Antitumor activity of a membrane lytic peptide cyclized with a linker sensitive to membrane type 1-matrix metalloproteinase

Jieying Zhong1,2 and Ying Chau1,2,3

1Department of Chemical and Biomolecular Engineering,  
2Bioengineering Program, and  
3Center for Cancer Research,  
Hong Kong University of Science and Technology,  
Clear Water Bay, Hong Kong

Abstract

Membrane lytic peptides are a novel class of anticancer agents that have the potential to overcome drug resistance. The limited selectivity against cancer cells, however, presents a major hurdle for the application. We aim to exploit the proteolytic activity of tumor-associated matrix metalloproteinases (MMP) to mediate the cytotoxicity of these peptides. We designed a membrane lytic peptide cyclized with a linker cleavable by membrane type 1-MMP (MT1-MMP). We showed that the cyclic peptide could be restored to the linear state on MT1-MMP digestion, and it preferentially killed MMP-overexpressing cells above a threshold concentration. Circular dichroism indicated that cyclization resulted in a more rigid structure, making it more difficult for the lytic peptide to transit from random coil to α-helix in a membrane-mimicking environment. Selective membrane activity of the cyclic peptide was shown by comparing cytotoxicity results on RBC and two human breast cancer cell lines of different malignancy and MT1-MMP expression: highly invasive MDA-MB-435 and noninvasive MCF-7. Above a concentration of 5 μmol/L, suppressed activity to MCF-7 and RBC was observed, whereas the toxicity against MDA-MB-435 was maintained. MMP inhibition experiments further showed that the membrane-lysing activity was enzyme dependent. [Mol Cancer Ther 2008;7(9):2933–40]

Introduction

Membrane lytic peptides serve as an innate immune mechanism against pathogens in many species in nature (1, 2). These peptides have a simple structure yet rapid and potent activity against a broad spectrum of microbes, representing a new class of antibiotics (3, 4). More recently, their potential as novel anticancer reagents has been explored (5–7). Most of the membrane lytic peptides are short (14–40 amino acids), linear, and cationic. In a membrane environment, they organize into ordered secondary structures such as α-helix and β-sheet (8, 9). The mechanism behind the membrane-lysing function is under investigation and different models have been proposed (10–12). The fundamental consensus is that peptides are bound to cell surface through electrostatic interaction first. Above a threshold concentration, peptides insert into and disturb the cell membrane and ultimately cause cell death due to membrane disintegration. This process can be completed in minutes at a micromolar level. As the potency does not rely on cellular internalization, lytic peptides circumvent the problems of multidrug resistance, which causes inefficient drug uptake and presents a predominant hindrance to conventional chemotherapy (13).

However, the potential of this peptide family as therapeutic agents is limited by the low specificity. Most of lytic peptides lyse membranes without much discrimination. Considerable efforts have been made to render peptides selective for cancer cells while sparing normal cells (14–18). In this study, tumor-associated enzyme-controlled activation is used as our targeting strategy. Matrix metalloproteinases (MMP) are a family of over 25 secreted and membrane-bound zinc endopeptidases that participate in numerous normal and pathologic extracellular matrix remodeling events, including tumor progression, metastasis, and angiogenesis (19, 20). Membrane type-MMPs (MT-MMP) are a unique subset tethered to the cell membrane by a transmembrane domain or glycosylphosphatidylinositol linkage (21). The first identified MT-MMP, MT1-MMP, has been shown to play a central role in tumor cell invasion and migration (22–24). Because MT1-MMP is highly localized at the leading edge of invading cancer cells and is a powerful proteolytic enzyme, it is considered a promising target for cancer treatment. In previous studies, short peptide sequences susceptible to MT1-MMP were found (25, 26). Notably, substrates efficiently cleavable by MT1-MMP but not other MMPs have been identified (27). One of the reported sequences was used in the present study to mediate tumor targeting.

