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One New Acid-Activated Hybrid Anticancer Peptide by Coupling with a Desirable pH-Sensitive Anionic Partner Peptide

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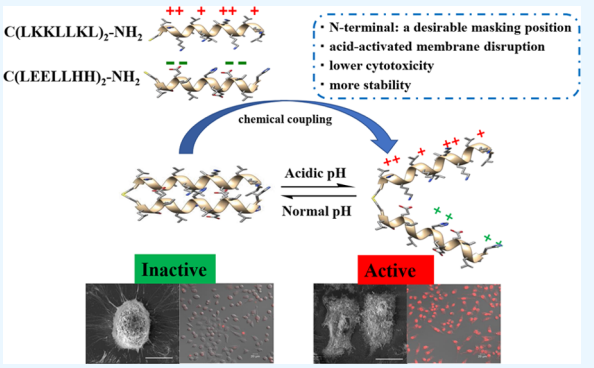
ABSTRACT: Anticancer peptides (ACPs) are promising antitumor resources, and developing acid-activated ACPs as more effective and selective antitumor drugs would represent new progress in cancer therapy. In this study, we designed a new class of acid-activated hybrid peptides LK-LE by altering the charge shielding position of the anionic binding partner LE based on the cationic ACP LK and investigated their pH response, cytotoxic activity, and serum stability, in hoping to achieve a desirable acid-activatable ACP. As expected, the obtained hybrid peptides could be activated and exhibit a remarkable antitumor activity by rapid membrane disruption at acidic pH, whereas its killing activity could be alleviated at normal pH, showing a significant pH response compared with LK. Importantly, this study found that the peptide LK-LE3 with the charge shielding in the N-terminal of LK displayed notably low cytotoxicity and more stability, demonstrating that the position of charge masking is extremely important for the improvement of peptide toxicity and stability. In short, our work opens a new avenue to design promising acid-activated ACPs as potential targeting agents for cancer treatment.



INTRODUCTION

In spite of tremendous progresses in cancer therapy, cancer remains a major public health problem worldwide, as the insufficient selectivity and multidrug resistance mostly produce adverse side effects and show resistance to almost available anticancer drugs.[1−3] Thus, it is more pressing to design and develop alternative anticancer agents with desirable therapeutic potential. Various targeting strategies have been applied to increase the therapeutic index of antitumor drugs by utilizing specific cell surface markers.[4,5] However, these targeting abilities have been compromised because of tumor heterogeneity.[6,7] It is known that the microenvironment around most tumors is more acidic compared with normal tissues due to the hypoxia and the abnormal tumor metabolic process.[8,9] The common acidic pH has been regarded as a desirable trigger to devise new pH-targeting strategies for tumor-specific therapy or drug delivery.[10−12]

Anticancer peptides (ACPs) have been introduced as alternative candidates to treat cancer with a certain degree of cancer cell selectivity, low tendency to drug resistance, good biocompatibility, and ease of synthesizing and modifying.[13−15] Generally, they are relatively small peptides with variable cationic and amphipathic characteristics. They exhibit broad-spectrum antitumor activity through multiple action mechanisms.[16−18] One prominent merit of ACPs is that these peptides could selectively disrupt the tumor cell membrane in a receptor-independent manner, which is different from that of conventional antineoplastic agents.[19,20] Despite this, the drawbacks of anticancer peptides, such as the toxicity at high concentrations and fast degradation in vivo, are still the main concerns for drug development.[14,15] To circumvent these limitations, many different strategies have been used to improve the therapeutic efficiency of ACPs, including amino acid substitution,[21−23] fatty acid modification,[24,25] glycosylation,[25,26] backbone conjugation or cyclization,[27,28] and utilization of functionalized carriers.[29,30] Histidine modification is a simple and effective approach to develop smart pH-responsive ACPs with better selectivity for cancer therapy based on the tumor acidic environment,[31] as histidine could protonate into a positive charge under the acidic tumor microenvironment from predominantly no charge in tissues with normal physiological conditions, thereby endowing peptides with a pH-activated charge conversion feature.[31,32] Hence, this type of histidine-based ACPs is inactivated under physiological conditions, while their antitumor activity can be

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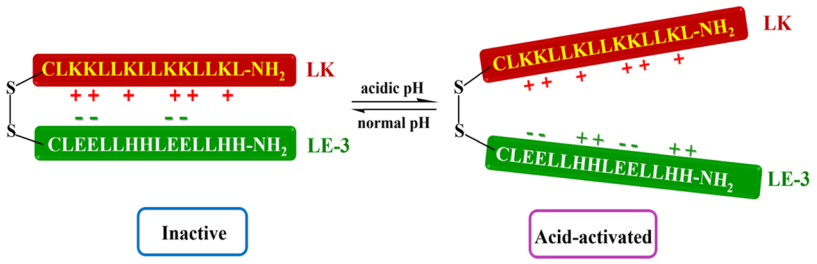

Figure 1. Structural rationale for the pH-activatable α-helical hybrid peptide LK-LE3.

