Constructing new acid-activated anticancer peptide by attaching a desirable anionic binding partner peptide

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ABSTRACT

Improving the cell selectivity of anticancer peptides (ACPs) is a major hurdle in their clinical utilisation. In this study, a new acid-activated ACP was designed by conjugating a cationic ACP LK to its anionic binding partner peptide (LEH) via a disulphide linker to trigger antitumor activity at acidic pH while masking its killing activity at normal pH. Three anionic binding peptides containing different numbers of glutamic acid (Glu) and histidine were engineered to obtain an efficient acid-activated ACP. The conjugates LK-LEH2 and LK-LEH3 exhibited 6.1- and 8.0-fold higher killing activity at pH 6.0 relative to at pH 7.4, respectively, suggesting their excellent pH-dependent antitumor activity; and their cytotoxicity was 10-fold lower than that of LK. However, LK-LEH4 had no pH-responsive killing effect. Interestingly, increasing the number of Glu from 2 to 4 increased the pH-response of the physical mixture of LK and LEH; conversely, they weakly decreased the cytotoxicity of LK, suggesting that the conjugate connection is required to achieve excellent pH dependence while maintaining minimum toxicity. LK-LEH2 and LK-LEH3 were more enzymatically stable than LK, indicating their potential for in vivo application. Our work provided a basis for designing promising ACPs with good selectivity and low toxicity.

Introduction

Cancer is the second main cause of death worldwide and remains a major public health concern \cite{1,2}. It is generally caused by genetic mutations in ‘driver genes’ that enable cancer cells to evade growth suppression and resist cell death and metastasis \cite{3,4}. Common cancer treatment methods mainly involve chemotherapy, radiotherapy, immunotherapy and surgery. Although treatment strategies have been improved, cancer treatment is still limited by the lack of selectivity to cancer cells, causing damage to normal tissues \cite{5–8}. Another main problem associated with currently available antineoplastic drugs is multidrug resistance \cite{9,10}. Thus, alternative anticancer agents with high selectivity and low drug resistance must be developed.

Many different strategies have been developed to improve the targeting of anticancer agents by using specific aspects of a tumour microenvironment \cite{11–15}. In addition to highly expressed surface markers in tumour cells, other features that distinguish tumours from normal tissues have been considered potential targets \cite{15–17}. One of the major differences between tumours and normal tissues is the decreased extracellular pH triggered by hypoxia and abnormal tumour metabolic processes \cite{18,19}. Thus, this unique feature of tumours can be utilised to develop pH-responsive targeting drugs that can be specifically activated to kill tumour cells in an acidic tumour microenvironment. Several successful attempts have been made to develop novel pH-dependent strategies based on tumour acidity \cite{20–25}.

Anticancer peptides (ACPs) are potential anticancer therapeutics because of their broad-spectrum anticancer activity, low tendency to develop resistance, minimal immunogenicity and ease of synthesis and modification \cite{26–28}. In general, they are short peptides with a net positive charge and hydrophobicity. They can exert an anticancer activity through direct membranolytic effects or programmed cell death \cite{29–31}. However, the cytotoxic effects of most ACPs on normal cells at high concentrations are the main factors limiting their further development as anticancer drugs \cite{26–28}. Hence, generating ACPs with enhanced selectivity and low cytotoxicity remains challenging. To date, many effective modifications have been made to increase the selectivity of ACPs and reduce their side effects \cite{31–35}. Developing acid-activated ACPs based on an acidic tumour microenvironment is a promising approach for selective tumour therapy \cite{35–38}. This type of ACP is inactive or less active under normal conditions, while its antitumor activity is activated by tumour acidity.

LK (LKKLLKKLLKKL-NH\textsubscript{2}) is a cationic \(\alpha\)-helical peptide of 14 amino acids. It is effective against tumour cells by disrupting their cell membrane \cite{37}. However, this peptide causes remarkable damage to normal cells at concentrations close to the effective one, which greatly narrows its therapeutic index. In a previous study, a new pH-responsive ACP was designed with a histidine modification strategy by using the acidic tumour microenvironment as a trigger to increase the selectivity of LK \cite{37}. Consequently, protonation could occur to obtain a net positive charge in the acidic tumour microenvironment, in contrast to the
predominance of no charge under normal physiological conditions. The newly designed histidine-rich peptide L9H5-1 showed an excellent pH-responsive antitumor activity with a reduced cytotoxic effect compared with that of LK [37]. Thus, pH-triggered charge conversion enabled by introducing histidine may be an effective method to improve LK selectivity.

