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CXCR4-Targeted Necrosis-Inducing Peptidomimetic for Treating Breast Cancer

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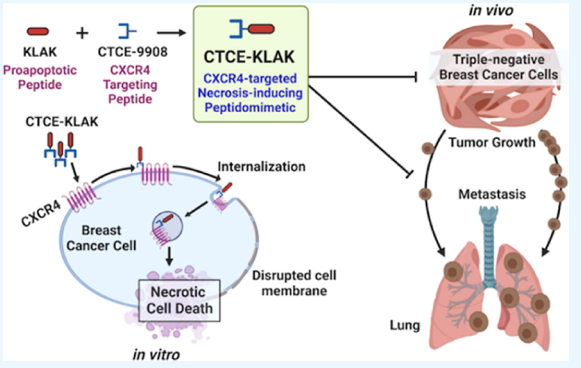
ABSTRACT: Triple-negative breast cancer is an aggressive subtype with a high recurrence rate, potential for metastasis, and a poor prognosis. The chemokine receptor, CXCR4, is a promising molecular target in breast cancer therapy. Here, we have developed a CXCR4-targeted antitumor peptidomimetic (named CTCE-KLAK), which is a fusion of the CXCR4 receptor antagonist CTCE-9908 and the D-form of proapoptotic peptide $(KLAKLAK)_2$, for the treatment of breast cancer. First, we investigated the *in vitro* antitumor activity of CTCE-KLAK against various breast cancer cells and noncancerous mammary epithelial cells. CTCE-KLAK showed cell-selective cytotoxicity and induced rapid necrotic cell death in breast cancer cells but not in normal cells. In contrast, unconjugated peptides such as the carboxylate analogues of CTCE-9908 and $_D(KLAKLAK)_2$ were not cytotoxic to these cells. The tumor selectivity of CTCE-KLAK for cytotoxic activity depends on its internalization into tumor cells. There was no cleavage of caspase-3, caspase-7, or PARP1 in CTCE-KLAK-treated cells. In addition, cell death by CTCE-KLAK was not prevented by z-VAD-fmk, a pan-caspase inhibitor that inhibits cisplatin-induced cell death. These data indicate that the CTCE-KLAK conjugate is a cell-selective inducer of necrosis. Furthermore, we evaluated the *in vivo* antitumor activity of CTCE-KLAK in the 4T1 mouse metastatic breast cancer model. Intravenous administration of CTCE-KLAK significantly inhibited tumor growth and lung metastasis. Together, these findings suggest that the necrosis-inducing peptidomimetic CTCE-KLAK is a promising CXCR4-targeted agent for treating triple-negative breast cancer.



INTRODUCTION

Breast cancer is the most common malignancy among women and the second highest cause of death in women worldwide.[1,2] Triple-negative breast cancer, which does not express estrogen receptors (ER), progesterone receptors (PgR), or human epidermal growth factor receptor type 2 (HER2), is an aggressive subtype of breast cancer with a poor prognosis. It shows a higher recurrence rate, more significant metastatic potential, and a lower overall survival rate than other breast cancers. For treating this subtype of cancer, molecular-targeted therapies are largely ineffective, and chemotherapy using cytotoxic agents is currently the primary systemic treatment option.[3] To effectively treat triple-negative breast cancer, new therapeutic targets need to be explored.

C-X-C chemokine receptor type 4 (CXCR4) is a chemokine receptor specific for stromal-derived factor-1α (SDF-1α). CXCR4 plays a critical role in tumorigenesis and cancer progression in various types of cancer.[4−6] Some reports showed that CXCR4 is expressed at a low level or absent in normal breast tissue but is highly expressed in primary and metastatic breast tumors.[4,7] Based on this concept, CXCR4 antagonists have been developed. For example, CTCE-9908 is a 17 amino acid peptide that consists of a dimer for the first eight amino acids of SDF-1α. CTCE-9908 competitively inhibits the interaction between the CXCR4 receptor and its ligand SDF-1α.[8−10] CTCE-9908 blocks the function of CXCR4, thus suppressing tumor growth and metastasis in different mouse tumor models.[9−11]