Table 1. Properties of LK and Newly Designed Peptides[a,b,c,d]

Peptide	Sequence	Molecular mass		Purity (%)[b]	t_R[c] (min)	Net charge	
		Calculated	Measured[a]			pH 7.4	pH 6.0
LK	LKKLLKLLKKLLKL-NH_2	1691.35	1691.32	97.32	15.73	+7	+7
LE-1	LHELLEHLHELLEH-NH_2	1761.01	1760.96	98.55	14.36	-3	+1
LE-2	LEHLLEHLEHLLEH-NH_2	1761.01	1760.95	98.55	15.11	-3	+1
LE-3	LEELLHHLEELLHH-NH_2	1761.01	1760.95	99.51	14.99	-3	+1
LK-LE-1	s-CLKKLLKLLKKLLKL-NH_2 | s-CLHELLEHLHELLEH-NH_2	3654.21	3655.32	100.00	18.73	+4	+8
LK-LE-2	s-CLKKLLKLLKKLLKL-NH_2 | s-CLEHLLEHLEHLLEH-NH_2	3654.21	3655.21	100.00	19.16	+4	+8
LK-LE-3	s-CLKKLLKLLKKLLKL-NH_2 | s-CLEELLHHLEELLHH-NH_2	3654.21	3655.22	100.00	19.23	+4	+8

[a]Measured: molecular mass of peptides was measured by ESI-MS. [b]Purity of peptides was determined by RP-HPLC analysis. [c]t_R: retention time (t_R) on analytical RP-HPLC. [d]Notes: the C-terminal was amidated in each peptide.

activated in an acidified tumor microenvironment. Notably, the combination of unique histidine with negatively charged glutamate (Glu) is also a promising approach to obtaining acid-responsive recombinant peptides.[33,34] The cationic charge of these peptides can be first shielded by anionic glutamate under normal physiological conditions to alleviate their indiscriminative effect. After exposure to an acidic tumor microenvironment, their activity can be efficiently activated because the negative charge of Glu residues is neutralized by protonated histidine.

LK (LKKLLKLLKKLLKL-NH_2), a cationic amphiphilic ACP containing two repetitive amino acid sequences of LKKLLKL, shows a typical α-helical structure upon membrane binding.[35] LK has been reported to exert a potent antitumor activity by rapid membrane disruption.[36,37] However, the low selectivity and poor serum stability severely hinder its application in biomedicine. To improve the therapeutic prospect of LK, a series of histidine-based analogues and conjugates have been previously designed and synthesized by histidine substitution and backbone conjugation.[36,37] Our results showed that the new pH-responsive histidine-based peptides could effectively increase the selectivity of LK and reduce its cytotoxic effect. In addition, conjugating LK with anionic binding peptide LEHs not only exhibited low cytotoxicity but also enhanced peptide stability.[37] In our previous study, we observed an interesting phenomenon: in the acid-activated conjugated peptide, when anionic peptide LEH contains the same number of histidine and glutamate, the conjugates displayed superior pH-responsive antitumor activity and low toxicity.[37] To facilitate the therapeutic application of acid-activated conjugate, it is essential to further explore the desirable substitution sites of histidine/glutamate, as we supposed that the charge shielding position in the conjugate might be a crucial factor in the pH-responsive ACP optimization. To the best of our knowledge, there are comparatively few studies to elucidate the effects of the charge shielding position on the pH response, toxicity, and enzymatic stability of the acid-activated conjugate.

According to our previous study, a new anionic binding peptide LE was designed by introducing histidine/glutamate to different sites in LK. After covalently coupling to LK via the same disulfide linker, we investigate their physicochemical properties, pH-responsive antitumor activity, toxicity, and enzymatic stability to explore the position effect of histidine/glutamate modification on the performance of acid-activated hybrid peptide.

RESULTS

Peptide Structures and Characteristics. In this work, novel acid-activated anticancer peptides were formed by

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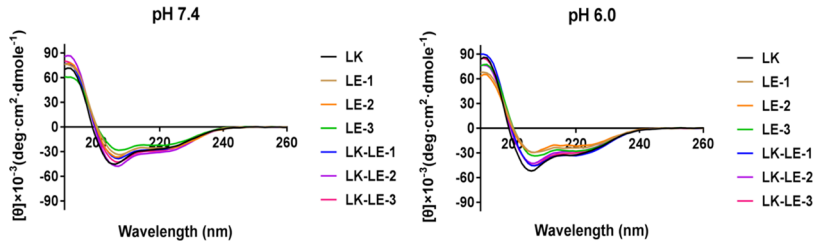

Figure 2. CD spectra of the newly designed peptides in a membrane-mimicking environment (50% TFE/water) at different pH values. All of the peptides were dissolved in 50% TFE/water (V/V) solution adjusted to pH 7.4 or 6.0 at a concentration of 50 μM.

Table 2. IC50 Values (μM) of the Peptides at Physiologic and Acidic pH Values Detected by the MTT Assay[a]

	HeLa cells		RG cells		4T1 cells		HK-2 cells	
peptide	pH 7.4	pH 6.0	pH 7.4	pH 6.0	pH 7.4	pH 6.0	pH 7.4	pH 6.0
LK	10.3	11.7	10.5	11.3	11.2	12.1	8.6	9.0
LE1	>100	>100	>100	>100	>100	>100	>100	>100
LE2	>100	>100	>100	>100	>100	>100	>100	>100
LE3	>100	>100	>100	>100	>100	>100	>100	>100
LK + LE1	11.9	14.1	9.5	10.1	12.3	14.1	14.0	13.4
LK + LE2	11.5	12.8	10.9	10.3	12.5	13.8	14.7	13.2
LK + LE3	12.0	12.6	9.3	10.6	12.9	13.9	11.2	12.5
LK-LE1	>100	11.3	>100	12.6	>100	13.1	>100	11.7
LK-LE2	>100	12.9	>100	13.4	>100	14.0	>100	12.2
LK-LE3	>100	10.2	>100	13.6	>100	15.3	>100	13.0

[a]Note: results are the mean of three independent experiments, each performed in duplicate.