Notably, pH-sensitive recombinant peptides have been successfully engineered on the basis of acidic tumor microenvironment [39–43]. They can specifically deliver macromolecules and nanoparticles to tumor sites in vivo and in vitro. In the design of pH-sensitive peptide constructs, a pH-sensitive oligopeptide sequence is typically used to mask the cationic charge and increase the specificity at target sites. Thus, the sequence editing of masking peptides may be a key point for tumor-targeting diagnosis and therapy. This masking effect has been utilised in several designs of new ACPs to reduce non-specific toxicity [38,44,45]. The masked ACPs can be activated efficiently in tumor microenvironment and show potential for therapeutic application. However, few studies have rarely investigated the effects of different anionic sequences on the activated feature of ACP, which will be useful for the design of an enhanced cationic peptide drug with targeted delivery to solid tumors.

Herein, the novel design of a pH-responsive ACP utilises a pH-responsive anionic binding partner peptide coupled to cationic LK via a disulphide linker to mask the cationic charge and prevent the cytotoxicity of the construct ACP under normal physiological conditions (Figure 1). The pH-responsive anionic peptides were obtained by substituting all lysines for different numbers of glutamic acids (Glu) and histidines. The histidine in the anionic peptide sequence is neutral at normal pH 7.4, allowing the electrostatic interaction between anionic Glu and cationic lysine. After exposure to an acidic tumor environment, histidine changes from neutral to cationic, and the anionic charge of Glu residues can be neutralised. Therefore, the antitumor activity of LK is rapidly activated in the acidic tumor environment. Different anionic peptides were designed to obtain an ideal acid-activated conjugate, and their effects on pH response and cytotoxicity were studied. This work provides new information regarding the effect of anionic structural changes on the acid-activated profile of ACP. It is useful for the design of a promising peptide drug targeting tumor tissues with an acidic extracellular environment.

Materials and methods

Peptide synthesis

All designed peptides were synthesised on an MBHA resin using the standard solid-phase method with Fmoc chemistry as described previously [37,46]. The crude peptides were purified and analysed by reversed-phase high performance liquid chromatography (RP-HPLC) on a C18 column and identified using electrospray ionisation mass spectrometry (ESI-MS). To synthesise construct (LK-LEH), Cys-LK was reacted for 48 h at RT with 10 equiv. of 2,2′-dipyridyl disulphide in MeOH/H2O (1:1) to get the thiolpyrindine protected peptide (thiolpyr-Cys-LK). Subsequently, the purified thiolpyr-Cys-LK was acquired by RP-HPLC, and reacted for 48 h at RT with 1.5 equiv. of purified Cys-LEH in MeOH/H2O (1:1) to get LK-LEH. Final reaction mixture was purified by RP-HPLC and then identified using ESI-MS.

Cell cultures

HeLa and CHO-K1 cells were cultured in RPMI-1640 medium (Gibco BRL, Carlsbad, CA) containing 10% neonatal bovine serum (NBS) (Sijiqing Biotech, Hangzhou, China). MCF-7 and A549 cells were grown in DMEM (Gibco BRL, Carlsbad, CA) with 10% FBS (Gibco BRL, Carlsbad, CA). All cell lines used in experiments were maintained in a 5% CO2 humidified atmosphere at 37 °C.

Circular dichroism analysis

All peptide samples were dissolved in 50% trifluoroethanol to a final concentration of 50 μM. The CD spectra were measured using a J-810 spectrometer and then converted to mean residue ellipticity to estimate the relative helicity of each peptide by using the previous method [37,47].

Cytotoxicity assays

Briefly, cells (1 × 10⁴ cells/well) were seeded in 96-well plates and cultured overnight, the cells were treated with medium adjusted to pH 7.4 or pH 6.0 containing different peptides with various concentrations for 0.5 h or 12 h, the medium adjusted to pH 7.4 or pH 6.0 without peptides was the control, respectively, followed by adding MIT solution (10 μL of 5 mg/mL in PBS). After 4 h, the medium was replaced with 150 μL of dimethyl sulphoxide (DMSO). The absorbance at 490 nm was detected by a microplate reader.

Lactate dehydrogenase (LDH) leakage assay

The LDH assay was performed to monitor the membrane integrity according to the manufacturer's instructions. HeLa cells (1 × 10⁴ cells/well) were plated into a 96-well plate and incubated for 24 h. Next, 100 μL of medium adjusted to pH 7.4 or pH 6.0 containing various concentrations of peptides was added and incubated for 0.5 h. Forty microlitres of medium was transferred from each well into a new 96-well plate, followed by the addition of 40 μL of reaction solution. After 30 min, 20 μL of stop solution was added into each well and then fluorescence was measured at 560/590 nm. Untreated cells were considered as no leakage, and cells exposed to 0.2% Triton X-100 represented 100% leakage.

