Effect of Hydrophobicity on the Anticancer Activity of Fatty-Acyl-Conjugated CM4 in Breast Cancer Cells

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ABSTRACT: Antimicrobial peptides (AMPs) are important anticancer resources, and exploring AMP conjugates as highly effective and selective anticancer agents would represent new progress in cancer treatment. In this study, we synthesized C4–C16 fatty-acyl-conjugated AMP CM4 and investigated its physiochemical properties and cytotoxicity activity in breast cancer cells. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and reversed-phase high-performance liquid chromatography (RP-HPLC) showed that long-chain fatty acyl (≥C12) conjugation prevented N-acyl-CM4 from trypsin hydrolysis. RP-HPLC and circular dichroism (CD) spectra showed that the hydrophobicity and helical content of N-acyl-CM4 increased with the acyl length. The acyl chain length was positively related to the cytotoxicity of C8–C16 conjugates, and C12–C16 fatty acyl conjugates exhibited significant cytotoxicity against MX-1, MCF-7, and MDA-MB-231 cells, with IC50 values <8 μM. Flow cytometry and confocal laser scanning microscopy results showed that N-acylated conjugation significantly increased the membrane affinity in breast cancer cells, and C12–C16 acyl conjugates were capable of translocating to the intracellular space, thereby targeting mitochondria and inducing apoptosis. N-acyl-CM4 showed low cytotoxicity against normal mammalian cells and erythrocytes, especially ≤C12 fatty acyl conjugates, exhibiting selective cytotoxicity to breast cancer cells. The current work indicated that increasing hydrophobicity by attaching long fatty acyl (≥C12) to AMPs may be an effective method to improve the anticancer activity, together with selectivity and resistance to trypsin hydrolysis. This finding provides a good strategy to develop AMPs as effective anticancer agents in the future.

INTRODUCTION

Cancer is a major public health problem worldwide and represents a major cause of death. Breast cancer is the most common cancer and the leading cause of cancer-related deaths among women worldwide, and approximately 2.1 million newly diagnosed female breast cancer cases were reported in 2018. With active screening for early detection and advancements in treatment, there has been a significant decrease in mortality from breast cancer. For nonmetastatic breast cancer, patients are increasingly choosing surgical resection (mastectomy and lumpectomy) or removal of axillary lymph nodes, with the purpose to eradicate tumor from the breast and regional lymph nodes. To minimize the risk of metastatic recurrence, radiation therapy, cytotoxic chemotherapy, and targeted therapy for different subtypes were considered. Chemotherapy regimens (such as anthracyclines and taxanes) are recommended in the vast majority of triple-negative, human epidermal growth factor receptor-2 (HER2)-positive breast cancers, high-risk luminal-like HER2-negative cancers, estrogen receptor (ER)-negative cancers, or ER-positive cancers that have become resistant to endocrine therapy, or cancers that are rapidly progressive and/or associated with high visceral tumor burden, and play an effective role in reducing mortality. However, potential resistance development and toxic side effects including gastrointestinal side effects, pain, fatigue, cardiomyopathy, and neuropathy caused by chemotherapy on nontumor cells in patients with breast cancer are common. Therefore, the development of novel and more effective and high-specificity anticancer agents for breast cancer is urgently needed.

Peptides have been widely investigated in cancer treatment because of their important advantages: they are small in size, easy to synthesize and modify, and have excellent tissue penetrability and minimal immunogenicity. They also may have high activity, specificity, and affinity; minimal drug–drug interaction; and biological and chemical diversity. One class of novel anticancer peptides with therapeutic potential is cationic antimicrobial peptides (AMPs). Naturally occurring AMPs are generally 10–40 amino acids in length and carry a net positive charge, which bestows them with antimicrobial, antitumor, and immunomodulatory properties. They are naturally present in biological tissues and are synthesized by the body as part of the innate immune system. AMPs are characterized by their small size, easy synthesis, and high efficiency in stabilizing membranes, thereby damaging them and causing cell death. Moreover, AMPs exhibit minimal toxicity to normal cells and require fewer doses than other agents. AMPs are composed of amino acids, which are classified as cationic or anionic based on their charge. Cationic AMPs are positively charged, which allows them to bind to negatively charged membrane surfaces, thereby increasing membrane permeability and inducing apoptosis.
charge, as well as hydrophobicity and amphipathicity. Currently, more than 230 natural and synthetic AMPs (http://aps.unmc.edu/AP/database/antiC.php) with cytotoxicity against cancer cells have been identified, representing an attractive anticancer resource. Some AMPs exert their anticancer activity by selective recognition of cancer cells via electrostatic interactions exerting a direct membranolytic effect on cancer cells, while other nonmembranolytic AMPs may simply traverse the membrane and access the intracellular compartment to disrupt the mitochondria and then induce programmed cell death of cancer cells. However, two main problems remained to be addressed for AMPs: (1) the limited anticancer activity and (2) specificity toward tumor cells. Thus, the successful generation of anticancer AMPs with high cytotoxicity to cancer cells and low cytotoxicity to normal cells (selectivity) remains challenging. Many AMPs show a negative correlation between anticancer activity and selectivity, which may result from the interaction of various structural parameters, such as net charge, secondary structure, amphipathicity, and hydrophobicity. To find a balance considering the effects of various structural parameters, AMPs are modified to enhance their antimicrobial activity, such as improving hydrophobicity through fatty acid conjugation.