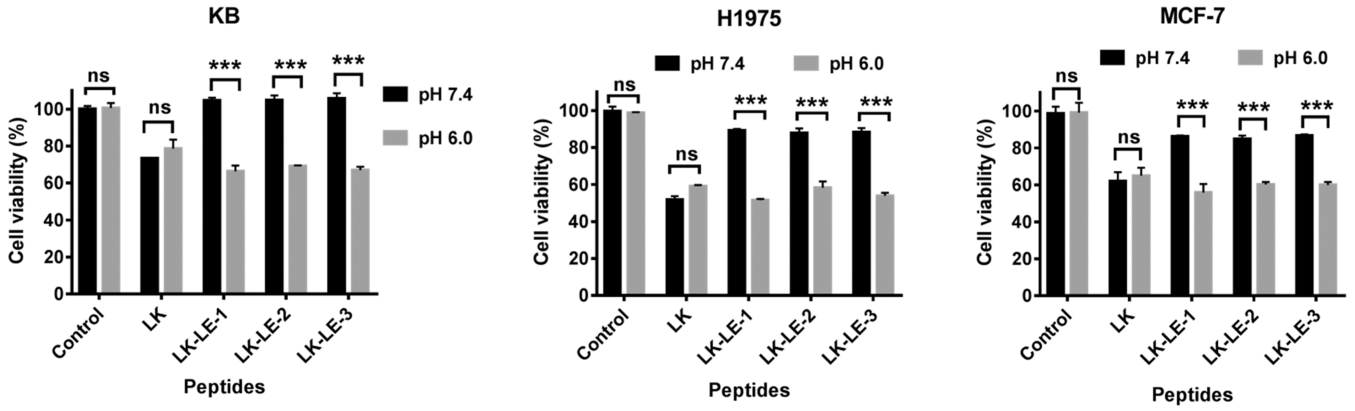

Figure 3. pH-dependent antitumor activity of peptides to different tumor cells at 10 μM. KB, H1975, and MCF-7 cells were treated with LK or hybrid peptides at pH values 7.4 and 6.0 for 30 min and then measured by the MTT assay. Results are representative of triplicate experiments. ***p < 0.001; ns indicates no significant difference.

conjugation of LK and anionic binding partner peptides (LEs) via a disulfide linker (Figure 1). We replaced all lysines and two leucines in LK with four histidines and four glutamates while keeping the C-terminal amidation of parent peptide LK constant to produce the anionic peptides LEs. The anionic peptides containing the same numbers of Glu and His would favorably reach a desirable shielding effect according to our previous report. Here, three anionic peptides containing Glu at different sites were designed to evaluate the influence of the positions of charge shielding on the pH-responsive activity, toxicity, and enzymatic stability of new peptides. The sequences, predicted 3D structures and properties of newly designed peptides, are summarized in Table 1 and Figure S1, respectively. According to structure prediction (Figure S1), each peptide presented an α-helical conformation. The hydrophobicity of peptides was evaluated by retention time measurement. The anionic peptides showed slightly decreased hydrophobicity compared with LK, but the hydrophobicity of three anionic peptides was not significantly changed when Glu was introduced to different sites. After coupling to LK, the hydrophobicity of the three conjugates was higher than that of LK, and no obvious difference was observed among the three conjugates. Moreover, the secondary structures of peptides were assessed in a membrane-mimicking environment (50% TFE/water) by using CD spectroscopy at pH 7.4 or 6.0. As shown in Figure 2, all peptides exhibited a typical α-helical structure at different pH values. The introduction of Glu slightly reduced the helical content of the anionic peptides. When the anionic peptides were coupled to LK, the helical content of the conjugates was comparable to that of LK and there was no significant discrepancy in the helical content of conjugates at different pH conditions compared to LK.

pH-Dependent Antitumor Activity *In Vitro*. The killing efficacy of peptides against tumor cells (HeLa cells, RG cells,

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and 4T1 cells) was examined at different pH values by an MTT assay. As shown in Table 2, LK showed significant cytotoxicity to tumor cells at both pH values 7.4 and 6.0, and similar IC_{50} values could be observed at both pH values. Three anionic peptides exhibited no cytotoxicity against tumor cells at different pH values. After the conjugation of LK with anionic peptides, the conjugates LK-LE1, LK-LE2, and LK-LE3 exhibited markedly decreased cytotoxicity at pH 7.4, whereas they exhibit a pH-dependent killing activity to three tumor cells. And their pH-dependent killing activity had no noticeable disparity as shown in Table 2, suggesting that the Glu substitution position plays a negligible role in the pH response of these conjugates. However, the physical mixture of each anionic peptide LE and cationic LK in a 1:1 ratio showed no obvious pH-dependent cytotoxicity to tumor cells compared with chemical conjugates LK-LE, and their killing effect on tumor cells is comparable to that of LK because similar IC_{50} values were observed after different treatments. This result suggested that the conjugate connection plays a crucial role in the pH-dependent antitumor activity of LK-LE. As shown in Table 2, after acid activation, hybrid peptides also exhibited toxicity against normal cells (HK-2) at acidic pH. However, the IC_{50} values of three hybrid peptides under pH 7.4 were remarkably higher relative to pH 6.0 when compared with those of LK and the physical mixtures, consistent with the significantly decreased cytotoxicity of hybrid peptides after 24 h of incubation measured by the MTT assay (Figure S3). Similar results were also shown in the previously reported acid-activated peptide.[31]

The killing activity of hybrid peptides to other tumor cells (KB cells, H1975 cells, and MCF-7 cells) at pH values 7.4 and 6.0 shown in Figure 3 indicated that LK-LE1/2/3 also exhibited a prominent pH-dependent antitumor activity compared to LK. In addition, to better study the pH-responsive profile of peptide, we further evaluated the cytotoxicity of LK and three hybrid peptides at 10 μM to HeLa cells at different pH conditions (pH values 7.4, 7.0, 6.5, 6.0, and 5.5). The results presented in Figure 4 revealed that hybrid peptides LK-LE1/2/3 could effectively kill tumor cells in a significant pH-dependent manner compared with LK, further confirming the marked pH-responsive killing activity of three hybrid peptides shown in Table 2 and Figure 3, and their acid-responsive profiles were consistent with that of earlier reported pH-dependent peptides.[32,36] Taken together, these data demonstrated that the coupling of the designed anionic peptides LE and LK could endow a hybrid peptide with an obviously pH-dependent antitumor activity. Moreover, changing the positions of Glu modification has no great influence on the pH response of these acid-activated hybrid peptides.