Propidium iodide (PI) uptake assays

HeLa cells (6 × 10⁴ cells/well) were grown on coverslips 24 h before treatment. The cells were treated with medium adjusted to pH 7.4 or pH 6.0 containing 20 μM of peptides for 30 min. Then, the cells were stained with 50 μg/mL of PI solution for 10 min in the dark. The PI fluorescence was visualised using laser confocal scanning microscopy.
Scanning electron microscope (SEM)

The cells were exposed to medium adjusted to pH 7.4 or pH 6.0 containing 20 μM of peptides for 30 min. After being rinsed with phosphate-buffered saline (PBS), cells were fixed with 2.5% glutaraldehyde at 4°C. Postfixation with 2% osmium tetroxide for 2 h was followed by dehydration in an ascending ethanol series and drying in a critical point dryer. After samples were coated with gold, SEM observation was performed in a scanning electron microscopy.

Haemolysis assay

Fresh mouse blood was collected in a centrifuge tube containing sodium heparin, and erythrocytes were obtained by centrifugation at 1000 × g for 10 min at 4°C. The erythrocytes were washed three times with PBS and then resuspended in PBS to 8% (v/v). A 100 μL portion of erythrocyte suspension was added to the wells of a 96-well plate and incubated with 100 μL of peptide solution containing various concentrations for 1 h at 37°C, followed by centrifugation at 1000 × g for 10 min. Then, the supernatant was transferred into a new 96-well plate, and the absorbance of the supernatant was determined at 450 nm by a microplate reader. For 0% and 100% haemolysis, PBS and 0.1% Triton X-100 were used, respectively.

Enzymatic stability

The stability of peptides was examined by an analytical RP-HPLC. A portion of 15 μL of peptide stock solution (10 mM) was incubated with 285 μL of trypsin or chymotrypsin solution (10 μg/mL) at 37°C. At 0.5, 1, 2, 4, and 6 h time points, a portion of 40 μL of aliquots was diluted with equal volume of water–acetonitrile (60:40 v/v) containing 0.1% TFA and then analysed using RP-HPLC.

Results

Peptide design and characteristics

In this study, novel acid-activated ACPs were designed by coupling with different anionic binding partner peptides (LEHs) via a disulphide linker. LEHs were obtained by replacing the lysines in LK with different numbers of Glu and histidines. Glu was introduced at the amphipathic interface between the end of the hydrophilic side and the start of the hydrophobic side because these positions in LK are crucial for its lytic activity [37], which would favourably decrease the cytotoxicity of LK under normal physiological conditions. The number of introduced Glu was optimised to obtain the desirable acid-activated ACPs. The peptide sequences and RP-HPLC retention times are shown in Table 1. The analysis of the hydrophobicity of the peptides through retention time measurement revealed that the hydrophobicity of the anionic peptides increased with the increasing numbers of Glu. Correspondingly, the hydrophobicity of the three hybrid peptides increased gradually when the anionic peptides were coupled to LK. In addition, the secondary structure of the new peptides was characterised using CD spectroscopy in TFE/water (membrane-mimicking environment). Figure 2 shows that the new peptides exhibited a typical α-helical structure in this environment. Increasing the amount of Glu gradually increased the helical content.

Table 1. Sequences and RP-HPLC retention times of the designed peptides.

| peptides | sequence | t_R (min) |
|----------|----------|-----------|
| LK       | LKLLLKLKKLLLKL-NH₂ | 20.3      |
| LEH2     | LEHELHLEHELHLEL-NH₂ | 21.7      |
| LEH3     | LEHELHELHELHEL-NH₂  | 22.5      |
| LEH4     | LEHELHELHELHEL-NH₂  | 24.7      |
| LK-LEH2  | s-CLKLLLKLKKLLLKL-NH₂ | 23.0      |
| LK-LEH3  | s-CLHELHELHELHEL-NH₂  | 23.6      |
| LK-LEH4  | s-CLHELHELHELHEL-NH₂  | 25.3      |

*aRetention time (t_R) was measured by analytical RP-HPLC on a C18 column.

Figure 2. CD spectra of the constructed peptides in 50% TFE/water solution.
content of the new anionic peptides, and the helical content of the hybrid peptides increased with the increasing number of Glu.

**pH-dependent antitumor activity**

The antitumor activity of the peptides was assessed at different pH values via the MTT assay. LK showed similar cytotoxicity in HeLa cells at pH 7.4 and 6.0 (Figure 3(A)). Three new anionic peptides exhibited a pH-dependent antitumor activity, and their pH response decreased with increasing Glu contents (Figure 3(A)). After the coupling of LK and anionic peptides, the conjugates LK-LEH2 and LK-LEH3 showed a more significant pH-dependent antitumor activity than the anionic peptides (Figure 3(A)). However, the antitumor activity of LK-LEH4 was not detected at either pH value, indicating that the pH-dependence of the conjugates was almost lost when the number of Glu residues reached four. Moreover, a mixture of LK and LEH in a 1:1 physical ratio displayed a pH-dependent antitumor activity (Figure 3(A)); particularly, LK+LEH3 and LK+LEH4 were more pH-sensitivity than

