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Constructing bioactive peptides with pH-dependent activities

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Abstract

Many bioactive peptides are featured by their arginine and lysine rich contents. In this study, lysine and arginine residues in lytic peptides were selectively replaced by histidines. Although resulted histidine-containing lytic peptides had decreased activity, they did show pH-dependent cytotoxicity. The activity of the constructed histidine-containing lytic peptides increased 2 ~ 8 times as the solution pH changed from of 7.4 to 5.5. More importantly, these histidine-containing peptides maintain the same cell killing mechanism as their parent peptides by causing cell lysis. Both the activity and pH-sensitivity of histidine-containing peptides are tunable by adjusting histidine substitution numbers and positions. This study has presented a general strategy to create bioactive peptides with desired pH-sensitivity to meet the needs of various applications such as cancer treatments.

Keywords

Peptide; pH sensitivity; histidine; bioactive; cell lysis

1. Introduction

Bioactive peptides are a group of peptides which have specific biological functions [10,14, 22,24]. Some well-known bioactive peptides include glucagon-like peptide-1 (GLP-1), this peptide is used for the control of diabetes [5], ghrelin, which is used to treat obesity [21], gastrin-releasing peptide is used in cancer treatments [9], and defensin, which is used as an antimicrobial agent [15]. At present, hundreds of these peptides have been isolated from different biological sources. The number of pharmacologically active peptides under development for the prevention and treatment of human disorders has increased rapidly due to their advantages over small molecules and proteins.

Many bioactive peptides are featured by their unique amino acid compositions such as proline-rich [11], cysteine-rich [3], and arginine/lysine-rich [1]. Lytic peptides are a group of peptides which have their primary targeting sites on cell membranes and demonstrate strong anti-bacterial and anticancer activities. Like antibacterial peptides, lytic peptides are featured by their high arginine and lysine contents. These two basic amino acids play a key role in peptide membrane partitioning and contribute greatly to the bioactivity of peptides. However, another basic amino acid, histidine, is hardly found in bioactive peptides. These histidine-containing antibacterial peptides have been reported in only Histatin and Clavanin [2,17]. Interestingly,

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these histidine-containing peptides demonstrate pH-dependent antimicrobial activity and become more active at acidic pHs.

In this study, we explored the possibility of creating bioactive peptides with pH-sensitivity by introducing the amino acid histidine into the peptide sequence. Our results suggest that selective substitution of corresponded lysine and arginine residues with histidines might present a general strategy to construct bioactive peptides with desired pH-sensitivity to meet the needs of various applications.

2. Materials and methods

2.1. Materials

Human alveolar basal epithelial cells (A549), Dulbecco's modified Eagle's medium (DMEM), and Kaighn's Modification of Ham's F-12 medium were purchased from American Type Culture Collection (ATCC). Calcein, Cholesterol, and Cell Growth Determination Kit (MTT kit) were from Sigma-Aldrich Company. The 1, 2-Dipalmitoyl-*sn*-Glycero-3-Phospho-L-Serine (DPPS) and 1, 2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine (DPPC) were purchased from Avanti polar lipids, Inc. Peptides were synthesized by GenScript. All other reagents used in this experiment were analytic grade.

2.2. Cytotoxicity measurement

The activity of peptides was determined by MTT assay as described previously [8]. Briefly, cells in complete medium were added into 96-well plates (5×10^3 cells/well) and cultured at 37 °C for 14–16 hrs. After being washed, cells were fed with serum-free DMEM medium containing various concentrations of peptides and incubated 37 °C for 2 hours. After that, 10 μ l of MTT (5 mg/ml) were added into each well. Cell viability was tested after being incubated for between 2 and 4 hrs at room temperature.

2.3. Lytic activity assay

Calcein loaded large unilamellar vesicles (LUVs) were prepared by extrusion method using a lipid mixture of DPPS and DPPC [19]. Prepared LUVs (5 μ l) were mixed with 180 μ l peptide solutions of various concentrations (from 1.0 to 20 μ M) and incubated at room temperature for five minutes. Peptide-induced calcein leakage, reflected by an increase in fluorescence intensity, was monitored using a fluorescent plate reader by setting the excitation and emission wavelengths at 485 nm and 525 nm respectively. Calcein release from LUVs was represented as F/F_0 , where F_0 and F represent the fluorescence intensity of calcein loaded LUVs in the absence and presence of peptides.