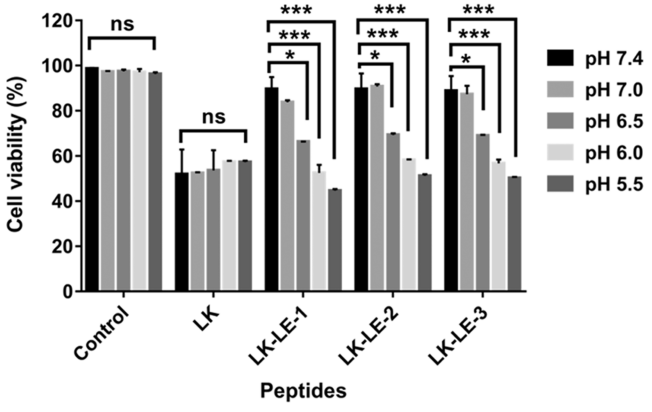

Figure 4. pH-dependent antitumor activity of peptides at different pH values. HeLa cells were treated with 10 μM of peptides at pH values 7.4, 7.0, 6.5, 6.0, and 5.5 for 30 min and then measured by the MTT assay. Results are representative of triplicate experiments. $^{*}p < 0.05$ and $^{***}p < 0.001$; ns indicates no significant difference.

pH-Dependent Membrane Disruption Mechanism. The PI uptake assay was used to investigate the membrane-disruptive effect of the newly designed peptides. PI is a membrane-impermeable fluorescent dye that can penetrate the damaged cell membrane and stain nucleic acids to excite red fluorescence. The PI uptake in HeLa cells was studied after peptide treatment at different pH values to evaluate the direct disturbance of the membrane. As shown in Figure 5, compared with the control cells without significant red PI fluorescence, the cells treated with LK presented obvious red fluorescence at both pH values 7.4 and 6.0 and the PI uptake had no significant differences at both pH values. In contrast, when the cells were exposed to three hybrid peptides LK-LE1/2/3, weak red fluorescence was detected in HeLa cells at pH 7.4, whereas the PI red fluorescence at pH 6.0 presented apparently higher than that at pH 7.4.

To further evaluate the pH-dependent membrane-lytic activity of hybrid peptides, SEM imaging was performed to observe the morphological change of the cell membrane after peptide incubation at pH values 7.4 and 6.0 (Figure 6). Similar to the untreated control cells, most of the cells displayed an intact cell shape and smooth surface with numerous microvilli after exposure to LK-LE1/2/3 at pH 7.4, while dramatic membrane damage with severe depression and loss of microvilli was found in HeLa cells following peptide treatment at pH 6.0. However, when cells were treated with LK, abnormal cell membrane rupture was observed at both pH values. This SEM result was consensus to the results derived from the PI uptake assay. Our results further confirmed that hybrid peptides LK-LE1/2/3 exerted a significant pH-dependent membrane-lytic activity compared with LK and they could efficiently kill tumor cells at acidic microenvironment by the rapid membrane-disruptive effect.

Hemolytic Cytotoxicity. The hemolytic activity of the peptides was determined to evaluate their cytotoxicity to normal red blood cells (RBCs) by measuring the hemoglobin release. As shown in Figure 7, LK induced significant hemolysis in a dose-dependent manner and relatively higher erythrocyte hemolysis (71.1%) was observed at the highest concentration. Three anionic peptides displayed negligible hemolytic toxicity even at the highest concentration of 200 μM. The hemolytic toxicity of the new hybrid peptides LK-LE1, LK-LE2, and LK-LE3 was significantly decreased relative to LK, for they only induced approximately 22.2, 13.8, and 11.3% hemolysis at 200 μM, respectively. And the hemolytic concentrations of 10% (HC_{10} values) for LK-LE1, LK-LE2, and LK-LE3 were around 85.5, 148.2, and 172.0 μM, respectively, and their HC_{10} values were individually more than 7.0, 12.1, and 14.0 times higher than that of LK (12.3 μM). It is suggested that the new hybrid peptide LK-LE3 exhibited an obviously reduced hemolytic cytotoxicity to normal red cells compared with other hybrid peptides. This result implied that charge shielding in the N-terminal of LK did facilitate the decrease of peptide cytotoxicity.

Peptide Stability. The serum stability of new hybrid peptides was evaluated by using HPLC analysis. Figure S2

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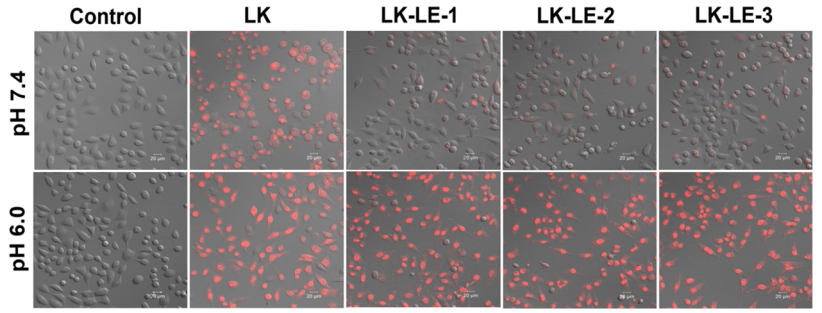

Figure 5. PI uptake in HeLa cells after treatment with LK or hybrid peptides at 10 μM for 30 min at different pH values. Scale bar is 20 μm.