Antimicrobial peptide CM4, isolated from the hemolymph of the Chinese silkworm Bombyx mori, is a cationic linear peptide with 35 amino acids. It is an effective AMP against bacteria and fungi by disrupting the plasma membrane. Besides antimicrobial activity, CM4 also has anti-inflammatory effects mediated by the neutralization of the endotoxin lipopolysaccharide. It has no hemolytic activity to human erythrocytes at a concentration of 200 μM and even shows very low cytotoxicity to normal mammalian cells. Although cytotoxicity to leukemia cells is observed, CM4 exhibits very limited anticancer activity. Its high selectivity and limited anticancer activity provide a proper template for modification. In a recent work from our lab, we attached myristoyl, a 14-carbon saturated fatty acid, to the N-ter minus of CM4 and found that myristoyl conjugation significantly enhances the anticancer activity of the CM4 against breast cancer cells (MX-1, MCF-7, and MDA-MB-231) and leukemia cells (K562/MDR and Jurkat) in vitro. Myristoyl conjugation also significantly enhanced the depression effect of tumor growth to triple-negative breast cancer cells MDA-MB-231 xenograft tumors. This may imply that increasing hydrophobicity by attaching fatty acid may be an effective method to enhance the anticancer activity of AMPs. In the meantime, we observed relatively low cytotoxicity of myristoyl-CM4 against healthy cells compared with breast cancer cells. Thus, a deeper understanding of the effects of modulating hydrophobicity by changing the acyl chain length on anticancer activity and selectivity is needed.

In this paper, fatty acyl chains ranging from 4 to 16 carbon units in length were conjugated to the N-terminus of the CM4. We explored the effect of fatty acid acylation of the peptide on its physiochemical properties and anticancer activity against breast cancer cells in vitro. The interaction between N-acylated CM4 and the breast cancer cell membrane was also investigated. The current paper indicated that increasing hydrophobicity by attaching long fatty acid (≥C12) to AMPs may be an effective method to improve the anticancer activity. The effect of N-acylated CM4 on the selectivity was also analyzed.

### RESULTS

#### N-Acyl-CM4 Sequence, Secondary Structure, and Hydrophobicity

Acyl chains of varying lengths (C4, C6, C8, C10, C12, C14, and C16) were conjugated to the N-terminus of the CM4 peptide, and the resulting peptides were cationic with a net charge of +5 at neutral pH (Table 1). Acyl chains were linked to the α-NH₂ of Arg 1 of CM4 by an amide bond (Figure 1A). The hydrophobicity of N-acyl-CM4 was compared by reversed-phase high-performance liquid chromatography (RP-HPLC) analysis. The percentage of acetonitrile (ACN%) corresponding to the elution peak of each peptide was calculated. The higher the ACN%, the higher the hydrophobicity of the peptide.

### Table 1. Physiochemical Properties of All Peptides

| name | sequence | net charge | % helix (50% TFE) | ACN% |
|------|----------|------------|------------------|------|
| CM4  | RWKIFKIEKVQNRDGIVKAGPAVAVGQAATI-NH2 | +6 | 30.4 | 39.85 |
| C4-CM4 | butyric acid-RWKIFKIEKVQNRDGIVKAGPAVAVGQAATI-NH2 | +5 | 31.1 | 44.43 |
| C6-CM4 | caproic acid-RWKIFKIEKVQNRDGIVKAGPAVAVGQAATI-NH2 | +5 | 37.9 | 46.56 |
| C8-CM4 | octanoic acid-RWKIFKIEKVQNRDGIVKAGPAVAVGQAATI-NH2 | +5 | 38.6 | 48.87 |
| C10-CM4 | decanoic acid-RWKIFKIEKVQNRDGIVKAGPAVAVGQAATI-NH2 | +5 | 41.7 | 51.20 |
| C12-CM4 | lauric acid-RWKIFKIEKVQNRDGIVKAGPAVAVGQAATI-NH2 | +5 | 42.7 | 53.73 |
| C14-CM4 | myristic acid-RWKIFKIEKVQNRDGIVKAGPAVAVGQAATI-NH2 | +5 | 53.5 | 55.95 |
| C16-CM4 | palmitic acid-RWKIFKIEKVQNRDGIVKAGPAVAVGQAATI-NH2 | +5 | 55.1 | 58.01 |

*ACN%: percentage of acetonitrile corresponding to the elution peak of the peptide by RP-HPLC analysis.