2.4. Tumor killing activity

Allograft tumor-bearing Nu/Nu nude mice were created according to the standard protocol [12]. The 4 ~6 week old Nu/Nu nude mice were injected subcutaneously in the middle of the left flank with 100 μ L of a single-cell suspension containing 1.7×10^6 A549 cells. Tumors were measured every other day with vernier calipers, and volumes were calculated according to the formula: $\text{Volume} = 0.52 \times W^2 \times L$, where W and L represent the width and length respectively. Treatment (intratumoral injection) started when the tumor size reached approximately 100 ~150 mm³. Three groups were included, they were: (i) saline (negative control); (ii) peptide L5c in saline solution; (iii) peptide L5c in pH=5.5 saline solution. Each group comprised 6 ~ 7 mice. Injections (20 nmol peptide/mouse) were repeated every three days. Tumor volume was monitored once every four days after injections. At the end of the observation (twenty-four days after the treatment) mice were sacrificed and autopsied. Tumors

were removed, weighed, and fixed with formalin. Paraffin sections were prepared, stained with hematoxylin and eosin, and then examined by light microscopy.

3. Results

3.1. Construction of pH-sensitive lytic peptides

We tried to construct bioactive peptides with pH-sensitivity by introducing histidines into peptide sequences. Well studied lytic peptides, peptide L5 [23], Citropin [18], and PTP-7 [7], were selected as templates to construct histidine-containing peptides. These three peptides are very potent and are able to induce cell death within a very short period of time (less than 30 mins). More importantly, they belong to the same category and both the targeting and acting sites of these peptides are restricted to cell membranes. The selection of lytic peptides as the peptide templates will simplify result interpretations and avoid the artifacts in cytotoxicity and pH-sensitivity assays.

To avoid or minimize amino acid substitution induced possible activity loss, only lysine and arginine residues in the lytic peptides were selectively replaced by histidines. Based on theoretical calculation, protonated histidine has very close interfacial free energy with lysine and arginine residues in peptides [6]. Unlike the amino group ($pK_a = 10.5$) in lysine and the guanidine group ($pK_a = 12.5$) in arginine, the pK_a value of imidazole group in histidine is about 6.0. Histidine residues with fully charged imidazole groups can only be found under acidic conditions when the solution pHs are below 6.0. At the physiological pH ($pH=7.2\sim7.4$), the side chains of histidine is only partially charged and thus each histidine residue in peptides or proteins carries less than one unit of positive charge. Therefore, a direct consequence of the replacement of lysine and arginine by histidine are the low isoelectric point (pI) values and significantly decreased positive charges in resulted histidine-containing lytic peptides (Table 1). Only under the acidic conditions and when the imidazole groups in histidines were protonated, these histidine-containing lytic peptides might have comparable pI values and positive charges with their parent peptides

As a reflection of all these chemical property changes, histidine-containing peptides showed reduced cytotoxicity at $pH=7.4$ (Table 1). The activity of a histidine-containing lytic peptide was mainly determined by histidine substitution numbers. For histidine-containing peptides from the same lytic peptide template, the more lysine and arginine residues in lytic peptides were replaced, the less active of resulted histidine-containing peptides. Peptide PTP-7c and Citropin-c in which all of the two lysine residues were replaced by histidines represented the lowest active peptide in the group. However, in some cases, the effect of histidine substitution positions was also profound. The replacement of the lysine residues at position 7 and 8 of Citropin yielded two peptides, Citropin-a and Citropin-b. Although these two peptides had the same histidine substitution number, peptide Citropin-a, was much more active than Citropin-b.