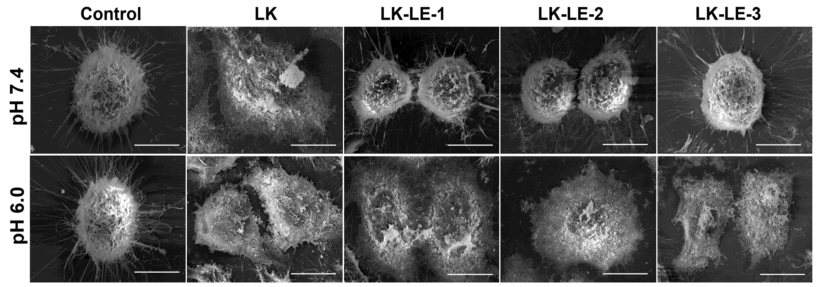

Figure 6. Membrane changes of HeLa cells treated with LK or hybrid peptides at 10 μM for 30 min at different pH values. Scale bar is 10 μm.

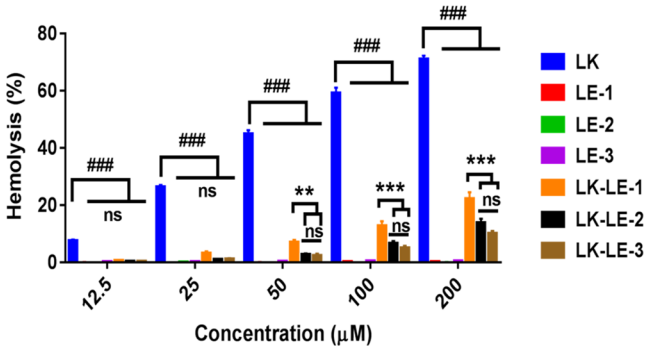

Figure 7. Hemolytic toxicity of LK and hybrid peptides toward red blood cells at various concentrations. Results are representative of triplicate experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{###}p < 0.001$; ns indicates no significant difference.

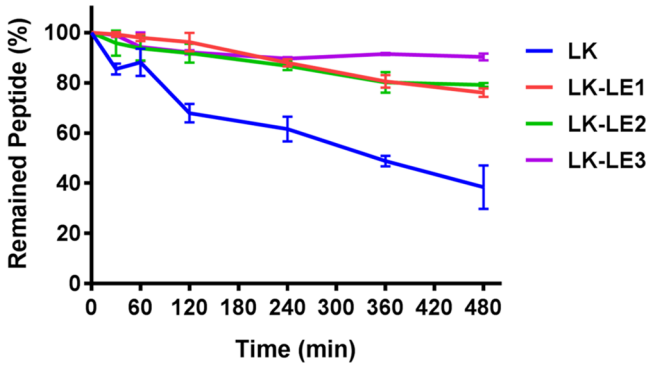

Figure 8. Serum stability of LK and hybrid peptides after incubation with serum at different times by RP-HPLC. Results are representative of triplicate experiments.

shows the degradation chromatogram of LK and three hybrid peptides incubated with serum. LK exhibited significant degradation in a time-dependent manner: about 38.5% of LK remained after incubation with serum for 480 min. In contrast, three hybrid peptides LK-LE1, LK-LE2, and LK-LE3 showed apparent resistance to degradation by serum with approximately 76.1, 79.2, and 90.4% of the peptide remaining after 480 min of incubation, respectively (Figure 8). Moreover, the serum stability of LK-LE1, LK-LE2, and LK-LE3 was 2.0-fold, 2.8-fold, and 7.1-fold compared to that of LK, respectively, demonstrating that the introduction of anionic peptide LE could efficiently enhance the resistance of the new peptides to protease degradation. Notably, the coupling of LE3 with Glu in sites 2 and 3 has an outstanding effect on improving the peptide stability.

DISCUSSION

ACPs have gained more and more focus due to their desirable therapeutic characteristics. Nevertheless, the clinical utilization of ACPs is greatly hindered by low selectivity, high systemic toxicity, and poor stability.[13−15] Therefore, it is extremely essential to develop effective methods to overcome current problems. Ingeniously masking positive charges of ACPs is a promising strategy to enhance selectivity and reduce toxicity.[31] Some acid-activated anticancer peptides or recombinant peptides have been developed using the protonation of histidine in an acidic environment, like substituting lysines/arginines for histidines or fusing with specific binding peptides.[32−34,38,39] Previously, we have also obtained a series of pH-responsive anticancer peptides by histidine substitutions and introduction of anionic binding peptides based on the short cationic α-helical ACP LK.[36,37] In addition, we found that the coupling of anionic binding peptides to cationic LK could play a major role in improving peptide stability. Thus, the anionic peptide fusion strategy might be a good approach to obtain the pH selectivity concomitant with enhancing the stability of ACPs. However, until now, it has not been reported whether the changing of the charge shielding position in anionic peptides produces an effect on pH response, toxicity, and stability of acid-activated ACP. Therefore, in this study, a series of new anionic binding peptides were designed to study

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the position effect of histidine/glutamate modification on the performance of acid-activated hybrid peptides.