In this paper, fatty acyl chains ranging from 4 to 16 carbon units in length were conjugated to the N-terminus of the CM4.
the ACN%, the stronger the hydrophobicity. As shown in Table 1, the ACN% increased with increasing acyl chain length, indicating that hydrophobicity was positively correlated with the fatty acid chain length. The order of hydrophobicity is as follows: CM4 < C4-CM4 < C6-CM4 < C8-CM4 < C10-CM4 < C12-CM4 < C14-CM4 < C16-CM4. The secondary structure of N-acyl-CM4 was determined by circular dichroism (CD) spectroscopy in water and 50% 2,2,2-trifluoroethanol (TFE, Figure 1B). In water solution, all of the peptides exhibited the characteristics of random coils, featuring a single large negative peak at approximately 198 nm. In 50% TFE, all of the N-acyl-CM4 peptides exhibited a positive band at 195 nm and two negative bands at 208 and 222 nm, indicating that N-acyl-CM4 underwent a significant structural rearrangement from random coil to α-helix upon the addition of 50% TFE. CDNN software calculations showed that acyl conjugation increased the helical contents of acyl-CM4, compared with those of CM4. Furthermore, the α-helix content increased in correlation with the increased length of the fatty acyl chain.

**Effect of N-Acylation on the Sensitivity to Trypsin.** An important obstacle to the application of AMPs is the degradation of peptides by innate proteases or those existing in the environment, which leads to low bioavailability. CM4 contains Arg 1, 16 and Lys 3, 6, 7, 10, and 21 residues, which makes it more sensitive to trypsin digestion. Whether N-acylation affects this biochemical property remains unknown. We therefore performed trypsin treatment for 60 min followed by Tricine/SDS-PAGE (A) and RP-HPLC (B) analyses.

![Figure 2](https://dx.doi.org/10.1021/acsomega.0c02093)

**Figure 2.** Effect of N-acylation on the sensitivity of N-acyl-CM4 to trypsin digestion. Peptides were incubated with trypsin (10 μg/mL) at 37 °C for 60 min followed by Tricine/SDS-PAGE (A) and RP-HPLC (B) analyses.
and C4-CM4 were degraded completely. C8-CM4 and C10-CM4 were degraded partly. C12-CM4, C14-CM4, and C16-CM4 bands remained in the gel after trypsin treatment. RP-HPLC analysis also confirmed that C12-CM4, C14-CM4, and C16-CM4 showed resistance to trypsin treatment (Figure 2B). However, after trypsin treatment, C4-CM4 and C6-CM4 were degraded completely and C8-CM4 and C10-CM4 were degraded partly. These data suggest that long-chain fatty acid (≥C12) conjugation protects N-acyl-CM4 from trypsin hydrolysis.

Chain-Length-Dependent Cytotoxicity of N-Acyl-CM4 in Breast Cancer Cells. Three breast cancer cell lines MX-1, MCF-7, and MDA-MB-231, derived from mammary gland epithelial cells, were used in the following study. MX-1 cells were an ER− cell line. MCF-7 cells were an ER+ cell line. MDA-MB-231 cells were an aggressive and invasive triple-negative breast cancer (ER−/PR−/HER2−) cell line. The cytotoxicity of N-acyl-CM4 peptides was assessed using the CCK-8 assay. CM4 (8 μM) showed no cytotoxicity in breast cancer cells (Figure 3A). C4-CM4 and C6-CM4 showed no significant cytotoxicity against all breast cancer cells at 8 μM (P < 0.05). However, conjugation of C8, C10, C12, C14, and C16 fatty acyl chains significantly increased their cytotoxicity against MX-1 cells (P < 0.001); the IC50 in MX-1 cells was <4 μM; and (3) the cell line showed sensitivity to N-acyl-CM4 in the following order: MX-1 > MCF-7 > MDA-MB-231. The trypan blue assay was performed to confirm the cytotoxicity in the most-sensitive cell line, MX-1 (Figure 3C,D). After treatment with 6 μM N-acyl-CM4 for 24 h, C8-CM4 treatment and C10-CM4 treatment resulted in death rates of 40 and 60% in MX-1 cells, respectively. The death rate was >80% after treatment with C12-CM4, C14-CM4, and C16-CM4.