The proapoptotic domain $_D(KLAKLAKKLAKLAK)$ (designated KLAK) was developed as an antimicrobial peptide.[12] The KLAK peptide forms α-helical conformation distributing the cationic residues to one side and the hydrophobic residues to the other, making it amphipathic. The positively charged KLAK peptide attracts the negatively charged mitochondrial membrane electrostatically, disrupting the mitochondrial membrane and releasing cytochrome *c*, which subsequently induces apoptotic cell death in a cell-free system.[12,13] Thus, intracellular delivery of KLAK peptide into tumor cells will exert antitumor properties. However, due to its high polarity, KLAK does not cross the plasma membrane or enter cells. The

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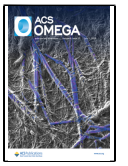

KLAK peptide has been fused with tumor-homing peptides to induce receptor-mediated endocytosis and to subsequently induce apoptosis. Many studies have reported that the KLAK peptide linked with tumor-homing peptides showed significant antitumor activity.[13−17] Previously, we also demonstrated that the KLAK conjugate of the leukemic cell-binding peptide from a phage-displayed random peptide library showed significant antitumor activity against most of leukemia cells.[14,18] The proapoptotic domain, KLAK, fused with the targeting ligands such as tumor-homing peptide, antibody, and natural ligand has gained much attention as one of the molecular-targeted therapies for cancer and is being developed for clinical translation.[3]

In this study, we exploited a CXCR4 antagonist CTCE-9908 and constructed the CXCR4-targeted KLAK conjugate (CTCE-KLAK). We investigated the *in vitro* cytotoxic activity and mode of cell death of the CTCE-KLAK conjugate in breast cancer cell lines. Furthermore, we examined whether the CTCE-KLAK conjugate suppresses tumor growth and metastasis in a syngeneic mouse triple-negative breast cancer model.

RESULTS

Design of a CXCR4-Targeted Proapoptotic Peptide Conjugate. CTCE-9908 consists of a dimer for the first eight amino acids of SDF-1α, where the second Pro residue is substituted with Gly. To design the potent antitumor agent, we used the CXCR4 antagonist CTCE-9908 [$(KGVSLSYR)_2$K-amide] as a breast tumor-homing motif and a proapoptotic domain, $_D(KLAKLKAK)_2$ peptide (Figure 1). CTCE-9908 was linked to the proapoptotic peptide $_D(KLAKLKAK)_2$ through a glycylglycine linker to form the CTCE-KLAK conjugate [$(KGVSLSYR)_2$K-GG-$_D(KLAKLKAK)_2$]. In addition, we prepared a CTCE-9908 equivalent analogue CTCE-9908c, whereby the amido group on middle lysine residue (residue 9) of CTCE-9908 is replaced with a carboxylate group [$(KGVSLSYR)_2$K]. For microscopic analysis, we synthesized green fluorescence analogues (Fam-CTCE-9908c and Fam-CTCE-KLAK), which attached 5(6)-carboxyfluorescein at the N-termini of the peptides through a glycylglycine linker.