As we had expected, all histidine-containing lytic peptides constructed in this study showed pH-dependent feature, this is, having significantly improved cytotoxicity at acidic pHs (Table 2). Most histidine-containing lytic peptides experienced 2 to 8 times activity increase as the solution pH dropped from $pH=7.4$ to $pH=5.5$. Since the pH change in this range hardly affect MTT assay results (Fig. 1A), it excluded the possibility that the pH sensitivity of histidine-containing lytic peptides was an artifact. Typical pH-sensitive profiles of histidine-containing derivatives from peptide PTP-7 and L5 measured on A549 cells were included in Fig. 1B. Although the activity of peptide PTP-7 was not be affected by solution pHs, all three histidine-containing PTP-7 derivatives demonstrated pH-dependent activity. Two peptides with one histidine substitution at position 7 (PTP-7a) and position 11 (PTP-7b) gave almost the same $pH \sim$ activity profiles. A dramatic activity drop in a narrow pH range from $pH=7.5$ to $pH=7.0$

was observed for these two peptides. In contrast, the pH ~ cytotoxicity correlation from another histidine-containing PTP-7, PTP-7c, in which both of the lysine residues were replaced by histidines, was a nearly linear. Therefore, like cytotoxicity, the pH sensitivity of a histidine-containing peptide is also mainly determined by histidine substitution numbers although effects from histidine substitution positions do exist (Fig. 1A and Table 2). Despite slightly different IC₅₀ values, similar pH-dependent activity profiles of histidine-containing peptides were observed on both A549 and CHO cells. In fact, we had also tested the cytotoxicity of histidine-containing peptides on other cell lines including MCF-7 cell, MCF-7/ADR, MCF-10A, MCF-12A, CCD-13Lu, and fibroblast (data not shown) and confirmed that the pH-sensitivity was a unique feature of histidine-containing peptides.

3.2. Cell lysis activities of histidine-containing peptides

Since all of the four lytic peptide templates used in this study are able to kill cells within a very short period of time (30 ~ 60 mins), we have kept the exposure time of cells to peptides for only 2 hrs in our MTT assay (see Materials and Methods). In this regard, the cytotoxicity measured in this study actually reflected the cell lysis ability of peptides. Since most histidine-containing peptides maintain considerable cytotoxic activity (Table 1) especially under acidic conditions, it suggests that constructed histidine-containing peptides may maintain the same acting mechanism as their parent peptides by causing membrane lysis and cell leakage.

The membrane disrupting activities of histidine-containing PTP-7 derivatives were further tested on a lipid micelle system by testing peptide induced fluorescence dye (calcein) release from LUVs. The dye release results from PTP-7 and its derivatives were given in Fig. 2A. Histidine-containing derivatives showed low but pH-dependent LUV lysis activity. More importantly, this membrane-disrupting ability and pH-sensitivity measured on LUVs were consistent with the cytotoxicity and pH-sensitivity results of the same peptides from cultured cells (Table 1 and Fig. 1B). The same membrane-disrupting ability was also found in other histidine-containing peptides from template L5 and citropin (data not shown). These results confirmed that histidine-containing peptides constructed through this lysine and arginine replacement approach may maintain the same cell lysis mechanism as their parent peptides. We had tested the cell lysis activities of histidine-containing peptides on live cells by observing peptides induced membrane damages and cell lysis under microscopy. Like their parent peptides, histidine-containing peptides constructed in this study could induce mild (forming holes on membranes) to severe (lipid capsule detachment) cell damages. Peptides PTP-7a induced membrane detachment from cultured cells was captured and shown in Figure 2B.

3.3. The pH-dependent anti-tumor activity

The pH-dependent anti-tumor activity of peptide L5c was demonstrated on allograft tumor-bearing Nu/Nu nude mice (Fig. 4). Peptide L5c at 20 μ M/mouse induced tumor tissue damages evidenced by inflated tumor cell structure and enlarged nuclei and obscured nucleolar structures (Fig. 4B). However, peptide L5c at this dose could not inhibit tumor growth (Fig. 4A). On the contrary, if an acidic vehicle solution was used, the same dose (20 μ M/mouse) of peptide L5 caused severe tumor tissue damages (Fig. 4B) and inhibited tumor growth completely (Fig. 4A). This result is consistent with pH-affected peptide L5c cytotoxicity as measured *in vitro* (Fig. 1B), suggesting the anti-tumor activity of histidine-containing peptides is also pH-dependent.