N-Acyl-CM4 Showed Low Cytotoxicity in Normal Cells. The cytotoxicity of N-acyl-CM4 peptides in normal mammalian cells (human liver cells L02 and normal mammary epithelial cells MCF-10A) was determined (Figure 4A). After treatment with 10 μM N-acyl-CM4, more than 90% of cells remained viable in L02 and MCF-10A. More than 60% of cells remained viable after treatment with 30 μM N-acyl-CM4. These data indicated that N-acyl-CM4 exhibited lower cytotoxicity against L02 and MCF-10A cells than MX-1, MCF-7, and MDA-MB-231 breast cancer cells. Hemolytic assays using mouse erythrocytes showed that C4-CM4, C6-CM4, C8-CM4, C10-CM4, and C12-CM4 showed low hemolysis (lower than 8%); C14-CM4 and C16-CM4 showed about 20 and 30% hemolysis at this concentration. At a concentration of 50 μM, C4−C12-conjugated CM4 showed <20%, hemolysis; however, C14-CM4 and C16-CM4 showed enhanced hemolysis, with about 60% hemolysis observed.
Chain-Length-Dependent Membrane Affinity of N-Acyl-CM4 in Breast Cancer Cells. C4-CM4, C6-CM4, C8-CM4, C10-CM4, C12-CM4, C14-CM4, and C16-CM4 were labeled with FITC (3 μM) and fluorescence was detected by fluorescence-activated cell sorting (FACS) analysis after incubation. The fluorescence intensity of 10⁶ cells was collected in this study. Compared with CM4, C4−C16 fatty acyl conjugation significantly increased the fluorescence intensity for all tested cells, including breast cancer cells MX-1, MCF-7, and MDA-MB-231 and normal mammalian cells L02 and MCF-10A (P < 0.001, Figure 5A). FITC fluorescence intensity significantly reached to a very high level after incubation with FITC-C14-CM4, and FITC-C16-CM4, especially in MX-1 and MCF-7 cells, increasing by 20−30 fold (Figure 5B). For C14-CM4 and C16-CM4, a higher affinity for MX-1 and MCF-7 cells than that for normal L02 and MCF-10A cells was observed, which showed approximately fourfold increased affinity. There were no significant differences between normal mammalian cells (L02 and MCF-10A) and breast cancer cells (MCF-7, MX-1 and MDA-MB-231) on the FITC fluorescence binding for C4−C8 fatty acyl conjugation (P < 0.05, Figure 5C). These data implied that (1) the membrane affinity of N-acyl-CM4 increased in a chain-length-dependent manner both for breast cancer cells and normal cells, (2) C14 and C16 fatty acid conjugation significantly enhanced the membrane affinity for breast cancer cells, and (3) the general trend indicated that the membrane affinity of N-acyl-CM4 was lower for normal cells than that for breast cancer cells.

Figure 4. Effects of N-acylation on the cytotoxicity of N-acyl-CM4 in normal cells. (A) Comparison of the cell viability of L02 and MCF-10A cells treated with N-acyl-CM4 by the CCK-8 assay. (B) Hemolytic activity was tested in mouse erythrocytes; melittin was used as a control. Results are the mean ± SEM of 4−6 experiments.

Figure 5. Affinity of FITC-acyl-CM4 in breast cancer cells and normal mammalian cells. (A) Cells were incubated with 3 μM FITC-labeled peptides for 30 min at 37 °C and analyzed by flow cytometry. Results are the mean ± SEM of three different experiments. The multiple relations of membrane affinity were analyzed for N-acylated CM4 vs CM4 (B) and breast cancer cells vs normal mammalian cells (C). *p < 0.05, **p < 0.01, ***p < 0.001 between N-acyl-CM4 treatment and CM4 treatment.
no intracellular FITC fluorescence was observed, indicating that strong binding did not necessarily lead to transmembrane transport. Generally, membrane affinity and transmembrane translocation capacity were positively correlated with the length of conjugated fatty acyl chains, and peptides conjugated with C12 and longer chains had the ability to translocate into breast cancer cells.

Detection of colocalization using MitoRed, a specific probe for localizing mitochondria in living cells, showed that FITC-C12-CM4, FITC-C14-CM4, and FITC-C16-CM4 localized to mitochondria in the three breast cancer cell lines. In MX-1 cells, obvious colocalization was also detected after FITC-C10-CM4 treatment in breast cancer cells. These results indicated that after transported into the intracellular compartment, N-acyl-CM4 had the ability to localize to mitochondria.

**Cytotoxic Effect Induced by Different Chain Lengths of N-Acyl-CM4.** Necrosis and apoptosis are the two main mechanisms of cytotoxicity. Propidium iodide (PI)/Annexin V staining was used to examine the effects of N-acylated-CM4 (Figure 7A). After 16 h of treatment, early-stage apoptotic cells (PI−/Annexin V+) were detected in response to treatment with C12-CM4, C14-CM4, and C16-CM4, indicating that C12, C14, and C16 fatty-acyl-conjugated CM4 had the ability to induce apoptosis in MX-1, MCF-7, and MDA-MB-231 cells. Especially in MX-1 cells, C10-CM4 also induced apoptosis. Hoechst 33342 staining was used further to identify apoptosis (Figure 7B). Apoptotic nuclei were observed in MX-1, MCF-7, and MDA-MB-231 lines after treatment with C12-CM4, C14-CM4, and C16-CM4. In MX-1 cells, apoptotic nuclei were also observed after treatment with C10-CM4.

**DISCUSSION**

Despite several beneficial characteristics of AMPs such as antibacterial, antifungal, and anticancer properties, there are several obstacles that limit the application of these peptides. One of the most important obstacles is the degradation of peptides by innate proteases or those present in the environment, which leads to low bioavailability. Thus, a bifunctional peptide possessing both anticancer and protease inhibitory activities would be an ideal template for future clinical use. AMPs are cationic peptides and contain several positively charged amino acids, such as Arg and Lys, which make them sensitive to digestion by serine proteinase trypsin. Many strategies have been proposed to overcome the sensitivity to degradation, including chemical modification of the peptide, incorporation of α amino acids, cyclization, and polymer or hyperbranched polyglycerol conjugation. However, some of these strategies affect the biological activity of the peptides. In this study, we incubated N-acyl-CM4 with trypsin for 1 h and characterized the peptide fragments generated by Tricine-SDS-PAGE and RP-HPLC. The results showed that conjugation with ≥C12 fatty acyl chains did not prevent proteolysis effectively. However, ≥C12 fatty-acyl-conjugated CM4 showed resistance to proteolysis by trypsin. This indicated that long fatty acyl modification may be an effective method to prevent trypsin-mediated degradation of AMPs.