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Figure 3. pH-dependent antitumor activity of peptides to different tumour cells. (A) Cell viability of HeLa cells treated with peptides for 0.5 h. (B) Cell viability of MCF-7 and A549 cells treated with 20 µM peptides for 0.5 h. Data are representative of triplicate experiments.
LK + LEH2. However, the cell viability of the mixtures significantly decreased at pH 7.4 compared with that of the conjugates. The effect of the mixture with a 1:1 physical ratio on reducing the cytotoxicity of the peptide was weaker than that of the conjugates. This result suggested that when LK was conjugated with its anionic peptides LEH2 and LEH3, LK would exhibit excellent pH-dependence and markedly reduced cytotoxicity compared with that of the anionic peptides and physical mixtures. Similar results were also obtained in other tumour cells (such as MCF-7 cells and A549 cells; Figure 3(B)). The antitumor activity of the new peptides at pH 7.4 and 6.0 was assessed by treating HeLa cells for 12 h to further confirm these results. In Figure 4, LK-LEH2 and LK-LEH3 also exhibited a more remarkable pH-dependent antitumor activity and low cytotoxicity than the other peptides did.

Cytotoxic effect on normal cells

The haemolytic activities of all the peptides against mouse red blood cells were assessed. LK showed a remarkable haemolytic activity at a low haemolytic concentration of 10% (HC10, 11.1 μM; Figure 5(A)). The anionic peptides had no haemolytic activity even at the highest concentration of 150 μM. The haemolytic activity of
Figure 5. The cytotoxicity of the new peptides to normal cells. (A) Haemolysis effect of peptides on normal blood cells. (B) The cell viability of CHO-K1 cells treated with peptides. Data are representative of triplicate experiments.

Figure 6. pH-dependent membrane disruption. (A) LDH leakage in HeLa cells treated with peptides at different pH values. (B) PI staining images in HeLa cells after peptides treatment at different pH values. (C) Membrane changes of HeLa cells exposed to peptides at different pH values.
the physical mixtures slowly decreased as the number of Glu residues increased. The HC10 values of the physical mixtures were 11.3, 14.3 and 22.6 μM as the number of Glu increased from 2 to 4. While the LK-LEH2 conjugate induced 10% haemolysis at a higher concentration of 131.0 μM, LK-LEH3 and LK-LEH4 displayed no detectable haemolysis even at 150 μM, indicating their markedly decreased haemolytic activity compared with that of LK. In addition, the cytotoxicity of peptides to the normal cell lines CHO-K1 was determined (Figure 5(B)). LK exhibited a high cytotoxic effect and resulted in 76.8%, 48.7% and 20.2% viability at 5, 10 and 20 μM, respectively. The cytotoxicity of the physical mixtures decreased as the Glu content increased compared with that of LK. After the coupling of LK and anionic peptides, all conjugates exerted a significant less cytotoxic effect than LK and physical mixtures did because the cell viability remained above 80% even at 80 μM; thus, the conjugates were safer than LK and physical mixtures. These results indicated that the coupling conjugates of LK and anionic peptides obviously reduced the cytotoxicity of LK to normal cells compared with that of their physical mixtures. Accordingly, LK-LEH2 and LK-LEH3 exhibited an excellent pH response and low cytotoxicity and could be desirable acid-activated ACPs for further studies.

**pH-dependent membrane disruption**

An LDH leakage assay was used to visualise the perturbation of the cell membrane after exposure to different pH values and to gain insights into the killing mechanism of the new conjugates. In Figure 6(A), LK, with an obvious membrane destruction ability [37], significantly released LDH in a concentration-dependent manner, but no significant difference in LDH release at pH 7.4 and pH 6.0 was observed. After treatment with the new conjugates, LK-LEH2 and LK-LEH3 exhibited weak and negligible LDH release at pH 7.4; conversely, LDH release markedly increased with increasing concentration at pH 6.0, indicating that the new conjugates had a more remarkable pH-dependent membrane-destructive activity than LK. This finding was further validated by visualisation with PI staining (Figure 6(B)). PI can pass through damaged cell membranes only to stain nucleic acids and show red fluorescence [37,38]. LK presented strong red fluorescent signals at pH 7.4 and 6.0 and had no pH dependence. LK-LEH2 and LK-LEH3 slightly destroyed the membrane without red fluorescent signals at pH 7.4; conversely, the red fluorescent signals at pH 6.0 were higher than those at pH 7.4. Moreover, SEM was performed to directly visualise the morphologic changes in the cell membrane after the treatment with LK-LEH2 and LK-LEH3 at pH 7.4 and 6.0 (Figure 6(C)). Similar to the untreated control cells, the cells treated with LK-LEH2 and LK-LEH3 at pH 7.4 showed normal cell morphology and intact cell membranes with numerous microvilli; at pH 6.0, the treated cells had irregular shapes with abnormal membrane disruption and loss of microvilli. These findings suggested that LK-LEH2 and LK-LEH3 could induce significant pH-dependent membrane disruption, which was in sharp contrast to the comparably heavy membrane rupture of HeLa cells exposed to LK at both pH values.