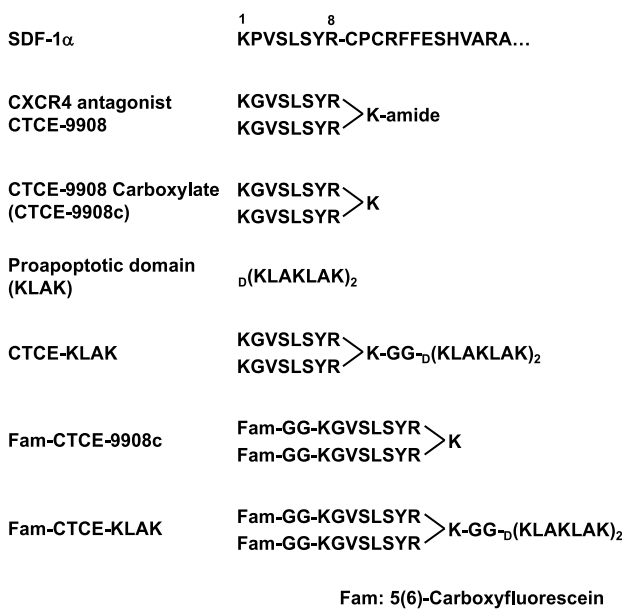

Figure 1. Structure of CXCR4-targeting peptides and the proapoptotic derivatives.

CTCE-KLAK Exhibits Cytotoxic Activity to Breast Cancer Cell Lines *In Vitro*. To evaluate the antitumor activity of the CTCE-KLAK conjugate, we selected three types of human breast cancer cell lines (triple-negative MDA-MB-231, ER-positive/PgR-positive MCF-7, and ER-positive/PgR-negative MDA-MB-361) and noncancerous mammary epithelial cell line MCF-10A. Western blot analysis indicated that all these cell lines express the CXCR4 protein at high levels (Figure 2A). First, we examined that CTCE-9908c can be taken up by these selected cell lines. As shown in Figure 2B, a fluorescent-labeled CTCE-9908c (Fam-CTCE-9908c) was rapidly internalized into all these cells. These data suggest that the CTCE-9908c peptide can serve as a cell-penetrating peptide, and it is convenient for intracellular payload delivery. Next, we evaluated the cytotoxic activity of the CTCE-KLAK conjugate on the breast cell lines. Cells were incubated with CTCE-KLAK at different concentrations for 24 h to determine its cytotoxic activity (Figure 3A). CTCE-KLAK exerted selective cytotoxicity toward MDA-MB-231 and MCF-7 cells in a concentration-dependent manner. However, it was not active against MDA-MB-361 and noncancerous MCF-10A cells. The unconjugated peptides, CTCE-9908c and KLAK, had no cytotoxic activity even at high concentration levels (>100 μM) on all the cell lines, except CTCE-9908c which significantly affected cell viability of the MCF-10A cells. This cytotoxicity did not correlate with the expression levels of CXCR4 in the cell line types (Figure 2A). Therefore, we

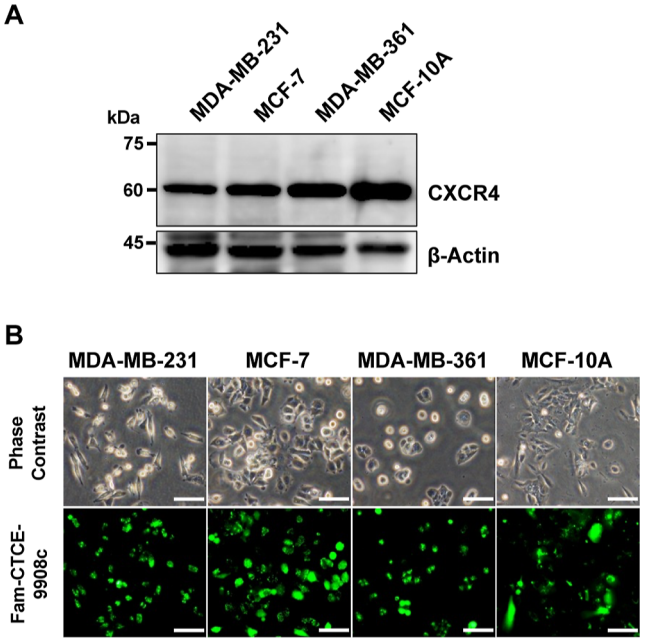

Figure 2. Internalization of the CTCE-9908c peptide in human breast cancer cells expressing CXCR4. (A) Protein expression of CXCR4 in human breast cancer (MDA-MB-231, MCF-7, and MDA-MB-361) and non-neoplastic epithelial mammary MCF-10A cells. Protein was separated on 10 or 12% SDS-PAGE gel, and the blot was probed with an anti-CXCR4 monoclonal antibody. (B) Internalization of CTCE-9908c in the breast cancer or normal cells. Cells were incubated with 20 μM Fam-CTCE-9908c for 3 h, fixed with 4% PFA, and green fluorescence was observed under a fluorescence microscope. Scale bars represent 50 μm.