The ability to adopt certain amphiphatic secondary structures is considered critical to the function of lytic peptides (3, 7, 10). Cyclization affects peptide behavior by imposing spatial restriction. Previous studies have revealed that helical structure was impaired and binding affinity to zwitterionic lipid bilayer was decreased on cyclization.
when compared with linear analogues (28–30). In the present study, an 18-amino acid membrane lytic peptide was cyclized with a MT1-MMP-sensitive linker (27), resulting in a MT1-MMP cleavable cyclic peptide consisting of 25 amino acids. The peptide structures in different environments were monitored by circular dichroism (CD) spectroscopy and the corresponding membrane activity was examined using cell lines of different MT1-MMP expression levels. We hypothesize that lysing activity of the peptide will be restored. We aim to show that the peptide will be suppressed by cyclization. On cleavage by MT1-MMP, the linear state and consequently the potency of the peptide will be restored. We aim to show that the newly designed peptide exhibits selectivity against tumor cells with overexpressed MT1-MMP.

Materials and Methods

Materials
All peptides were purchased from GL Biochem. DTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), SDS, trifluoroacetic acid, acetonitrile, and MMP inhibitor monocyicone were obtained from Sigma. Triton X-100 was purchased from USB Corp. Concanavalin A (Con A) and recombinant human MT1-MMP catalytic domain were purchased from Calbiochem. Human tissue inhibitor of metalloproteinase-2 was ordered from Chemicon International.

Cells
Human breast cancer cell lines MDA-MB-435 and MCF-7 were kindly provided by Prof. Kathy Luo (Hong Kong University of Science and Technology). Cells were cultured in DMEM (MDA-MB-435) or MEM (MCF-7) supplemented with 10% fetal bovine serum and 0.1% penicillin-streptomycin. Trypsin-EDTA (0.05%) was used to detach cells in subculturing. All the cell culture media and reagents were purchased from Invitrogen. Rabbit blood was collected and heparinized at the Animal and Plant Care Facility of the Hong Kong University of Science and Technology.

Peptide Cyclization and Purification
Peptide was dissolved in water at a final concentration of 0.1 mg/mL and then air-oxidized by slow stirring at room temperature for 24 h to afford the cyclic peptide with an intramolecular disulfide bond. The reaction process was monitored by reverse-phase high-performance liquid chromatography (HPLC). On completion, the entire solution was lyophilized. All the peptides were analyzed by gradient reverse-phase HPLC and were shown to be homogeneous (~95%). Briefly, peptide was injected into a C18 column (Vydac218TP; 0.21 × 25 cm). After loading, column was washed with mobile phase A (water with 0.2% trifluoroacetic acid) at a flow rate of 0.5 mL/min for 5 min. Mobile phase B (acetonitrile with 0.2% trifluoroacetic acid) was subsequently increased from 0% to 100% in 20 min at the same flow rate. Peak elution was detected by UV absorption at 220 and 280 nm. The identity of cyclization of each peptide was confirmed by electrospray ionized mass spectroscopy. Disulfide bond can be broken by reduction in 0.1% DTT aqueous solution at room temperature for 1 h. The reaction was examined by reverse-phase HPLC and mass spectroscopy analysis.

Enzymatic Digestion
A cyclic peptide solution at a final concentration of 50 μmol/L was incubated with recombinant human MT1-MMP catalytic domain at a final concentration of 40 nmol/L in the digestion buffer [50 mmol/L NaCl, 50 mmol/L Tris-HCl, 10 mmol/L CaCl2, 0.025% Brij35 (pH7.5)] at 37°C. At the designated time points, the digestion mixture was sampled and analyzed by reverse-phase HPLC. Analysis was done using the same chromatographic conditions as described above. Eluted fractions were collected for mass spectroscopy analysis. Digested peptides were applied to MCF-7 and RBC to examine the effect of enzymatic cleavage on the cytotoxicity of the peptides.

Cytotoxicity Measurement by MTT Assay
MTT assay was conducted to examine the peptide cytotoxicity against breast cancer cell lines with and without MT1-MMP expression. MDA-MB-435 and MCF-7 (31–33) were grown in a humidified atmosphere with 5% CO2 at 37°C. On reaching 80% confluence, cells were detached and resuspended in fresh medium and then seeded unto a 96-well plate for overnight incubation. For MDA-MB-435, cells were cultured in serum-free DMEM containing Con A (20 μg/mL) for additional 12 h or in serum-free DMEM containing tissue inhibitor of metalloproteinase-2 (2.5 μg/mL) for additional 1 h before the addition of peptides. Cells were incubated with peptide for 12 h. For MCF-7, cells were incubated with peptide in serum-free MEM for 12 h. At the end of peptide incubation, MTT was added to each well at a final concentration of 0.5 mg/mL. The plate was left in a dark humidified environment at 37°C for 4 h, and 100 μL of 10% SDS in 0.01 mol/L HCl were added to dissolve the formazan purple crystals. After overnight incubation, the absorbance at 595 nm of each well was measured by a microplate reader (Wallac Victor® 1420). The percentage of cell viability was determined as follows:

\[
\text{Viability} (%) = \frac{(A_s - A_0)}{(A_c - A_0)} \times 100\%
\]

where \(A_s\) is the absorbance value of the sample, \(A_c\) is the absorbance of control, and \(A_0\) is the background absorbance. Each experiment was repeated at least three times independently.

Hemolysis of RBC
Heparinized fresh rabbit blood was rinsed three times by centrifugation at 2,000 rpm for 10 min and resuspended in warm PBS. Peptides were added to RBC suspension to obtain the designated peptide concentrations and a final erythrocyte concentration of 4% (v/v). The resulting suspensions were incubated at 37°C for 1 h under agitation at 300 rpm. Samples were then centrifuged at 2,000 rpm for 10 min. The absorbance of supernatant at 540 nm was measured to monitor the release of hemoglobin and indicate RBC membrane damage. Zero hemolysis and 100%
hemolysis consisted of RBC suspended in PBS and 1% Triton X-100, respectively. The percentage of hemolysis was determined as follows:

\[
\text{Hemolysis(\%)} = \frac{(A_s - A_0)}{(A_{100} - A_0)} \times 100\%
\]

where \(A_s\) is the absorbance value of sample, \(A_{100}\) is the absorbance of completely lysed RBC in 1% Triton X-100, and \(A_0\) is the absorbance of zero hemolysis. Each experiment was repeated at least three times independently.

**Secondary Structure Determination by CD Spectroscopy**

The peptide ellipticity was determined by CD spectroscopy by a J-810 spectropolarimeter (Jasco). Peptides were dissolved in water and a membrane-mimicking solution (1% SDS in water) separately at a final concentration of 0.25 mg/mL. Peptide solutions were loaded into a quartz cell and exposed to ellipticity scan from 190 to 260 nm at 25°C.

**Fractional Helicity Calculation**

The output of CD measurement was processed to provide the mean residue ellipticity \(\theta\) as follows:

\[
\theta (\text{deg} \cdot \text{cm}^2/\text{decimole}) = \frac{\text{Ellipticity (mdeg)}}{\text{peptide concentration (M)} \times \text{pathlength (cm)} \times \text{number of residues} \times 10}
\]

Fractional helicity (FH) is an index to quantify peptide helicity. \(\Delta FH_{\text{SDS-WATER}}\) represents the difference of fractional helicities in the two environments. These variables are calculated by the following equations (34):

\[
FH = \frac{\theta_{222,n,T,\text{exp}}}{\theta_{222,H,n,T}} \times 100\%
\]

\[
\theta_{222,H,n,T} = \frac{60500 + 260(T-2)}{(1 - X/n)}
\]

\[
A[\theta]_{222SDS-WATER} = [\theta]_{222} \text{ in SDS} - [\theta]_{222} \text{ in water}
\]

\[
\Delta FH_{\text{SDS-WATER}} = FH \text{ in SDS} - FH \text{ in water}
\]

where \(\theta_{222,n,T,\text{exp}}\) is the experimental mean residue ellipticity of peptide consisting of \(n\) residues at \(T\)°C at 222 nm. \(\theta_{222,H,n,T}\) is the mean residue ellipticity of a theoretical standard helical peptide of the same length at the same temperature at 222 nm. X is a constant used to correct non-hydrogen-bonded carbonyl at terminals that does not contribute to \(\theta\). When \(T = 25°C, X = 6.5\). Equations are valid when 18 ≤ \(n\) ≤ 45. The presented result is the average of results from three independent experiments.