We next determined whether N-acylation had an effect on the anticancer activity of the peptides. Acyl chain conjugation improved hydrophobicity, as demonstrated by RP-HPLC analysis, and the length of the acyl chain was positively correlated with the hydrophobicity of N-acyl-CM4. The effect of fatty acyl conjugation on improving hydrophobicity has been widely investigated in antimicrobial research. In a study on the effect of acylation of AMPs on the antibacterial activity, fatty acyl chains of different lengths (C12–C20) were introduced at the N-terminal end of AKK- and LKK-motif AMPs, and the results indicated that the conjugates, including C14–C18 fatty acyl chains, dramatically improved the antibacterial activity for several bacterial strains. Here, we found that improving hydrophobicity through C4 and C6 conjugation did not
significantly improve the anticancer activity of the peptide in breast cancer cells. Long acyl chain conjugation (C12, C14, and C16) resulted in effective anticancer activity in all tested breast cancer cells, with IC\textsubscript{50} < 8 μM. In addition, compared with native CM4, C14 and C16 conjugates showed strong membrane binding abilities in MX-1, MCF-7, and MDA-MB-231 cells. Short acyl (C4 and C6) and medium acyl chain (C8 and C10) conjugation also increased the membrane-binding capacity of CM4; however, these peptides did not show effective anticancer activity in MCF-7 and MDA-MB-231 cells. Further experiments showed that long acyl chain (≥C12 fatty acyl) conjugation conferred peptides the ability to translocate to the intracellular compartment of MX-1, MCF-7, and MDA-MB-231 cells. The helical wheel diagram of CM4 showed the N-terminus to be a typical amphipathic.\textsuperscript{18} We speculate that the N-terminal fatty acid chain conjugation may locate on the hydrophobic face of CM4, which would contribute to the stronger peptide–membrane interaction. Hydrophobic moieties in the AMP sequence play a significant role in binding and in promoting the antibacterial activity of the peptide.\textsuperscript{28} Peptides with high hydrophobicity can penetrate deeper into the hydrophobic core of the cell membrane.\textsuperscript{29} Thus, strong binding did not necessarily lead to transmembrane transport. Besides the binding activity, enough hydrophobicity is needed for transmembrane transport. Our study indicated that ≥C12 fatty acyl conjugation had stronger hydrophobicity, which may lead to a stronger interaction with the cell membrane and then result in the transport of the peptide into the intracellular space and play effective anticancer activity.

In addition to hydrophobicity, the helical structure of peptides is another important parameter modulating activity. Both CM4 and N-acyl-CM4 peptides displayed “random coil” structures in an aqueous buffer. In the membrane-mimetic environment of the TFE solution, the N-acyl-CM4 underwent an α-helical conformational transition. The α-helix content increased in correlation with the increase in the length of the fatty acyl chain. For example, the helical content increased from 30.4% in CM4 to 55.1% in C16-CM4. This indicated that the hydrophobicity from the acyl chain conjugation drove the peptide to form a helix, leading to higher helicity. Other groups have observed that

Figure 7. Apoptosis and necrosis in breast cancer cells induced by N-acyl-CM4. (A) After treatment by N-acyl-CM4 for 16 h, cells were stained by PI/Annexin V and then analyzed by FACS. (B) Hoechst 33342 staining was detected by fluorescence microscopy. The concentrations of N-acyl-CM4 were the same as in the PI/Annexin V assay.
acylation in peptides or proteins can also enhance the helical structure.\(^ {29} \) Indeed, helicity plays a crucial role in the activity of AMPs, and the formation of a secondary structure is considered as a driver for peptide insertion into the membrane.\(^ {30} \) We assume that the higher hydrophobicity and helicity resulting from long acyl chain (C12–C16) conjugation may contribute to more effective interaction between the peptide and the breast cancer cell membrane, resulting in the cellular entry of these peptides into breast cancer cells. A previous report showed that high hydrophobicity generated from tryptophan end-tagging of the GRR10 peptide efficiently mediated its internalization in melanoma cells.\(^ {31} \) Several similar reports showed that acylation drastically increased in vitro intestinal peptide flux and conferred transient permeability.\(^ {32,33} \) Our recent report showed that C14 acyl can efficiently mediate peptide internalization in leukemia cells.\(^ {18} \) Lipid modification was also previously shown to enhance the passive transport of some drugs mediated by the lipid moiety, and lipid-conjugation strategies can increase antitumor efficacy by increasing cell permeability and the retention of anticancer agents.\(^ {34} \) Covalent attachment of C14 myristate or C16 palmitate is a post-translational protein modification that can promote membrane binding of the modified protein, and palmitate is sufficiently hydrophobic to achieve strong association with the lipid bilayer by itself or in combination with additional lipids.\(^ {34,35} \) C14 and C16 fatty acyls are used in conjugation with antibiotics as a lipid-antibiotic prodrug.\(^ {36,37} \) The current findings suggest that there is a threshold level of hydrophobicity for N-acyl-CM4 and a certain helical content. When it exceeds the threshold level, it drives the N-acyl-CM4 to cross the membrane into the intracellular space. Long acyl chain (≥C12 fatty acyl) conjugation appears to be above the threshold level, and long acyl chain conjugates, therefore, efficiently mediated the internalization of the peptide in three breast cancer cell lines. Furthermore, in MX-1 cells, ≥C10 fatty acyl conjugation conferred the peptide the ability to translocate into the cell, indicating that the membrane of MX-1 cells was more sensitive to N-acyl-CM4.