**Enzymatic stability**

The stability of LK-LEH2 and LK-LEH3 was evaluated by RP-HPLC. Figure 7 shows that they exhibited resistance to trypsin treatment and were slightly degraded after 6 h of incubation. However, LK showed obvious degradation; approximately 50% of LK remained after 2 h of incubation with trypsin, but it was completely degraded after 6 h. In addition, LK-LEH2 and LK-LEH3 were more remarkably stable in chymotrypsin than LK (Figure 7). Approximately, 40% of LK was degraded after incubation for 6 h, while LK-LEH2 and LK-LEH3 hardly exhibited any degradation even after 6 h of incubation. These data suggested that the coupling of anionic peptides greatly increases the stability of the new conjugates against protease degradation.

**Discussion**

ACPs have emerged as alternative antitumor candidates with many beneficial characteristics [26–28]. However, one of the major obstacles is the lack of tumour specificity, which causes serious side effects [8,29,31]. Thus, peptides with enhanced selectivity will be a desirable template for future tumour treatments. Most ACPs generally contain positively charged amino acids [26–28], which facilitate strong membrane binding and membrane disruption by peptides because of efficient electrostatic attraction; however, their high positive charge contributes to the binding of peptides to normal cells, easily causing severe toxicity. Masking positive charges has been shown to be a good approach to enhance peptide specificity [36–45]. Our group also demonstrated previously that effective shielding the positive charges of ACPs increased their selectivity by altering the histidine distribution of single-chain peptides [37]. In the present study, a new acid-activated ACP was designed by chemical conjugation with different anionic binding partner peptides (LEHs) to the ACP LK. In addition, the effect of the physical mixture at a ratio of 1:1 on the pH response and cytotoxicity of peptides was compared. The study was conducted to investigate an ideal acid-activated peptide for future cancer therapy by optimising the coupled anionic partner peptides.
When the anionic peptides were coupled to cationic LK, the constructed conjugates had noticeably decreased cytotoxicity at normal pH compared with that of LK; this decrease was attributed to the charge shielding of lysines in the key amino acid sites, revealing the importance of the amphipathic interface for a strong membrane interaction. At acidic pH, the histidine residues were protonated, and the glutamate residues in anionic peptides were neutralised, disrupting the association of LEH and LK; thus, LK-LEH2 and LK-LEH3 could effectively kill tumour cells (Figures 3 and 4). Notably, LK-LEH4 lost its antitumor activity compared with those of LK-LEH2 and LK-LEH3. It is speculated that the protonation level of the anionic peptide LEH4, with fewer histidines, was too low to disrupt its binding to LK and prevented peptide dissociation; as a result, antitumor activity was markedly decreased or even lost [37,38]. This observation might also be attributed to the ability of increased amounts of α-helical structures to stabilise and enhance the peptide-peptide interaction of LK-LEH4, which impairs its pH-response [39]. In general, LK-LEH2 and LK-LEH3 showed a remarkable pH-responsive antitumor activity, which was further confirmed by evaluating their antitumor activity at different pH values (pH 7.4, 6.7 and 6.0; Figure S1). Our results suggested that the number of histidines and Glu introduced into the anionic peptides is a critical factor providing the new conjugates with a pH-dependent activity [39-43]. Interestingly, the physical mixture of anionic peptides and LK in a 1:1 ratio could also exhibit a pH-dependent antitumor activity, which was positively correlated with the number of introduced Glu residues. Indeed, charge shielding was necessary to reduce the cytotoxicity of LK. Notably, the introduction of additional Glu residues did not impair the pH sensitivity of the physical mixture, indicating that LK and anionic peptides would be more easily dissociated from the physical mixture than from the chemical conjugates. However, the physical mixture weakly reduced peptide cytotoxicity compared with that of the chemical conjugates; this finding was further supported by their cytotoxicity to normal cells. One possible explanation was that chemical conjugation could establish stronger electrostatic interactions between anionic peptides and cationic LK than the physical mixture; it could also facilitate the shielding of the positive charges of LK, contributing to their obviously low cytotoxicity at normal pH. In addition, the designed anionic peptides were dependent on pH, but their antitumor activity was significantly compromised upon reducing positive charges. Fewer substitutions of His in turn influenced the protonation level of the peptides and reduced the net cationic charge of the peptides at pH 6.0; as a result, the antitumor activity of the peptides was apparently lower than that of LK. When anionic peptides were mixed with LK, the killing activity of the mixture was comparable with that of LK; these findings suggested that anionic peptides mainly endowed LK with pH-sensitive charge-conversion ability and slightly enhanced its antitumor activity. Therefore, charge shielding and efficient conversion played a major role in the pH-responsive antitumor activity of the new peptides.