4. Discussion

Despite proposed different acting models, the interaction of lytic peptides with cell membranes has common features and can be divided into two thermodynamic steps: 1) the electrostatic attraction of cationic peptides to anionic cell membranes; 2) the transition of the peptides into

the plane of binding and insertion into the lipid bilayer of cell membranes. Among these two steps, the electrostatic attraction of peptides to cell membrane is the critical one. For example, the concentration of a peptide of charge $Z = +3$ at the membrane surface is about 350-fold larger than that in bulk solution [16]. This is the reason why almost all lytic peptides are positively charged with very few exceptions. Replacement of lysine and arginine residues in lytic peptides with histidine will greatly decrease the cells affinity of these peptides and thus explains significantly reduced cytotoxicity of histidine-containing lytic peptides (Table 1). As the imidazole groups in histidines are protonated under acidic conditions, histidine-containing lytic peptides can partially resume their cell affinity. For example, the membrane-disrupting and cell lysis activities of PTP derivatives are in the order of $PTP-7 > PTP-7a \sim PTP-7b > PTP-7c$ at $pH=5.5$. In fact, even at $pH=5.5$ and when the pH is less than pK_a ($pK_a = 6.0$) of imidazole groups, histidine residue will carry less than one unit of positive charge. Therefore, histidine-containing peptides have less net positive charges (1.8 units for PTP-7a and PTP-7b, and 1.5 units for PTP-7c, respectively) compared to their parent peptide PTP-7 (Table 1). It seems that net positive charges affected peptide binding to negatively charged cell membranes can explain the activity and also the pH -sensitivity of histidine-containing peptides. However, since not all histidine-containing lytic peptides give the expected linear $pH \sim$ cytotoxicity correlations in the pH range from 7.4 to 5.5 (Fig. 1B), the pH induced the charge and the cells affinity changes in peptides can not be the only reason for the pH sensitivity of histidine-containing lytic peptides.

It should be noted that histidine substitution at positions other than arginine and lysine may result in permanent activity loss (data not shown). The transition of the peptides into the plane of binding and insertion into the lipid bilayer of cell membranes depends greatly on the hydrophobic/hydrophilic balance of the molecule groups and forces involved [20]. Under acidic conditions and when the imidazole group is protonated, histidine has very close interfacial free energy ($G = 0.96$ kcal/mol) with lysine ($G = 0.99$ kcal/mol) and arginine ($G = 0.81$ kcal/mol). Therefore, replacement of lysine and arginine residues with histidines ensures that resulted histidine-containing lytic peptides will have the same or very similar membrane partitioning property as their parent peptides at acidic pH s (Fig. 3). This may explain why only lysine and arginine replacements will lead to pH -sensitive peptides with reasonable cell lysis activity.

Finally, the activity and pH selectivity of histidine-containing peptides are mainly determined by histidine substitution numbers, derivatives from a lytic peptide with the same histidine substitution numbers but at different positions give neither the same cytotoxicity nor the same pH sensitivity profiles (Table 1 and Fig.1). Therefore, theoretically, we should be able to tune the pH -sensitivity of histidine-containing peptides by manipulating histidine substitution numbers and positions. This guarantees the construction of histidine-containing bioactive peptides with desired activity and pH -sensitivity to meet the needs of various applications. For example, it has been found that the extracellular pH s of some solid tumor are consistently lower as compared to the $pH=7.2 \sim 7.4$ in normal tissues/organs [4,13]. The origins of this extracellular acidity lie in the chaotic nature of tumor vasculature, increased glycolytic flux in tumor cells, increased export of protons from tumor cells, diminished buffering capacity of tumor interstitial fluid, and diffusion-limited rates of transport of lactic acid from the interstitium into the vasculature. To this regard, if lytic peptides with appropriate pH sensitivity that perfectly fit the extracellular pH difference between tumor and normal tissues are constructed, they should be able to kill tumors in acidic environments selectively but spare normal tissues with physiological pH .

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Fig. 1.

A) The pH-affected cell respiration as measured by MTT assay; **B)** The pH-affected cytotoxicity changes in histidine-containing peptides. Cells were treated with various peptides at fixed concentration (IC₅₀) for 2 hrs at various pHs. Cell respiration was measured by MTT assay.

B

Fig. 2.