**Results**

**Peptide Design and Construction**

Three linear peptides and one cyclized sequence were designed and synthesized to investigate the relation between structure and anticancer activity (Table 1). The 18-residue lytic sequence was designed based on a \(\alpha\)-helical amphipathic model peptide initially developed by Steiner et al. (35). According to the Schiffer-Edmundson wheel model (36) and software simulation (37), the sequence of L-18 and D-18 in our study is a perfect amphipathic structure when exposed to a hydrophobic environment, with polar and nonpolar residues accumulated in opposite faces (Fig. 1; Supplementary Fig. S1). L-18, D-18, and H-18 are stereoisomers of identical amino acid composition and sequence: the former two are of opposite chirality to each other, whereas H-18 is a hybrid sequence with five residues randomly replaced by the corresponding D-amino acids. cycl-L-25 is a 25-residue sequence composed of a segment preferentially cleavable by MT1-MMP (27) and a membrane-lytic segment identical to L-18. The amino acids at the substrate sites \(P_1'\) and \(P_2'\) also contribute to the amphipathic structure such that the structural disturbance from linkers amino acids after cleavage will be minimized. Two cysteines are positioned at both ends for cyclizing the peptide through disulfide bond formation. Cyclization was confirmed by HPLC and mass spectrometry analysis (data not shown).

**Recovery of Linear Structure of cycL-25 by MT1-MMP Cleavage**

According to HPLC and mass spectrometry analysis, we found that cycL-25 could be converted to the linear form on cleavage by the tumor-associated enzyme MT1-MMP (Supplementary Fig. S2). The major cleavage site was as expected and shown in Table 1. We differentiated the cyclized and linear form by their difference in elution time over the reverse-phase HPLC column. As shown in Supplementary Fig. S2, intact and cleaved cycL-25 eluted at 20.6 and 20.9 min, respectively. The cleavage was further confirmed by mass spectrometry analysis of the eluted fractions (Supplementary Fig. S3). After 1 h incubation with MT1-MMP, 52% peptides were converted to linear form. This number increased to 82% in 5 h. Some minor peaks were observed after 2 h of incubation, indicating the presence of side reactions. Mass spectrometry analysis revealed that an additional site for slower cleavage existed in the lytic sequence (CGRIGFLRLALKLALKAA/LKLC-NH₂).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-18</td>
<td>LRLALKLALKAALKL-NH₂</td>
</tr>
<tr>
<td>D-18</td>
<td>LRLALKLALKAALKL-NH₂</td>
</tr>
<tr>
<td>H-18</td>
<td>LRLALKLALKAALKL-NH₂</td>
</tr>
<tr>
<td>cycL-25</td>
<td>CGRIGFLRLALKLALKAA/LKLC-NH₂</td>
</tr>
</tbody>
</table>

**NOTE:** D-amino acids are in italics. The MT1-MMP cleavage site is underlined. The cleavage position is indicated by ‘/’.

---

5 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
Random Coil to $\alpha$-Helix Transition of Lytic Peptides on Environment Alteration

It was reported that the ability to adopt a certain amphipathic secondary structure is critical to the membrane activity of a lytic peptide (3, 7, 10). CD spectroscopy reveals peptide structural transition during its translocation from an aqueous environment to a hydrophobic membrane-like environment. By comparing the spectra of linear and cyclic peptides, the influence of cyclization on the secondary structure transition was also studied. Because the enzymatic digestion mixture interferes with the CD measurement, 0.1% DTT reduction was used instead to retrieve the linear form from cycL-25.

For peptides of $L$-chirality, the CD spectrum of a typical $\alpha$-helix exhibits double minima at 208 and 222 nm and a maximum at 195 nm, whereas the spectrum with a single minimum at 200 nm is indicative of random coil. The ellipticity at 222 nm is conventionally used to quantify the extent of helicity (34). Spectra of D-peptides are symmetrical with their enantiomers about the $X$ axis. L-18, D-18, H-18 and reduced cycL-25 exhibited random coil or slightly helical structure in water, whereas the intact cycL-25 was an exception, which maintained a certain degree of helicity (Table 2; Supplementary Fig. S4). When peptides were transferred to SDS, a membrane-mimicking solution, highly helical structures were observed for L-18, D-18, and reduced cycL-25 but not for H-18 and intact cycL-25. The hybrid peptide H-18 did not show any significant helical structure in either an aqueous or membrane-mimicking environment. This is consistent with previous reports in which arbitrary $D$-amino acid replacement could abolish the capability of peptides to form ordered structures (10).