In addition to net charge, hydrophobicity and α-helical content are correlated with the antitumor potency of the new peptides [26-28]. However, the antitumor activity of LK-LEH4, with higher hydrophobicity and α-helical content, was compromised or even lost but not enhanced, indicating that hydrophobicity and α-helical structure had a lower contribution to the antitumor activity of the new peptides than the net charge. Furthermore, the antitumor activity of the anionic peptides was in the order of LK-LEH2 > LK-LEH3 > LK-LEH4, contrary to the order of the hydrophobicity and α-helical content of these peptides. These findings suggested that net charge is the most critical factor in improving not only peptide selectivity but also peptide antitumor potency.

A major restriction of cationic ACPs as promising candidates for future clinical use is the cytotoxicity of these peptides to mammalian cells, especially red blood cells. Cationicity and hydrophobicity are important structural determinants of the antitumor and toxic effects of ACPs [27-29]. However, excess cationicity and hydrophobicity usually strengthen electrostatic attractions and favour enhanced interactions with zwitterionic phospholipids, inadvertently leading to an increase in peptide cytotoxicity and loss of cell selectivity [9,26-28]. In the present study, although the designed conjugates had higher hydrophobicity than LK, all conjugates were slightly cytotoxic; this result suggested that the masking of positive charges efficiently hampered electrostatic attractions and prevented fewer peptides from binding to zwitterionic phospholipids, concomitantly facilitating the decrease in cytotoxicity. Our research also indicated that conjugate connection was required to achieve excellent pH dependence with minimum toxicity because the conjugation of anionic peptides and LK significantly reduced their haemolytic activity compared with that of the physical mixture that could slightly decrease peptide cytotoxicity.

Trypsin and chymotrypsin are two endogenous human proteases that usually affect the biological activity of peptides by predominantly cleaving peptides at the C-terminal amide bonds of cationic residues and hydrophobic residues, respectively [38,47]. However, in the present study, LK-LEH2 and LK-LEH3 displayed resistance to degradation in the presence of trypsin and chymotrypsin compared with that of LK, which did not effectively prevent proteolysis. The designed conjugates showed resistance possibly because the coupling of LK to the anionic LEH would mask the cleavage sites of LK and make it more difficult for enzyme recognition; thus, this coupling could enhance resistance to enzyme degradation [38,48]. Our observations suggested that LK-LEH2 and LK-LEH3 are promising therapeutic agents against cancer because of their lower cytotoxicity and enhanced enzymatic stability.

Our previous study indicated that membrane disruption is the major mechanism of cell death in response to LK [37]. LK induced remarkable LDH release without pH dependence, confirming that LK had a strong membrane lytic activity. The rapid killing effect induced by LK-LEH2 and LK-LEH3 suggested that they also exhibited the membrane-disruptive activity of LK, as evidenced in subsequent studies. Indeed, LK-LEH2 and LK-LEH3 showed evident pH-dependent LDH release, indicating the pH-dependent membrane disruption caused by the new conjugates. At acidic pH, the reduced LDH release produced by activated LK-LEH2 and LK-LEH3 was consistent with their decreased killing activity compared with that of LK. This result indicated that the attachment of anionic peptides might reduce the membrane interaction and disruption abilities of conjugates, thus exhibiting a decreased antitumor activity after acid activation. Notably, the coupling of LK with anionic peptides helped enhance the selectivity of peptides, which exhibited significantly reduced cytotoxicity to normal cells (more than a 10-fold decrease compared with that of LK). The PI uptake results and more direct SEM observations were consistent with the LDH release results, further confirming that the acid-activated conjugates could kill tumour cells via a membrane disruption mechanism. Due to this unique pH-dependent membrane-lytic mechanism, the new conjugates could overcome multidrug resistance and could kill common multidrug resistant tumour cells [9,49]. Moreover, tumour cells would experience difficulty in
developing resistance to conjugates, thus decreasing the risk of resistance development [8,27,29].

Conclusions

In this study, a new acid-activated ACP was developed by optimising the sequence of anionic peptides (LEHs). The conjugates LK-LEH2 and LK-LEH3 with a moderate Glu content exhibited an excellent pH-responsive antitumor activity and minimum toxicity compared with those of their physical mixtures and other peptides, which makes them promising drugs targeting the acidic tumour microenvironment. Our work provides new information regarding the interaction between LK and LEH and opens a new avenue to design efficient acid-responsive ACP with low toxicity.

Disclosure statement

The authors declare no potential conflict of interest.