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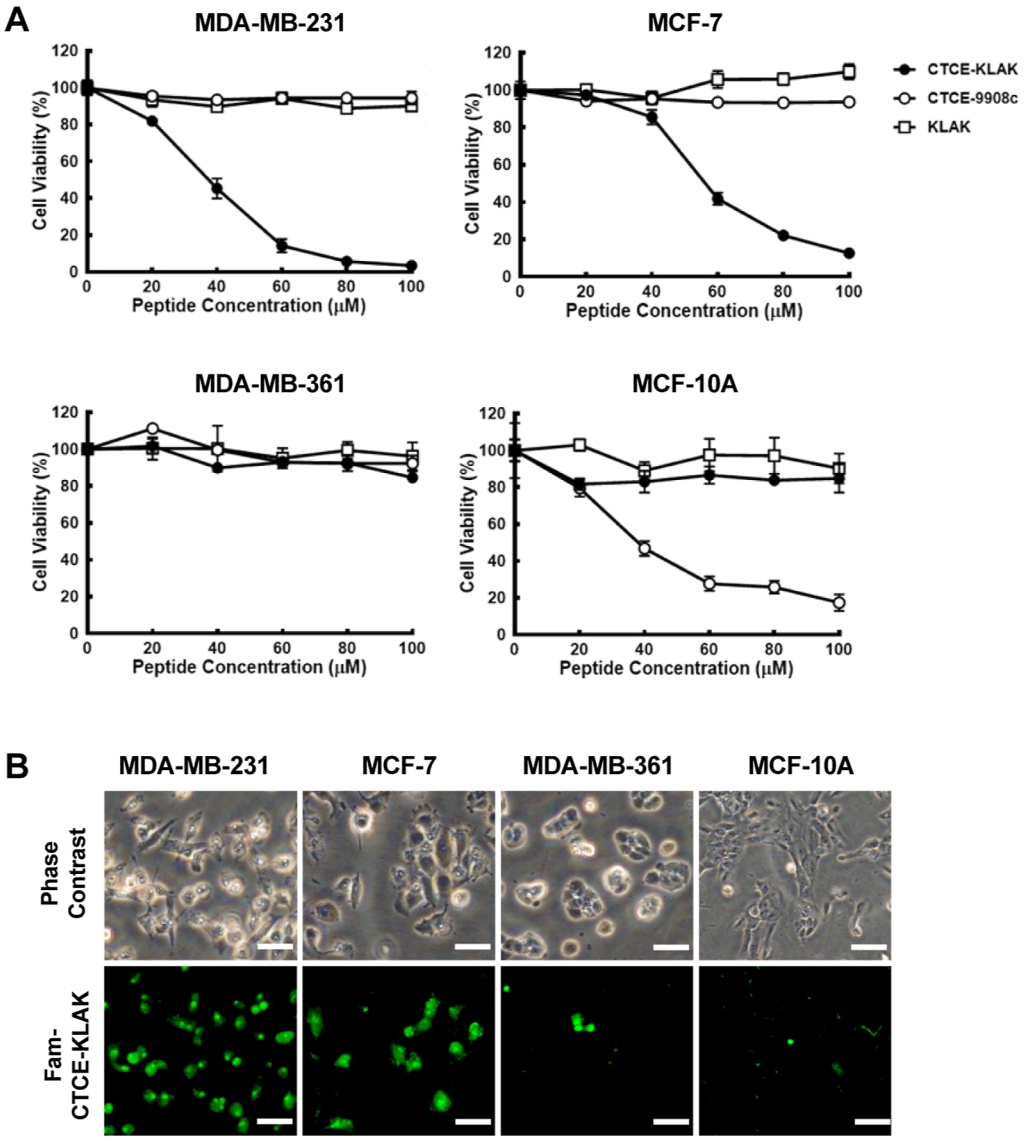