Normally, increasing the hydrophobicity of AMPs decreases their selectivity for the bacterial membrane, as hydrophobic forces contribute to nonselective binding because the peptide cannot distinguish eukaryotic from prokaryotic cell membranes on the basis of hydrophobicity.\(^ {17,25} \) In a previous study, we showed that CM4 is very less toxic to normal mammalian cells and erythrocytes.\(^ {18} \) After N-acylation, the toxicity to erythrocytes and normal mammalian cells (L02 cells and MCF-10A) increased. Among C4–C16 conjugates, hemolysis was still very low in C4–C12 conjugates. C14 and C16 conjugates showed relatively increased hemolysis. Peptides with higher hydrophobicity can readily enter zwitterionic membranes and cause hemolytic effects.\(^ {20} \) A higher hydrophobicity by fatty acyl conjugation increases the membrane affinity to normal mammalian cells. However, N-acyl-CM4 showed low cytotoxicity, as >90% of L02 and MCF-10A cells remained viable under 10 μM N-acyl-CM4 treatment, and more than 60% were viable under 30 μM N-acyl-CM4 treatment, indicating selective cytotoxicity to breast cancer cells. Affinity analysis revealed that the lower affinity for normal mammalian cells than breast cancer cells, especially for C14-CM4 and C16-CM4. We speculated that the net positive charge was the main factor that modulated the anticancer selectivity of N-acyl-CM4. The upregulation of sialic acid-containing molecules or elevated levels of phosphatidylserine on the outer membrane leaflet of leukemia cells may contribute to the higher binding of C14-CM4 to cancer cells than to normal cells.\(^ {19,20} \) Our current study suggests that increasing the hydrophobicity of AMPs may not decrease their selectivity for the cancer cell membrane, given that there is an electronegativity difference between cancer cells and normal mammalian cells that contributes to the selective binding of N-acyl-CM4 on the basis of charge. Our data showed that increasing the hydrophobicity of AMPs may enhance the membrane affinity significantly both to breast cancer cells and normal mammalian cells. The affinity of C4–C10 fatty acyl conjugates was similar to that of both breast cancer cells and normal mammalian cells. However, N-acyl-CM4 showed lower cytotoxicity to L02 and MCF-10A cells than MDA-MB-231 cells. Together with CLSM observation, our results indicated that strong binding did not necessarily lead to transmembrane transport. Therefore, even though the binding of C12–C16 fatty acyl conjugates to MCF-10A was not much lower than MDA-MB-231 cells, C12–C16 fatty acyl conjugates exhibited much lower cytotoxicity than MDA-MB-231 cells. Actually, strong binding of C12-CM4 and C14-CM4 was observed in MCF-10A cells by CLSM observation but no distributed in the cellular space (data not shown). Thus, in addition to the difference in binding capacity, there may be other factors that determine the selective cytotoxicity in breast cancer cells, such as unknown membrane structure differences between cancer cells and normal mammalian cells. Further study is needed in the future. In addition, erythrocytes were more sensitive than normal mammalian cells to N-acyl-CM4. Especially for C14–C16 acyl conjugation, obvious hemolytic effects were observed at a relatively high concentration, indicating that hydrophobicity is important for the interaction of N-acyl-CM4 with the membrane of erythrocytes.

Necrosis and apoptosis are the two main mechanisms mediating the anticancer activity of α-helical AMPs. Strong direct interaction with the plasma membrane could lead to necrosis. Entry of AMPs into cells often leads to apoptosis.\(^ {40,41} \) Our previous study showed that when C14-CM4 is internalized into leukemia cells and breast cancer cells, it induces mitochondria-dependent apoptosis.\(^ {19,20} \) In this study, we showed that C12-CM4 and C16-CM4 could enter into cells and target mitochondria. Accordingly, C12–C16 fatty-acyl-conjugated CM4 induced apoptosis in all tested breast cancer cell lines. C8-CM4 and C10-CM4 also had the ability to enter into MX-1 cells, which caused apoptosis of MX-1 cells. These data suggested that N-acyl-CM4 with the ability to enter into cells would induce apoptosis as a mechanism mediating its cytotoxicity to breast cancer cells. Future studies need to elucidate the detailed anticancer mechanisms.