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References

[1] Siegel RL, Miller KD, Fuchs HE, et al. Cancer statistics, 2021. CA Cancer J Clin. 2021;71(1):7–33.
[2] Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021;71(3):209–249.
[3] Martínez-Jiménez F, Muiños F, Sentís I, et al. A compendium of mutational cancer driver genes. Nat Rev Cancer. 2020;20(10):555–572.
[4] Chen DS, Mellman I. Elements of cancer immunity and the cancer-immune set point. Nature. 2017;541(7637):321–330.
[5] Gharwan H, Groninger H. Kinase inhibitors and monoclonal antibodies in oncology: clinical implications. Nat Rev Clin Oncol. 2016;13(4):209–227.
[6] Galluzzi L, Humeau J, Buqué A, et al. Immunostimulation with chemotherapy in the era of immune checkpoint inhibitors. Nat Rev Clin Oncol. 2020;17(12):725–741.
[7] Minutolo NG, Hollander EE, Powell DJ. The emergence of universal immune receptor T cell therapy for cancer. Front Oncol. 2019;9:176–190.
[8] Hadianamrei R, Tomeh MA, Brown S, et al. Rationally designed short cationic z-helical peptides with selective anticancer activity. J Colloid Interface Sci. 2022;607(Pt 1):488–501.
[9] Lin LM, Chi JY, Yan YL, et al. Membrane-disruptive peptides/peptidomimetics-based therapeutics: promising systems to combat bacteria and cancer in the drug-resistant era. Acta Pharm Sin B. 2021;11(9):2609–2644.
[10] Yang LP, He J, Tao ZC, et al. GSH-responsive poly-resveratrol based nanoparticles for effective drug delivery and reversing multidrug resistance. Drug Deliv. 2022;29(1):229–237.
[11] Vyasa D, Patel M, Wairkar S. Strategies for active tumor targeting—an update. Eur J Pharmacol. 2022;915:174512–174522.
[12] Deng XT, Song QC, Zhang YR, et al. Tumour microenvironment-responsive nanoplatform based on biodegradable liposome-coated hollow MnO2 for synergistically enhanced chemotherapy and photodynamic therapy. J Drug Target. 2022;30(3):334–347.
[13] Zorko M, Jones S, Langel U. Cell-penetrating peptides in protein mimicry and cancer therapeutics. Adv Drug Deliv Rev. 2022;180:114044–114060.
[14] Shim MK, Yang S, Sun IC, et al. Tumor-activated carrier-free prodrug nanoparticles for targeted cancer immunotherapies: preclinical evidence for safe and effective drug delivery. Adv Drug Deliv Rev. 2022;183:114177–114198.
[15] Hu MY, Huang L. Strategies targeting tumor immune and stromal microenvironment and their clinical relevance. Adv Drug Deliv Rev. 2022;183:114137–114158.
[16] Dutta D, Zhou QH, Mukerabigwi JF, et al. Hypoxia-responsive polypropdug nanocarriers for near-infrared light-boosted photodynamic chemotherapy. Biomacromolecules. 2021;22(11):4857–4870.
[17] Ding MB, Zhang YJ, Li JC, et al. Bioenzyme-based nanomedicines for enhanced cancer therapy. Nano Converg. 2022;9(1):7–26.
[18] Zhang YM, Yang LJ, Yang CH, et al. Recent advances of smart acid-responsive gold nanoparticles in tumor therapy. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2020;12(4):e1619–e1634.
[19] Boedtkjer E, Pedersen SF. The acidic microenvironment as a driver of cancer. Annu Rev Physiol. 2020;82:103–126.
[20] Dharmaratne NU, Kaplan AR, Glazer PM. Targeting the hypoxic and acidic tumor micro-environment with pH-sensitive peptides. Cells. 2021;10(3):541–554.
[21] Svoronas AA, Engelman DM. Pharmacokinetic modeling reveals parameters that govern tumor targeting and delivery by a pH-low insertion peptide (pHLIP). Proc Natl Acad Sci U S A. 2021;118(1):e2016605118–e2016605126.
[22] Nam SH, Jang J, Cheon DH, et al. pH-Activatable cell penetrating peptide dimers for potent delivery of anticancer drug to triple-negative breast cancer. J Control Release. 2021;330:898–906.
[23] Bayram NN, Ulu GT, Topuzoğulları M, et al. HER2-targeted, degradable core cross-linked micelles for specific and dual pH-sensitive DOX release. Macromol Biosci. 2022;22(1):e2100375–e2100391.
[24] Ding GB, Zhu CC, Wang Q, et al. Molecularly engineered tumor acidity-responsive plant toxin gelonin for safe and efficient cancer therapy. Bioact Mater. 2022;18:42–55.
[25] Wei TT, Zhang Y, Lei M, et al. Development of oral curcumin based on pH-responsive transmembrane peptide-cyclodextrin derivative nanoparticles for hepatoma. Carbohydr Polym. 2022;277:118892–118901.
[26] Chiangjong W, Chutipongtanate S, Hongeng S. Anticancer peptide: physicochemical property, functional aspect and trend in clinical application (review). Int J Oncol. 2020;57(3):678–696.
[27] Jafar A, Babajani A, Foroooshani RS, et al. Clinical applications and anticancer effects of antimicrobial peptides: from bench to bedside. Front Oncol. 2022;12:819563–819581.
Liscano Y, Oñate-Garzón J, Delgado JP. Peptides with dual antimicrobial–anticancer activity: strategies to overcome peptide limitations and rational design of anticancer peptides. Molecules. 2020;25(18):4245–4264.