Figure 3. Cytotoxic activity of CTCE-KLAK and cell-selective internalization of Fam-CTCE-KLAK in breast cancer cells or normal cells. (A) Cytotoxicity in human breast cancer or normal cells after 24 h of peptide treatment. Cell viability was determined by WST-8 assay. Values are presented as mean ± SD (n = 3). (B) Internalization of the CTCE-KLAK conjugate in the breast cancer cells (MDA-MB-231 and MCF-7). Cells were incubated with 20 μM Fam-CTCE-KLAK for 3 h, fixed with 4% PFA, and green fluorescence was observed under a fluorescence microscope. Scale bars represent 50 μm.

examined the internalization activity of CTCE-KLAK using a fluorescent probe Fam-CTCE-KLAK. Fam-CTCE-KLAK was internalized into MDA-MB-231 and MCF-7 cells but could not penetrate MDA-MB-361 and MCF-10A cells (Figure 3B). These data indicate that the cell selectivity of CTCE-KLAK for cytotoxic activity depends on its internalization into cells.

CTCE-KLAK Induces Non-apoptotic Cell Death in Breast Cancer Cells. To examine whether CTCE-KLAK induces apoptosis or necrosis in breast cancer cells, we investigated morphological features and caspase activation of the breast cancer cells, as compared with that of apoptosis inducer cisplatin. First, morphological changes were observed using a microscope (Figure 4A). Typical apoptotic blebbing and DNA fragmentation were observed in the cisplatin-treated cells, whereas in the CTCE-KLAK-treated cells, neither blebbing nor chromatin condensation was observed, suggesting necrotic cell death. Next, we investigated the time course of cell death in the CTCE-KLAK-treated MDA-MB-231 and MCF-7 cells. At 100 μM of CTCE-KLAK, cell viability reduced to less than 30% after 0.5 and 3 h in MDA-MB-231 and MCF-7, respectively (Figure 4B). This indicates that the CTCE-KLAK-treated cells die rapidly, suggesting their cell death mode is necrosis. Therefore, we measured lactate dehydrogenase (LDH) released from necrotic cells to confirm this possibility. The results showed that CTCE-KLAK-treated cells released significant amounts of LDH within 60 min after treatment, but the cisplatin-treated cells did not (Figure 4D), implying that the peptide conjugate induced necrotic cell death. Furthermore, we analyzed a biomarker of apoptosis; activation of caspase-3 and caspase-7 and subsequent cleavage of PARP1 enzyme.[19,20] Western blot analysis showed that cisplatin produced the cleaved fragment of caspase-3, which indicates activation of the caspase-3 (Figure 5A), but caspase-3 cleavage was not observed in the CTCE-KLAK-treated MDA-MB-231 cells. Similarly, in the caspase-3-*null* MCF-7 cells, CTCE-KLAK did not activate caspase-7 (Figure S2A). In