**CONCLUSIONS**

The results of this study indicate that conjugating fatty acyl chains to the peptide CM4 can increase its hydrophobicity, α-helix content (helicity), and cytotoxicity against breast cancer cells in relation to the length of the fatty acyl chain. Acyl conjugation increased the interaction with the plasma membrane, and ≥C12 fatty acyls were efficient in mediating the cellular entry of N-acyl-CM4 into breast cancer cells and the induction of apoptosis. The present findings indicate that the higher hydrophobicity and helicity resulting from N-acylation may contribute to more effective interaction between N-acyl-CM4 and the breast cancer cell membrane. Compared with normal mammalian cells and erythrocytes, N-acyl-CM4 shows selective cytotoxicity to breast cancer cells, especially for ≤C12 conjugates. In addition, C12–C16 conjugates showed resistance...
to trypsin-mediated proteolysis. Taken together, the present results suggest that increasing hydrophobicity by attaching long fatty acyl chains would be an effective method to improve the anticancer activity of CM4, and achieving appropriate hydrophobicity is important to develop N-acyl-CM4 as a selective anticancer drug. Long-chain fatty acyl (≥C12) conjugation is an effective strategy for developing N-acyl-CM4 as a bifunctional peptide that is both resistant to trypsin hydrolysis and an effective intracellular targeting anticancer drug, and C12 conjugation seems more effective to balance the efficiency and selectivity. This finding may potentially improve next-generation acylated AMPs as effective anticancer drugs in the future.

**MATERIALS AND METHODS**

**Peptide Synthesis and Reagents.** CM4, N-acylated CM4, and FITC-labeled N-acylated CM4 were synthesized using solid-phase Fmoc methods by Synpeptide Inc. (Nanjing, China). The synthesized peptides were all >95% homogeneous as indicated by C18 reverse-phase HPLC and electrospray ionization (ESI) mass spectrometry analyses.

RPMI-1640 and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Thermo Fisher Scientific (Waltham, MA), fetal bovine serum (FBS) was purchased from Capricorn Scientific (Hessen, Germany). DMEM/F12 and horse serum were purchased from Invitrogen. TFE was purchased from Sigma-Aldrich (St. Louis, MO). Melittin was purchased from Synpeptide, Inc. (Nanjing, China). Hoechst 33342 staining kit was purchased from Beyotime Institute of Biotechnology (Shanghai, China). CCK-8 kit, Annexin V/PI assay kit, and MitoRed (mitotracker red) were purchased from KeyGEN BioTECH, Inc. (Nanjing, China). A predestined protein marker (3.1–20.1 kDa) was purchased from Solarbio (Beijing, China). All other reagents were of analytical grade and produced in China. All of the reagents were used by the rules of standard biosecurity and safety procedures of Nanjing Normal University.

**Circular Dichroism (CD) Spectra.** CM4 and N-acyl-CM4 were diluted in 50% TFE to a final concentration of 250 μg/mL. CD spectral measurements were performed in a quartz cuvette with a 0.1 cm path length at room temperature. Samples were scanned from 180 to 270 nm at 0.1 nm/min with a Chirascan CD spectral measurements were performed in a quartz cuvette. The CDNN program was used to calculate the helical content of each peptide.

**RP-HPLC Analysis.** Peptide samples were analyzed on a Waters Alliance 2695 HPLC using a SHIMADZU Inertsil ODS-SP column (4.6 × 250 mm², 5 μm) with linear A/B gradient elution at 40 °C. Solvent A was 0.1% aqueous trifluoroacetic acid (TFA) in acetonitrile, and solvent B was 0.1% TFA in 100% water. For peptide runs, the elution linear A and B gradient was 0–25 min: started at 20% solvent A and 80% solvent B, followed by 25 min at 80% solvent A and 20% solvent B, at a flow rate of 1 mL/min. Signals were detected at 220 nm. The percentage of acetonitrile (ACN%) for each peptide was calculated by the elution peak and gradient elution curve.

**Protease Sensitivity Assay.** Peptides (final concentrations, 1 mM) were incubated with 10 μg/mL protease trypsin at 37 °C for 60 min. Then, each sample was incubated at 100 °C in a water bath for 5 min with 0.1% SDS and 1% 2-mercaptoethanol. Tricine/SDS-PAGE was performed with a 4% stacking gel, 10% spacer gel, and 16.5% resolving gel. The gel was stained with Coomassie blue R-250. Samples were also loaded on a Shimadzu Inertsil ODS-SP column to conduct RP-HPLC analysis as described above. Briefly, the elution linear solvent A (0.1% aqueous TFA in acetonitrile) and solvent B (0.1% TFA in 100% water) gradient was conducted from 0 to 25 min at a flow rate of 1 mL/min. Solvent A was increased from 20 to 80%; solvent B (0.1% TFA in 100% water) was decreased from 80 to 20%. Signals were detected at 220 nm.

**Cell Lines and Cell Culture.** The breast cancer cell lines MCF-7, MX-1, and MDA-MB-231 and the normal mammalian cells L02 and MCF-10A were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. All of the cell lines used in experiments were cultured at 37 °C in a 5% CO₂ humidified atmosphere. MCF-7 and MDA-MB-231 cells were maintained in DMEM with 10% FBS. L02 and MX-1 cells were maintained in RPMI-1640 medium with 10% FBS. All of the media used in experiments were supplemented with 100 μg/mL streptomycin, 100 U/mL penicillin, and 10% FBS. MCF-10A cells were maintained in DMEM/F12 with 5% horse serum.