The behavior of cycL-25 was interesting. Unlike the linear analogues, the degree of helicity of cycL-25 remained relatively constant in both water and membrane-mimicking solution. According to the change of FH during translocation ($\Delta$FH$_{SDS-WATER}$) in Table 2, an index to evaluate the structure inducibility of peptides, the structure of cycL-25 is much less inducible than L-18 and D-18. It is worth noting that the inducibility was almost completely recovered on the split of disulfide bond, shown by the reduced cycL-25. The results suggested that cyclization imposed a rigid structure on the peptide.

**In vitro Cytotoxicity against Tumor Cell Lines**

To study the relation between peptide activity and MT1-MMP cleavage, two human breast cancer cell lines were selected. They are different in their malignancy and MT1-MMP expression level. MDA-MB-435 is known as a highly invasive cancer cell line, which overexpresses MT1-MMP when exposed to Con A in vitro or extracellular matrix components such as collagen in vivo (31, 38). MCF-7 is noninvasive and does not express MT1-MMP (31–33). Figure 2 shows the change of MDA-MB-435 morphology 5 min after exposure to L-18. Cells were swollen after being treated with 7 nM L peptide (Fig. 2B). When the peptide concentration was increased to 20 nM/L, cell membranes were completely disintegrated (Fig. 2C). Similar results were observed for D-18 and cycL-25. Cells remained intact under microscope after H-18 treatment (data not shown).

### Table 2. Peptide helicity in water and 1% SDS and structural inducibility on environment change

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Water</th>
<th>SDS</th>
<th>$\Delta$[$\theta$]$_{222}$ SDS-WATER</th>
<th>$\Delta$FH$_{SDS-WATER}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>L18</td>
<td>$-1,693.31 \pm 446.31$</td>
<td>$4.86 \pm 1.28$</td>
<td>$-12,168.08 \pm 1119.91$</td>
<td>$34.93 \pm 3.22$</td>
</tr>
<tr>
<td>D18</td>
<td>$1,584.71 \pm 3.23$</td>
<td>$4.55 \pm 0.01$</td>
<td>$11,069.18 \pm 243.68$</td>
<td>$31.78 \pm 0.70$</td>
</tr>
<tr>
<td>H18</td>
<td>$-17.08 \pm 64.73$</td>
<td>$0.05 \pm 0.19$</td>
<td>$-1,434.63 \pm 279.22$</td>
<td>$4.12 \pm 0.80$</td>
</tr>
<tr>
<td>cycL-25</td>
<td>$-6,688.78 \pm 182.35$</td>
<td>$16.58 \pm 0.45$</td>
<td>$-8,118.56 \pm 576.77$</td>
<td>$20.12 \pm 1.43$</td>
</tr>
<tr>
<td>cycL-25 + 0.1% DTT</td>
<td>$-3,808.01 \pm 87.83$</td>
<td>$9.45 \pm 0.23$</td>
<td>$-14,094.44 \pm 1,197.30$</td>
<td>$34.98 \pm 2.91$</td>
</tr>
</tbody>
</table>

**NOTE:** Ellipticities were measured in water or in 1% SDS solution by CD spectroscopy at 222 nm. $[\theta]_{222}$ and FH were derived to evaluate the degree of peptide helicity. The difference of helicity in two solvents was derived to evaluate peptide structural inducibility on environment change. All the data were calculated as described in Materials and Methods. Results are the average of three independent experiments.

Abbreviation: FH, fractional helicity.

$^*\Delta$FH$_{SDS-WATER}$: the difference of ellipticity of a peptide in two environments.

$^[\theta]_{222}$: the mean residual ellipticity at 222 nm.

$^\dagger$FH: the helicity (in %) of a peptide relative to a standard helical peptide of same length.