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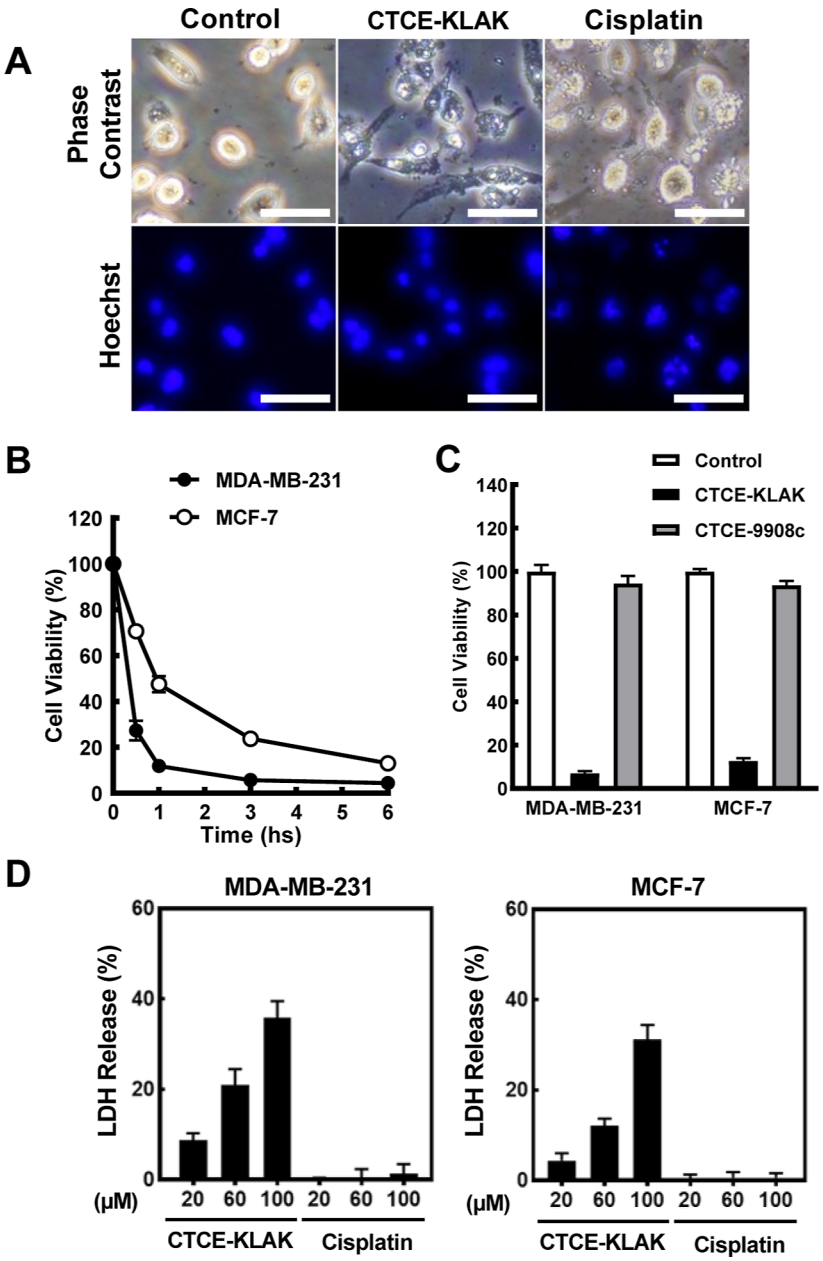

Figure 4. CTCE-KLAK conjugate induces necrotic cell death in the breast cancer cells. (A) Phase contrast and Hoechst 33342-stained images of MDA-MB-231 cells treated with 80 μM of CTCE-KLAK or cisplatin for 3 or 24 h, respectively. Scale bars represent 50 μm. (B) Time course of the CTCE-KLAK-induced cell death in MDA-MB-231 or MCF-7 cells. Cells were treated with 100 μM CTCE-KLAK, and then, cell viability was determined by WST-8 assay. (C) Cytotoxicity of peptides after prolonged exposure. Cell viability was determined after treatment with 100 μM of CTCE-KLAK or CTCE-9908c for 48 h. (D) LDH release assay in the CTCE-KLAK-treated MDA-MB-231 or MCF-7 cells. Cells were treated with 20, 60, and 100 μM of CTCE-KLAK or cisplatin at 37 °C for 60 min, and 0.2%(w/v) Tween 20 was used as a control for 100% LDH release.

addition, cisplatin treatment led to the cleavage of PARP1 into two fragments of 89 and 24 kDa but not CTCE-KLAK (Figures 5B and S2B). This result indicates that CTCE-KLAK could not activate the effector caspases (caspase-3 and caspase-7) and its substrate PARP1 during the cell death process. To confirm no caspase activation in the CTCE-KLAK-treated cells, we examined the inhibition of caspase activation with a pan-caspase inhibitor z-VAD-fmk. Pre-treatment of z-VAD-fmk did not affect the cell death rate in the CTCE-KLAK-treated MDA-MB-231 cells (Figure 5C), while it significantly reduced that of cisplatin (Figure 5D). The same results were obtained using MCF-7 cells (Figure S2C,D). These data suggest that caspase activation is not involved in the mechanism of cell death by the CTCE-KLAK conjugate.