**Cell Viability Assay.** Cells were cultured in 96-well plates at a density of 1 × 10⁵ cells/mL in medium supplemented with 1% FBS and then treated with different concentrations (2, 4, and 8 μM) of the peptides with six replicates for each treatment. After treatment for 24 h, the CCK-8 stock solution was added to each well for 2 h. The absorbance was measured at 450 nm using a Synergy H1 multifunction microplate reader.

**Hemolytic Activity Assay.** Erythrocytes were isolated from fresh mouse blood cells by centrifugation at 1000g for 10 min and washed three times with PBS. The hemolytic activity was evaluated using a method described previously. Briefly, erythrocytes (final concentration, 4% v/v) were treated with N-acyl-CM4 for 1 h at 37 °C, followed by centrifugation at 1000g for 5 min. The absorbance of the supernatants was measured at 414 nm. For 100% hemolysis and 0% hemolysis, 0.1% Triton X-100 (v/v) and PBS were used, respectively. Melittin, a hemolytic peptide from bee *Apis mellifera*, was used as a control. The percentage of hemolysis was calculated as follows: \((A_{\text{peptide}} - A_{\text{PBS}})/(A_{\text{TritonX-100}} - A_{\text{PBS}})\) × 100%. Data are reported as the mean ± SEM of 4–6 independent experiments.

**Peptide Binding Assay.** Cells (2 × 10⁵ cells/mL) were collected and resuspended in PBS. The binding activities of the peptides were assessed using 3 μM FITC-acyl-CM4. Briefly, after incubation with the peptide at 37 °C for 30 min in the dark, cells were washed with PBS and analyzed with a FACS Vantage SE flow cytometer at 530 nm emission. The mean fluorescence of 10⁴ cells was analyzed for each sample using BD flow cytometry software. The autofluorescence of untreated cells was subtracted. Data are reported as the mean ± SEM of three independent experiments.

**Confocal Laser Scanning Microscopy (CLSM) Observation.** Cells (MX-1, MCF-7, and MDA-MB-231) were seeded in a glass-bottom culture dish (10⁵ cells/dish) and maintained in DMEM or RPMI-1640 medium at 37 °C for 12 h. After being rinsed by PBS for three times, cells were incubated with 500 nM MitoRed for 30 min in the dark, rinsed three times with PBS, and then maintained in the DMEM or RPMI-1640 medium containing 1 μM FITC peptide at 37 °C for 4 h in the dark. Then, the cells were rinsed three times with PBS and analyzed immediately by a Nikon ECLIPSE CLSM with 488 nm excitation and 580 nm emission wavelengths for FITC and 560 nm excitation and 580 nm emission wavelengths for MitoRed signal detection.

**Annexin V/PI Staining and Hoechst 33342 Staining Assay.** The effects of apoptosis induced by N-acyl-CM4 in MX-
three times by PBS, cells were observed and photographed. Cells were stained with Hoechst 33342 reagent. The stained cells were analyzed by percentage of cells in different Annexin V binding buffer quadrants. Annexin V was collected and then washed with PBS. Some cells were suspended in Annexin V binding buffer and then added to an Annexin V-FITC solution and PI for 10 min at room temperature in the dark. The stained cells were analyzed by flow cytometry with 525 nm emission wavelength for FITC and 620 nm emission wavelength for PI signal detection. Annexin V−/PI− indicates live cells, Annexin V+/PI− indicates early-stage apoptotic cells, Annexin V+/PI+ indicates late-stage apoptotic cells and necrosis cells, and Annexin V−/PI+ indicates necrosis cells. The percentage of cells in different quadrants was calculated using Cell Quest software. Some cells were detected by Hoechst 33342 staining. Cells were stained with Hoechst 33342 reagent (v/v at 1:200) at 37 °C for 20 min in the dark. After washing three times by PBS, cells were observed and photographed under a Leica DMI6 fluorescence microscope.

**Statistical Analysis.** Values are expressed as the mean ± SEM of 3–6 independent experiments. Two-tailed Student’s t-test and one-way ANOVA with Dunnett’s multiple comparison test were used to determine the significance of differences. P < 0.05 was considered statistically significant. Statistical analysis was assessed using the Statistical Package for the Social Sciences (SPSS/PC 20.0, Chicago, IL).

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**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS**

DMEM, Dulbecco’s modified Eagle’s medium; PI, propidium iodide; FITC, fluorescein-5-isothiocyanate; TFE, 2,2,2-trifluoroethanol; TFA, trifluoroacetic acid; HER-2, human epidermal growth factor receptor-2; ER, estrogen receptor; RP-HPLC, reversed-phase high-performance liquid chromatography; PBS, phosphate-buffered saline; MitoRed (mitotracker red); 9-[(4-chloromethyl)phenyl]-2,3,5,6,7,12,13,16,17-octahydro-1H,5H,11H,15H-xantheno[2,3,4-ij:5,6,7-ij′]quinolinolizin-18-um chloride

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