A) Peptides induced calcein release from lipid micelles. Lipid micelle leakage associated fluorescence intensity increases (F/F_0) were recorded on a spectrofluorimeter by setting excitation and emission wavelengths at 480nm and 530 nm, respectively. Lipid micelle =120 μ M and peptide =20 μ M; **B)** Histidine-containing induced cell lysis. Cells were incubated with 20 μ M PTP-7a at pH=7.4 for 30 mins. **B)** Cell membrane detachment (indicated by arrows) from adhered cells were observed under microscopy.

Fig. 3.
Comparison of pH affected membrane partitioning of peptide PTP-7 and PTP-7a. Cross, PTP-7a at pH=7.4; Light grey, PTP-7a at pH=5.5; Black, PTP-7 at pH=7.4 or pH=5.5.

Fig. 4. Allograft tumor-bearing Nu/Nu nude mice (6~7 mice/group) were injected with (i) saline (negative control); (ii) peptide L5c in saline solution; (iii) peptide L5c in pH=5.5 saline solution. **A**, Tumor regression assay; **B**, Histology studies.

Physical and chemical properties of histidine-containing peptides

Peptide	Sequence	No. of histidine	No. of Arginine & lysine	^a Net charge			^b Charge ratio	^a pI
				pH=7.4	pH=5.5			
PTP-7	FLGALFKALSKLL	0	2	2.0	2.0		1.0	10.6
PTP-7a	FLGALFHALLSKLL	1	1	1.1	1.8		1.6	10.0
PTP-7b	FLGALFKALSHLL	1	1	1.1	1.8		1.6	10.0
PTP-7c	FLGALFHALLSHLL	2	0	0.1	1.5		1.5	7.6
L5	PAWRKAFRWAWRMLKKAA	0	6	6.0	6.0		1.0	12.7
L5a	PAWHHAFHWAWRMLKKAA	3	6	3.2	5.4		1.6	11.6
L5b	PAWRKAFRWAWHMLHHAA	3	6	3.2	5.4		1.6	12.4
L5c	PAWRHAFHWAWHMLHKAA	4	6	2.1	5.0		2.4	11.4
Citropin	GLFAVIKKVASVIGGL	0	2	2.0	2.0		1.0	10.6
Citropin-a	GLFAVIHKVASVIGGL	1	1	1.1	1.8		1.6	10.0
Citropin-b	GLFAVIKHVASVIGGL	1	1	1.1	1.8		1.6	10.0
Citropin-c	GLFAVIHHVASVIGGL	2	0	0.1	1.5		1.5	7.6

^aTheoretical calculation [7]

^bPositive charge ratios of peptides at pH=5.5 verse at pH= 7.4

Tab. 2
The cytotoxicity and pH-sensitivity of histidine-containing peptides

Peptide	A549 Cell		IC ₅₀ ratio ^a	CHO Cell		IC ₅₀ ratio ^a
	pH=7.4	pH=5.5		pH=7.4	pH=5.5	
PTP-7	9 ± 1	9 ± 1	1.0	10 ± 2	12 ± 2	0.9
PTP-7a	52 ± 3	27 ± 1	2.0	29 ± 1	17 ± 1	1.7
PTP-7b	64 ± 3	23 ± 2	2.8	41 ± 2	20 ± 1	2.1
PTP-7c	>160 ^b	42 ± 2 ^b	>4.0 ^b	>120	>120	NA
L5	3 ± 1	4 ± 1	1.0	5 ± 1	5 ± 1	1.0
L5a	19 ± 3	10 ± 2	1.9	13 ± 2	8 ± 1	1.6
L5b	12 ± 2	7 ± 2	1.7	12 ± 1	6 ± 2	2.0
L5c	>80	12 ± 2	>8	67 ± 3	7 ± 2	9.5
Citropin	28 ± 1	25 ± 2	1.1	19 ± 2	21 ± 1	0.9
Citropin-a	56 ± 3	44 ± 3	1.3	78 ± 2	47 ± 3	1.6
Citropin-b	97 ± 4	61 ± 3	1.6	76 ± 4	41 ± 5	1.8
Citropin-c	156 ± 7 ^b	36 ± 2 ^b	4.3 ^b	>120	>120	NA

^a IC₅₀ ratio = [IC₅₀]pH=7.4/[IC₅₀]pH=5.5

^b IC₂₀ ratio = [IC₂₀]pH=7.4/[IC₂₀]pH=5.5