CTCE-KLAK Inhibits Tumor Growth in Mice. To evaluate the *in vivo* antitumor activity of CTCE-KLAK, we examined whether the CTCE-KLAK conjugate inhibits tumor growth and lung metastasis in a 4T1 breast tumor-bearing mouse model.[21] Mouse 4T1 cells express the same level of CXCR4 protein as human breast cancer cells (Figure 6A). Like human MDA-MB-231 cells, CTCE-KLAK exhibited potent cytotoxicity against 4T1 cells in a dose-dependent manner (Figure 6B). In contrast, the unconjugated CTCE-9908c and KLAK peptides were not cytotoxic to the cells. Furthermore, CTCE-KLAK did not produce cleaved forms of caspase-3,

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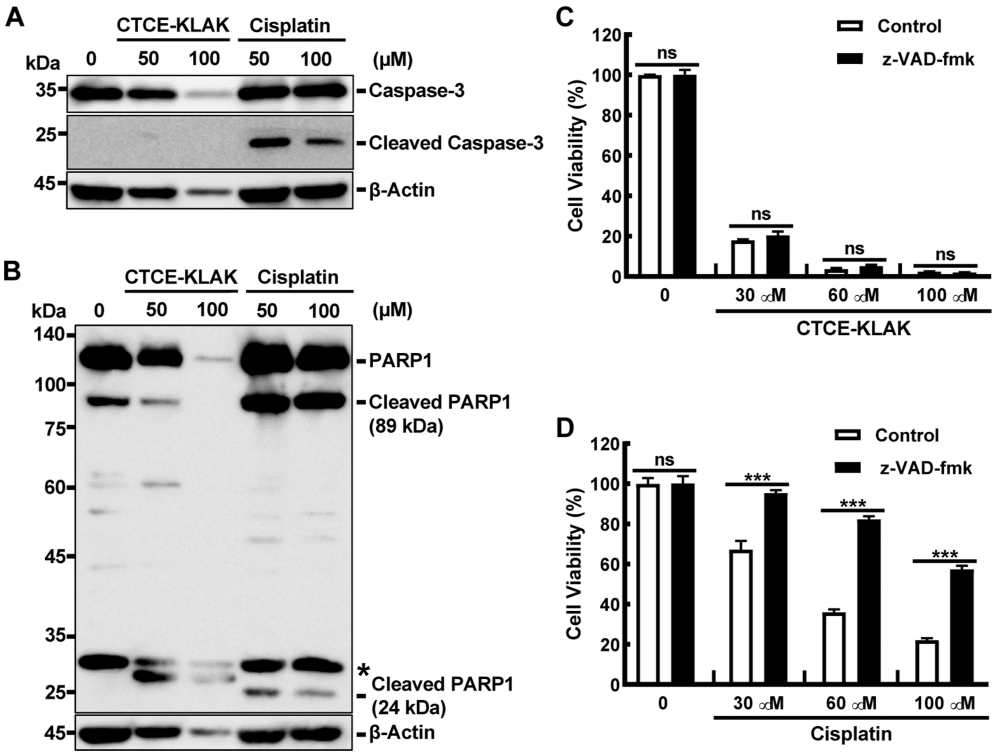