$^1\Delta$FH$_{SDS-WATER}$: the difference of helicity of a peptide in two environments.
The peptide cytotoxicity was accessed by MTT assay. On both MDA-MB-435 and MDF-7 (Fig. 3A and B), L-18 and D-18 were very potent with LC50 lower than 5 \( \mu \text{mol/L} \), whereas H-18 exhibited slight or no effect on cell viability, which was consistent with secondary structure measure result and our hypothesis. Unlike the linear analogues, the curves of cycL-25 obtained in two cell lines were quite different from each other. In MDA-MB-435 stimulated with Con A, the cytotoxicity of cycL-25 was slightly lower than linear L-18 and D-18, which might be due to the extra linker residues. However, the potency was still comparable with linear analogues with LC50 at 3 \( \mu \text{mol/L} \) (Fig. 3A). In the case of MT1-MMP-deficient MCF-7, the potency of cycL-25 was significantly impaired at most concentrations when compared with L-18 and D-18 (Fig. 3B). The increased potency at low concentration was unexpected (see Discussion). The results indicated that cycL-25 show preferential cytotoxicity against MT1-MMP-expressing cells.

**Effect of MT1-MMP on the Cytotoxicity of cycL-25**

To further study the relation between the enzyme activity and peptide toxicity, MT1-MMP inhibitor was applied to MDA-MB-435. Tissue inhibitor of metalloproteinase-2 selectively inhibits certain members of the MMP family including MT1-MMP (19, 22). The inhibitor partially preserved cell viability over the whole range of concentrations tested (Fig. 3C). This implies that the toxicity of cycL-25 at least partially depends on the presence of active MT1-MMP. To further illustrate the role of MT1-MMP in cycL-25 toxicity activation, cycL-25 was digested in vitro before being applied to MCF-7. It is shown in Fig. 3D that cycL-25 becomes more toxic to MCF-7 at most concentrations. The above results show that MT1-MMP increases the toxicity of cycL-25.

**Hemolytic Activity Measurement**

Hemolysis is one of the major side effects in lytic peptide therapy. Both L-18 and D-18 were hemolytic in dose-dependent manner and H-18 had no effect at all (Fig. 4A). Compared with L-18 and D-18, the hemolytic effect of cycL-25 was significantly suppressed at high concentrations but increased at low concentrations. To find out whether this was caused by cyclization, two reagents, MT1-MMP and 0.1% DDT, were applied separately to retrieve the linear structure. As shown in Fig. 4B and C, both treatments reduce the hemolytic effect of cycL-25 at low concentrations but enhance hemolysis activity at high concentrations.

**Discussion**

Since the discovery of defensin two decades ago (1, 2, 39), membrane lytic peptides have been considered as promising antibiotics (3, 4) and, lately, a novel class of anticancer agent (5–7). Because lytic peptides have the potential to lyse and kill cells almost immediately, they can potentially overcome the drug resistance problem. However, most membrane lytic peptides do not differentiate normal cells and cancer cells. Research has been conducted to render peptides more selective and some success has been achieved (3, 14, 15, 17, 18, 40). Structure-function investigations enable the rational design of peptides that preferentially kill microbes (3) or cancer cells (17). D-amino acid replacement is the most common sequence-modifying method. Conjugating peptides with targeting ligands such as...
as antibodies was also reported (18). Another strategy is via activation by tumor-associated enzymes or lower pH in the pathologic microenvironment of tumor tissues. In these examples, enzymatic or acidic digestion restored the peptide activity previously suppressed by oppositely charged residues or steric hindrance (14–16). Lytic peptides that self-assemble into pH-responsive fibers could improve the stability and selectivity of these potential therapeutic agents (40).

In this study, we explored a novel approach to control the activity of membrane lytic peptides via MMP mediation. Cyclic peptide was constructed by joining the two ends of a lytic peptide with a linker cleavable by MT1-MMP. MT1-MMP has been found overexpressed on the cell membranes of cancer cells and cancer vasculature (22–24, 41). This enzyme stands out because its unique location is suitable for activating membrane lytic peptides. We expect spatial synergy between enzymatic activation and peptide activity from the colocation. Because the formation of secondary structure is critical to membrane lysing, we reasoned that cyclization would suppress the cytotoxic activity by imposing conformational restriction. On contact with MT1-MMP-expressing cells, the embedded linker should be cleaved, restoring the functional linear state and hence the peptide activity.