Our earlier reports demonstrated that the anionic binding partner peptide containing four glutamate residues could largely block the lytic activity of LK at normal pH.[37] Moreover, the anionic peptides containing the same numbers of Glu and His would favorably reach a desirable acid-activated effect. Here, four glutamic acids were introduced to the anionic binding peptide by replacing two lysines in each repetitive unit (LKKLLKL) of LK. Furthermore, additional histidine introduction was essential to ensure the efficient dissociation of LK from LE and exhibit an acid-activated profile at acidic conditions. Therefore, apart from the substitution of the remaining single lysine in the repetitive unit with histidine, anionic peptide LE was generated by further replacing leucine in the C-terminal of each repetitive unit with histidine to increase the positive charges of LE at acidic pH and avoid excessive electrostatic attraction between LE and LK. The reason for introducing histidine at the C-terminal position of each repetitive unit is that the amphipathic interface positions are crucial for the membrane interaction of the α-helical peptide LK.[36] The replacement of the leucine in LK to histidine could weaken the membrane interaction of anionic peptide LE and avoid the increase of peptide cytotoxicity after the conjugation of LE to LK. Therefore, new acid-activated hybrid peptides were constructed by the coupling of LK and anionic binding peptide containing four His and four Glu (Table 1). In this study, we obtained three anionic binding peptides by changing charge distribution and adopted the same disulfide linker to investigate the position effect of histidine/glutamate modification on the pH response, toxicity, and enzymatic stability of acid-activated hybrid peptides.

Based on the previous study,[27,37,40] new hybrid peptides were obtained by coupling the anionic peptides LE to LK via the same disulfide linker and the physical mixture was generated by mixing LK with LE in a 1:1 ratio. Our results indicated that LK displayed a potent antitumor activity but no selectivity at pH values 7.4 and 6.0. The anionic peptides LE1, LE2, and LE3 had no cytotoxicity at both pH values. However, when the anionic peptides were conjugated with LK via a disulfide bond, the hybrid peptides LK-LE1, LK-LE2, and LK-LE3 with a disulfide bond exhibited a marked pH-responsive antitumor activity compared to LK (Figures 3 and 4). At normal pH, the cytotoxicity of LK was noticeably alleviated because the cationic charges of LK were shielded by the coupling of anionic peptides. In contrast, the hybrid peptides recovered their antitumor activity and effectively killed tumor cells in an acidic environment. The acid-activated ability was attributed to the protonation of histidines.[32−34] At acidic conditions, the protonated histidine can neutralize the negative charge of glutamates in anionic peptides, thereby disrupting the association of LE and LK and activating the antitumor activity of LK. The results showed that three hybrid peptides had no significant difference in the pH-dependent antitumor activity. This similarity might result from their considerable positive charges, hydrophobicity, and helical contents (Table 1), which are the three vital factors for peptide activity.[14−19] This observation might also imply that the modification position of glutamate residues has negligible influence on the acid-activated antitumor activity of hybrid peptides. In addition, we found that the mixture of LK and LE in a 1:1 ratio did not show pH-dependent antitumor activity and their killing effect was similar to that of LK at pH values 7.4 and 6.0, suggesting that the physical mixture of anionic peptides and LK was too insufficient to shield the positive charges of LK and prevent its cytotoxicity at normal conditions compared with their coupling effect. One possible explanation was that a conjugate connection via disulfide linker could establish stronger electrostatic interactions between cationic LK and anionic peptides than the physical mixture; it could also contribute to the efficient shielding of the positive charges of LK, resulting in their remarkably reduced cytotoxicity at normal pH.[40] The results highlighted the importance of the chemical conjugation for pH-dependent cytotoxicity of LK-LE compared to the physical mixture, which is also consistent with the earlier reported acid-activated peptides.[40]

Rapid membrane disruption is the primary antitumor mechanism of LK, just like many cationic ACPs.[36,37] The cationic LK can bind to anionic cell membranes via the electrostatic interaction and induce cell death by lysing the cell membrane. In this study, when cationic LK was coupled with anionic LE, the membrane-lytic activity of LK was compromised at normal pH because the killing activity of LK is alleviated by the anionic LE, whereas at acidic pH, LK-LE1/2/3 would recover the rapid membrane-lytic activity due to the dissociation of LK from LE. Indeed, three hybrid peptides exhibited evident pH-dependent PI uptake, indicating the pH-dependent membrane-disruptive activity caused by the new peptides. However, LK induced significant PI uptake without pH dependence and showed a similar membrane disruption at both pH values. Drastic changes in membrane morphology visualized by SEM further directly confirmed that the designed hybrid peptides could kill the tumor cells by pH-dependent membrane destruction, which was consistent with their rapid killing activity. Many studies demonstrated that the rapid membrane-lytic activity would endow ACP a great potential to battle multidrug resistance in cancer,[41,42] because they could kill common multidrug-resistant tumor cells with low susceptibility to resistance development compared to traditional chemotherapeutics.