Hadianamrei R, Tomeh MA, Brown S, et al. Correlation between the secondary structure and surface activity of β-sheet forming cationic amphiphilic peptides and their anticancer activity. Colloids Surf B Biointerfaces. 2022;209(Pt 2):112165–112175.

Guo FL, Zhang Y, Dong WB, et al. Effect of hydrophobicity on distinct anticancer mechanism of antimicrobial peptide chensinin-1b and its lipoolanalog PA-C1b in breast cancer cells. Int J Biochem Cell Biol. 2022;143:106156–106165.

Chen XL, Ji SS, Li A, et al. Toggling preassembly with single-site mutation switches the cytotoxic mechanism of cationic amphipathic peptides. J Med Chem. 2020;63(3):1132–1141.

Hu CH, Huang YB, Chen YX. Targeted modification of the cationic anticancer peptide HPRP-A1 with iRGD to improve specificity, penetration, and tumor-tissue accumulation. Mol Pharm. 2019;16(2):561–572.

Lv SX, Sylvestre M, Song KF, et al. Development of D-melittin polymeric nanoparticles for anti-cancer treatment. Biomaterials. 2021;277:121076–121086.

Permpoon U, Khan F, Vadevoo SMP, et al. Inhibition of tumor growth against chemoresistant cholangiocarcinoma by a proapoptotic peptide targeting interleukin-4 receptor. Mol Pharm. 2020;17(11):4077–4088.

Wang AQ, Zheng Y, Zhu WX, et al. Melittin-based nanodelivery systems for cancer therapy. Biomolecules. 2022;12(1):118–135.

Tanishiki N, Yano Y, Matsuzaki K. Endowment of pH responsiveness to anticancer peptides by introducing 2,3-diaminopropionic acid residues. ChemBioChem. 2019;20(16):2109–2117.

Chang LL, Bao HX, Yao J, et al. New designed pH-responsive histidine-rich peptides with antitumor activity. J Drug Target. 2021;29(6):651–659.

Song JJ, Zhang W, Kai M, et al. Design of an acid-activated antimicrobial peptide for tumor therapy. Mol Pharm. 2013;10(8):2934–2941.

Sun CM, Shen WC, Tu JS, et al. Interaction between cell-penetrating peptides and acid-sensitive anionic oligopeptides as a model for the design of targeted drug carriers. Mol Pharm. 2014;11(5):1583–1590.

Zaro JL, Fei L, Shen WC. Recombinant peptide constructs for targeted cell penetrating peptide-mediated delivery. J Control Release. 2012;158(3):357–361.

Fei L, Yap LP, Conti PS, et al. Tumor targeting of a cell penetrating peptide by fusing with a pH-sensitive histidine–glutamate co-oligopeptide. Biomaterials. 2014;35(13):4082–4087.

Jong H, Bonger KM, Löwik DWPM. Activatable cell-penetrating peptides: 15 years of research. RSC Chem Biol. 2020;1(4):192–203.

Yu YL, Zu C, He DS, et al. pH-dependent reversibly activatable cell-penetrating peptides improve the antitumor effect of artemisinin-loaded liposomes. J Colloid Interface Sci. 2021;586:391–403.

Yin J, Liu DK, Bao LC, et al. Tumor targeting and microenvironment-responsive multifunctional fusion protein for pro-apoptotic peptide delivery. Cancer Lett. 2019;452:38–50.

Yang SC, Leong JY, Wang YM, et al. Drug-free neutrally charged polypeptide nanoparticles as anticancer agents. J Control Release. 2022;345:464–474.

Zhang TY, Ouyang X, Gou SH, et al. Novel synovial targeting peptide-sinomenine conjugates as a potential strategy for the treatment of rheumatoid arthritis. Int J Pharm. 2022;617:121628–121639.

Zhu NY, Zhong C, Liu TQ, et al. Newly designed antimicrobial peptides with potent bioactivity and enhanced cell selectivity prevent and reverse rifampin resistance in gram-negative bacteria. Eur J Pharm Sci. 2021;158:105665–105680.

Rodríguez-Alvarez Y, Cabrales-Rico A, Diago-Abreu D, et al. D-amino acid substitutions and dimerization increase the biological activity and stability of an IL-15 antagonist peptide. J Pept Sci. 2021;27(3):e3293–e3305.

Ahmed S, Khan H, Fakhri S, et al. Therapeutic potential of marine peptides in cervical and ovarian cancers. Mol Cell Biochem. 2022;477(2):605–619.