From the CD spectroscopy (Table 2), we found that L-18 and D-18 underwent a secondary structure transition from random coil to helix in a nonpolar environment, which mimics the interior of cell membrane. However, the situation for cycL-25 is different because its structure barely changed, remaining almost the same helicity in both polar and nonpolar environments (Table 2; Supplementary Fig. S4D). This rigid structure of cycL-25 supports our hypothesis that cyclization imposes restriction to the peptide conformation. In addition, the cyclic peptide was freed from the structural constraint and the structural inducibility was restored by the reduction of DTT (Table 2; Supplementary Fig. S4E). Because the secondary structure formation has been reported as a driving force for cell membrane lysing (42), the distinct structures in response to MT1-MMP enable cycL-25 to behave differently in cells of different enzyme expression.

Figure 3. Cytotoxicity of peptides against tumor cells and effect of MT1-MMP on the potency of cycL25. The peptide cytotoxicity was evaluated in two human breast cancer cell lines of different MT1-MMP expression: Con A-stimulated MDA-MB-435 (A) and MCF-7 (B). To examine the effect of MT1-MMP on peptide activity, MDA-MB-435 was treated with either Con A or MMP inhibitor tissue inhibitor of metalloproteinase-2 before cycL-25 addition (C). MCF-7 was treated with either intact cycL25 or MT1-MMP digested cycL25 (D). Experiments in each condition above were repeated at least three times.
In agreement with our hypothesis, cyclL-25 exhibited MT1-MMP-related selectivity. The aggressive MDA-MB-435 was sensitive to cyclL-25 (Fig. 3A), whereas the MMP-deficient MCF-7 was relatively resistant (Fig. 3B), suggesting that the membrane activity of cyclL-25 was enzyme dependent. This was supported by the facts that MMP inhibitors were able to protect MDA-MB-435 from the cytotoxicity of cyclL-25 (Fig. 3C) and MT1-MMP predigestion could increase the activity of cyclL-25 against MCF-7 (Fig. 3D). In addition, the hemolytic activity against RBC is also significantly decreased above the concentration of 10 μmol/L (Fig. 4A). The results agreed with our expectation and previous reports that hemolytic activity of peptide could be suppressed by cyclization (28, 29). Taken together, cyclL-25 is more toxic against MMP-positive cells than MMP-negative cells.

It is interesting that, in the MMP-negative cells such as MCF-7 and RBC, cytotoxicity of cyclic peptide was slightly enhanced instead of being suppressed at low concentration (Figs. 3B and 4A). Secondary structure measurement provides a possible explanation to this phenomenon. As stated above, the functional peptides L-18 and D-18 undergo a random coil to α-helix transition when transferred from water to a membrane-mimicking solution. This transition is absent for H-18 and cyclL-25. According to the “two-state” model (11, 43, 44), the disturbance of the lipid membrane by peptides is attained through three sequential stages: interfacial binding in an unfolded state followed by the ordered secondary structure formation and finally by the insertion of ordered units into the lipid bilayer. Because the entropy decreases when ordered structure is formed, the penetration is free energy favored only when the entropy penalty is sufficiently compensated by the hydrogen bonding in helix formation (10, 43). Therefore, the secondary structure formation is considered as a major driving force for peptide penetration into the membrane. As the penetration proceeds, the structure becomes more and more ordered. This process is driven by and coupled with the helix formation. As revealed in Table 2 and Supplementary Fig. S4D, intact cyclL-25 is partially structured due to the stabilizing effect of cyclization. Thus, less energy needs to be invested in initial folding, which facilitates the membrane disturbance at low concentration. The activity of cyclL-25 is suppressed at high concentration because the rigid structure prevents further folding. The lack of enthalpic driving force hinders the penetration of cyclL-25 into the lipid membrane.

In conclusion, a lytic peptide that could be activated by tumor-associated enzyme has been constructed. Encouraging results with regard to the specificity against MT1-MMP-expressing cells has been obtained. By incorporating linkers sensitive to various disease-associated enzymes, this kind of enzyme-activated lytic peptides have the potential to become targeted therapeutic agents.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


Antitumor activity of a membrane lytic peptide cyclized with a linker sensitive to membrane type 1-matrix metalloproteinase

Jieying Zhong and Ying Chau

Mol Cancer Ther 2008;7:2933-2940.

Updated version
Access the most recent version of this article at:
http://mct.aacrjournals.org/content/7/9/2933

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2008/09/05/7.9.2933.DC1

Cited articles
This article cites 42 articles, 11 of which you can access for free at:
http://mct.aacrjournals.org/content/7/9/2933.full.html#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.