Generally, high cationicity and hydrophobicity of ACPs could increase their antitumor activity;[14−19] however, they always cause high cytotoxicity to normal cells due to the nonselective membrane binding and disruption, which hampers their use in cancer therapy. In the present study, LK showed significant hemolytic toxicity, which is closely associated with excess cationicity and potent hydrophobicity. After the introduction of anionic LE, three hybrid peptides showed higher hydrophobicity than LK, but the hemolytic toxicity of the new peptides was obviously diminished compared with that of LK. This may be attributed to the shielding of the positive charges of LK by the anionic peptide at normal pH. After LK was combined with anionic LE1/2/3, its positive charges could be efficiently shielded and fewer LKs bind with zwitterionic phospholipids because of the weak electrostatic attraction, resulting in remarkably reduced toxicity. This finding revealed that the masking of positive charge is the most key factor in decreasing peptide cytotoxicity. Here, the hemolytic toxicity of three hybrid peptides was in the order of LK-LE3 < LK-LE2 < LK-LE1, which indicates that the N-terminal of LK is a desirable masking position to reduce the hemolytic toxicity of LK. One possible explanation is that the positive Lys located in the N-terminal of LK may promote a more potent membrane interaction of LK. When LE3 was combined with LK, the charge shielding of lysines at the key positions 2 and 3 could efficiently weaken membrane binding

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and disruption of LK, leading to much lower hemolytic toxicity than LK-LE2 and LK-LE1.

Previous reports have shown that ACPs usually lost their activity due to enzymatic instability, which is one primary obstacle for ACP to be developed as anticancer drugs.[14,15,43] To increase the peptide stability, many effective strategies have been used in the ACP designs, such as the incorporation of unusual amino acids,[21−23] backbone cyclization,[27,28] and modification of polymer.[29,30] In this study, our results suggested that the coupling of anionic peptide LE to LK via a disulfide linker could bestow a new peptide with high enzymatic stability to resist proteolysis compared to LK. The increase in α-helix generally stabilizes the structure of the peptide and enhances resistance to enzyme degradation.[37] However, in this study, the helical content of new hybrid peptides had no remarkable change relative to LK, which suggested that the α-helical structure had little influence on peptide stability. The improved stability might be because the coupling combination of LK and anionic LE masks the cleavage sites of LK and hinders more enzyme recognition, thus contributing to the increase of peptide stability.[37,40] Interestingly, we found that conjugate LK-LE3 exhibited more stability than LK-LE1 and LK-LE2, indicating that the charge shielding of lysines at sites 2 and 3 was more conducive to improving the peptide stability than other sites. Therefore, our results demonstrated that the N-terminal of LK is an alternative modification position to strengthen the enzymatic stability.

CONCLUSIONS

In conclusion, we designed a novel kind of acid-activated ACP by optimizing the charge masking position of the hybrid peptides LK-LE. We showed here that three hybrid peptides exhibited a considerable pH-responsive antitumor activity and they could preferentially kill tumor cells in acidic conditions by rapid and drastic membrane disruption. Gratifyingly, LK-LE3 with the charge masking in N-terminal stood out from other hybrid peptides as it had minimum toxicity concomitant with enhancing the stability. The excellent performance makes LK-LE3 a promising drug targeting tumor acidic microenvironment. The present work is preliminary work; further studies on optimizations and targeting applications of this type of acid-activated hybrid peptides are underway in our laboratory. Nevertheless, our work provides a new avenue to develop new acid-activated ACPs with optimum therapeutic potential for future tumor therapy.

EXPERIMENTAL SECTION

Materials. Human cervical cancer cell line (HeLa), human hepatoma cell line (RG), mouse breast cancer cell line (4T1), human proximal tubular epithelial cell line (HK-2), human non-small cell lung adenocarcinoma cell line (H1975), human oral epidermoid carcinoma cell line (KB), and human breast cancer cell line (MCF-7) were obtained from the Laboratory Center for Medical Science of Lanzhou University. Dulbecco's modified Eagle's medium (DMEM), RPMI-1640, and fetal bovine serum (FBS) were purchased from Gibco. Neonatal bovine serum (NBS) was obtained from Sijiqing Biotech (Hangzhou, China).

All Fmoc-protected amino acids, 1-hydroxybenzotriazole (HOBT), O-benzotriazole-*N*,*N*,*N*′, and *N*′-tetramethyl-uronium-hexafluoro-phosphate (HBTU), were purchased from GL Biochem (Shanghai, China). Rink amide MBHA resin was obtained from Hecheng (China). Triisopropylsilane (TIS), *N*,*N*-diisopropylethylamine (DIEA), and trifluoroacetic acid (TFA) were supplied by J&K (China). 1,2-Ethanedithiol and 2,2′-dithiodipyridine were purchased from Energy Chemical. Propidium iodide (PI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were ordered from Sigma-Aldrich. Triton X-100 was obtained from Solarbio (China).

Peptide Synthesis. All peptides were synthesized by using the standard Fmoc solid-phase synthesis strategy on MBHA resins. The conjugated peptides LK-LE were synthesized according to our previous method.[37] The crude peptides were purified and identified by reversed-phase high-performance liquid chromatography (RP-HPLC) and electrospray ionization mass spectrometry (ESI-MS), respectively. The purity of the peptides was above 95% confirmed by HPLC.

Cell Cultures. HeLa cells were cultured in RPMI-1640 (Gibco BRL) supplemented with 10% neonatal bovine serum (NBS) (Sijiqing Biotech, Hangzhou, China). 4T1, H1975, and KB cells were incubated in RPMI-1640 (Gibco BRL) containing 10% fetal bovine serum (Gibco BRL). RG, HK-2, and MCF-7 cells were grown in DMEM (Gibco BRL) containing 10% fetal bovine serum (Gibco BRL). All cell lines were maintained in a 5% CO_2-humidified atmosphere at 37 °C.

