxicity and at most 66% immunogenicity decrease.

Although PEGlation of anti-tumor peptide has been widely regarded as an effective method to enhance it stability and solubility, the specific mechanism of PEGlation is still unknown. Towards this goal, Kaneda et al. [127] studied the mechanism of PEGlation; they supposed that the improvement of anticancer activity of peptide can attribute to the prolongation of the half-life time of peptide in the blood.

Imura et al. [128,129] also investigated the biological activity of PEGylated tachayplesin 1, a membrane- acting β-sheet cyclic anticancer peptide. The results demonstrated that the PEGylation of tachayplesin 1 decreased the activity and the cytotoxicity *in vitro* and increased the specificity of peptide. It is interesting that compared with free peptide; PEGylation did not alter the basic mechanism of membrane-permeabilizing. Furthermore, Imura and coworkers got the similarly results when they alter the β-sheet peptide of tachayplesin 1 to α-helical ACPs of magainin 2. These data also provide some useful information for the peptide designing, particularly for the design of prodrug.

Other polymers modification

Except the PEG, other ploymers also were used to modify the ACPs to improve the activity, stability and specificity, for example, Dextran, SMA, PVP, etc. In addition, several polymers have been used in peptide targeting delivery and controlled releasing research. Based on the modification, they can enhance the delivery of peptides to the target site and improve the therapeutic efficacy, while minimizing side effects [130-132]. Due to the enhanced permeability and retention effect (EPR), high molecular weight polymers and nano-sized particles prone accumulate in solid tumors with higher concentrations [133].

Dextran derivative of carboxymethyl dextran (CMD) is frequently used as a drug deliver carrier due to its low glomerular filtration rate and lower hepatic uptake [134,135]. An CMD-peptide conjugates of CMD-s-s-peptide was prepared through the disulfide bond between CMD and EGFRZR-lytic peptide by Gaowa et al. [136,137]. The obtained conjugate could be stimulate-responsive by GSH and release to lytic peptide. Compared to the free peptide, CMD-peptide conjugates were highly accumulated in tumor tissue and the attachment of CMD prolonged the elimination half-life and more effective anticancer activity of peptide after intravenous injection.

Mu et al. [138] modified YIGSR with SMA [poly (styrene comaleic anhydride)] to improve anti-tumor activity of YIGSR and the studies *in vivo* indicated that bio- conjugate SMA-YIGSR have a prolonged plasma half-time and higher binding affinity than merely YIGSR. Mu et al. [139] further modified YIGSR with Polyvinyl pyrrolidone (PVP). The results showed that the activity and half-life time in plasma of PVP-conjugated YIGSR (PVP-YIGSR) was more than 100-fold greater and 15-fold longer than the free YIGSR, respectively.

Gelatin, which is usually obtained from collagen, has been extensively explored for its biocompatibility and biodegradation in the last few decades. Recently, the combination of antitumor hybrid peptide with anionic gelation was developed [137]. The electrospinning fabrication technique is emerging in biomedical application such as cancer therapies and wound healing treatments. Recently, a recombinant silkworm ACP of Bmattacin2 was used to load into PLLA nanofibrous membrane by this technique, PLLA [Poly (L-lactic Acid)] and Bmattacin2 conjugate was successfully prepared, which exhibited significant anti-tumor activity and a good compatibility with the normal cells [140].

Peptide Amphiphiles (PAs) is an amphiphilic molecule composed of alkyl chain and hydrophilic β-sheet forming sequence, which can deliver the AMPs to tumor cells effectively. Standley et al. [141] introduced a novel self-assembled nanoparticle that conjugated KLAK to Pas. It can kill the breast cancer cells not only by disrupting cell membrane, but also through the way of caspase independent and Bax/Bak independent apoptosis pathway. Zha et al. [142] further researched nanoparticle and they reported on the complex of hyaluronic acid and positively charged PA to bear KLAK peptide. Deng et al. [143] conjugated aliphatic acid of various lengths to anticancer peptide of B1, a novel ACPs derived from Cathelicidin-BF15. All results revealed that the modified ACP obtained a higher bioactivity towards tumor cells and indicated that the conjugated aliphatic acid enhanced hydrophobicity and helicity of peptides, which subsequently resulted in higher membrane-lyting capability of ACPs. Thus, this method is suitable for the modification of membrane activity ACPs.

In this part, we focus on the polymer modification of ACPs. From the above data, we think maybe PEGyation is mainly to increase the stability of ACPs, it suitable for the modification of cellular adhesion molecules such as RGD or YIGSR or proapoptotic peptide of $_{D}$-$(KLA)_{2}$. While the design of peptide amphiphiles (PAs) maybe a better choice for the modification of membrane activity ACPs because biophysical parameters play an important role for the biological activity of ACP.

Conclusion

In summary, peptide anticancer therapeutics is a promising field against cancer problems. The amino acid substitution, cyclization, hybridization, fragmentization, modification of ACPs are great potential methods and provide many advantages such as increased the half-life time in plasma, enhanced its stability and activity, reduced its toxicity, which could improve the therapeutic efficacy of ACPs. Of course, these methods do not exist lonely; they can be used to modify different ACPs or combined several methods to modify same ACP at the same time. We believe that ACPs as novel anticancer drugs will be play an important role for the clinical practices.

Conflict of Interest

The authors confirm that this article has no conflicts of interest.

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