Circular Dichroism Analysis. The CD spectra of all peptides were determined using a JASCO J-810 CD spectrophotometer. Peptides were dissolved in 50% trifluoroethanol solution adjusted to pH 7.4 or pH 6.0 to a concentration of 50 μM. The CD spectra were measured from 190 to 260 nm. The relative helicity of each peptide was estimated by using a previous method.[40]

Cytotoxicity Assays. Cells were seeded onto 96-well plates at a density of 1×10^4 cells/well and then cultured for 24 h. The original medium was replaced with medium adjusted to pH 7.4, 7.0, 6.5, 6.0, or 5.5 containing different peptides at various concentrations. After 0.5 h of incubation, 10 μL of MTT (5 mg/mL) was added to each well, followed by further incubation for 4 h. Subsequently, the supernatant was removed, and 150 μL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the precipitated formazan. The control cells were treated with medium adjusted to relevant pH values without peptides. The absorbance was measured at 490 nm by a microplate reader. The cell viability of each group was calculated using the following formula

$$\text{cell viability}(\%) = (A_{\text{sample}} - A_{\text{blank}})/(A_{\text{control}} - A_{\text{blank}}) \times 100\%$$

where A_{control} represents the absorbance of the control group exposed to the medium adjusted to the respective pH values without peptides, A_{sample} is the absorbance of the peptide-treated group exposed to the medium adjusted to the parallel pH values containing various concentrations of peptides, and A_{blank} indicates the absorbance of the blank group.[33]

Propidium Iodide (PI) Uptake Assays. Hela cells were seeded into a culture dish at a density of 6×10^4 cells/dish and cultured for 24 h. The cells were treated with medium adjusted to pH 7.4 or pH 6.0 containing 10 μM of peptides for 30 min. Then, the cells were washed with phosphate-buffered saline (PBS) gently and incubated with PI solution (50 μg/mL) for

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10 min in the dark. The PI uptake was analyzed on a laser confocal scanning microscope.

Scanning Electron Microscope (SEM). HeLa cells (2×10^5 cells/well) were grown on coverslips under the bottom of the 24-well plate and incubated for 24 h. After treatment with medium containing 10 μM of peptides at pH 7.4 or pH 6.0 for 30 min, cells were washed with PBS twice and fixed with 2.5% glutaraldehyde solution overnight, followed by postfixation with 1% osmium tetroxide for 2 h. After further dehydration in an ascending ethanol series, the cells were dried in a critical point dryer and then coated with gold plating. The samples were imaged with a scanning electron microscope (JSM-6380Lv).

Hemolysis Assays. The hemolytic activity of the peptides was evaluated by monitoring the release of hemoglobin from mouse erythrocytes. All mice were obtained from the Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Lanzhou, China). All mice experiments have strictly followed the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. In addition, the official procedure was approved by the Ethics Committee of Lanzhou University (Permit Number: SYXK Gan 2018-0002). Briefly, the erythrocytes were obtained by centrifugation of fresh mouse blood at 1000g for 10 min at 4 °C. The erythrocytes were then washed three times with PBS and resuspended in PBS to a final concentration of 8% (v/v). One hundred microliters of the erythrocyte suspension was incubated with 100 μL of peptide solution at concentrations ranging from 12.5 to 200 μM for 1 h at 37 °C. Cells exposed to 2% (v/v) Triton X-100 and PBS were defined as positive and negative controls, respectively. After centrifugation for 15 min at 1200g, 100 μL of supernatant was transferred to a new 96-well plate and the absorbance at 490 nm was measured with a microplate reader. The hemolysis rate was calculated by the following formula

$$\text{hemolysis rate} = (\text{OD}_{\text{peptide}} - \text{OD}_{\text{negative control}}) / (\text{OD}_{\text{positive control}} - \text{OD}_{\text{negative control}})$$

Peptide Stability Assay. The serum stability of peptides was tested according to our previous method.[44,45] The peptide solution (10 mM) was prepared with saline and incubated with mouse serum in a ratio of 1:2 ratio at 37 °C. A portion of 40 μL of mixture was taken at 0, 30, 60, 120, 240, 360, and 480 min, and 40 μL of ice-cold acetonitrile was added immediately to quench the enzyme reaction in an ice bath for 10 min. The samples were subsequently centrifugated at 13 000g at 4 °C for 15 min, and the supernatant was taken and stored at −80 °C. All samples were collected and then analyzed by RP-HPLC with a sample volume of 20 μL, varying from 5 to 95% with ACN/H_2O (containing 0.1% TFA) in a gradient over 30 min at a flow rate of 1 mL/min. The obtained peak areas were used to determine the remaining full-length peptide concentrations. The percentage of the remaining full-length peptide was calculated based on the initial concentration. The stability test was carried out in triplicate.

Statistical Analysis. Data were expressed as mean ± standard deviation and analyzed by GraphPad Prism 7.0 software. One-way ANOVA and Student's t-test (two-tailed) were used for statistical analysis with the following p-values: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{\#\#\#}p < 0.001$; ns indicates no significant difference.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c06766.

Predicted 3D structure of all peptides using chimera software (Figure S1); degradation chromatogram of LK and three hybrid peptides in serum (Figure S2); cytotoxicity of peptide against HK-2 cells at various concentrations for 24 h; results are representative of triplicate experiments (Figure S3); and ESI mass spectra and analytical reversed-phase HPLC chromatogram for purified peptides (Figures S4−S10) (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ACP, anticancer peptide; His, histidine; Glu, glutamate; TFE, 2,2,2-trifluoroethanol; PI, propidium iodide; HC_{10}, the hemolytic concentrations of 10%; FBS, fetal bovine serum; NBS, neonatal bovine serum; HOBT, 1-hydroxybenzotriazole; HBTU, O-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluorophosphate; TIS, triisopropylsilane; DIEA, *N*,*N*-diisopropylethylamine; TFA, trifluoroacetic acid; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; ESI-MS, electrospray ionization mass spectrometry; RP-HPLC, reverse-phase high-performance liquid chromatography; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide

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