Figure 5. CTCE-KLAK does not activate apoptosis-related enzymes (caspase and PARP1). (A) CTCE-KLAK conjugate did not activate caspase-3, but cisplatin did in MDA-MB-231 cells. (B) Cleavage of PARP1 in the CTCE-KLAK- or cisplatin-treated MDA-MB-231 cells. (A,B) Cell lysates were subjected to Western blot with anti-caspase-3, anti-cleaved caspase-3, anti-PARP1, and anti-cleaved PARP1 monoclonal antibodies. An asterisk (*) represents nonspecific band. (C,D) Effect of the caspase inhibitor z-VAD-fmk on the cytotoxicity of the CTCE-KLAK (C) or cisplatin (D) in MDA-MB-231 cells. Cells were preincubated with or without z-VAD-fmk (50 μM) for 45 min, then treated with CTCE-KLAK or cisplatin for 48 h, and cell viability was determined by WST-8 assay. Values are presented as mean ± SD (n = 3). p values were determined using multiple t-test, ***p < 0.0001; ns, not significant.

caspase-7, and PARP1 in mouse 4T1 cells (Figure 6C), but cisplatin treatment generated cleavage of all enzymes. These results are consistent with observations in human breast cancer cells (MDA-MB-231 and MCF-7), indicating that CTCE-KLAK induces necrotic cell death, not apoptotic, in mouse 4T1 cells.

To investigate the inhibitory effect of the CTCE-KLAK conjugate on tumor growth *in vivo*, we used the 4T1-bearing syngeneic BALB/c mice. We started the peptide administration when tumors in mammary fat pads reached a palpable size (approximately 120 mm^3). CTCE-KLAK peptide conjugate (10 mg/kg body weight/mouse) and peptide mixture (equimolar mixture of unconjugated CTCE-9908c and KLAK) were administered *via* tail vein to mice (intravenous injection) or directly into the tumor mass (intratumoral injection) according to the scheme shown in Figure 7A. Neither the peptide conjugate nor its unconjugated motif induced hemolysis of red blood cells (RBCs) in mouse blood (Figure S4). Therefore, administration by the intravenous route was reasonable. After being intravenously administered 5 times, CTCE-KLAK-treatment groups significantly suppressed tumor growth compared to control mice that received saline (Figure 7B). In contrast, the peptide mixture-treated groups showed no significant inhibitory effect on tumor growth. Correspondingly, isolated tumor size was significantly reduced in the CTCE-KLAK-treatment group (Figure 7C,D). Similarly, intratumoral administration of CTCE-KLAK inhibited tumor growth in the 4T1-bearing mice but not the mixture (Figure S3A−C).

CTCE-KLAK Inhibits Pulmonary Metastasis in Mice. 4T1 cells have been used to study breast cancer metastasis. We examined the inhibitory effect of the CTCE-KLAK conjugate on pulmonary metastasis by counting the number of metastatic nodules on the surface of the isolated lungs. One month after 4T1 tumor transplantation, an increase in metastases was observed in saline-treated control mice (Figure 7E). A significant reduction (p < 0.01) of 4T1 lung metastases was observed in the CTCE-KLAK-treated group using intravenous administration (Figure 7E,F). In contrast, the group administered with unconjugated peptide mixture did not significantly reduce lung metastasis. On the other hand, the reduction in lung metastases was not statistically significant in the intratumorally injected groups, even in the CTCE-KLAK-treated group (Figure S3D,E).

DISCUSSION

Here, we demonstrated that the CXCR4-targeted proapoptotic conjugate, CTCE-KLAK, exhibited potent cytotoxicity against breast cancer cell lines *in vitro* and significantly suppressed tumor growth and metastasis in the 4T1 tumor-bearing mouse model *in vivo*. These results strongly suggest that CXCR4 ligand-directed intracellular delivery of a cytotoxic drug is an effective strategy for the therapy of triple-negative breast cancer.

Treatment of triple-negative breast cancer is challenging due to limited treatment options, as molecular-targeted therapies are ineffective, and its significant metastatic potential and poor prognosis.[1−3] In the clinical setting, cytotoxic agents such as

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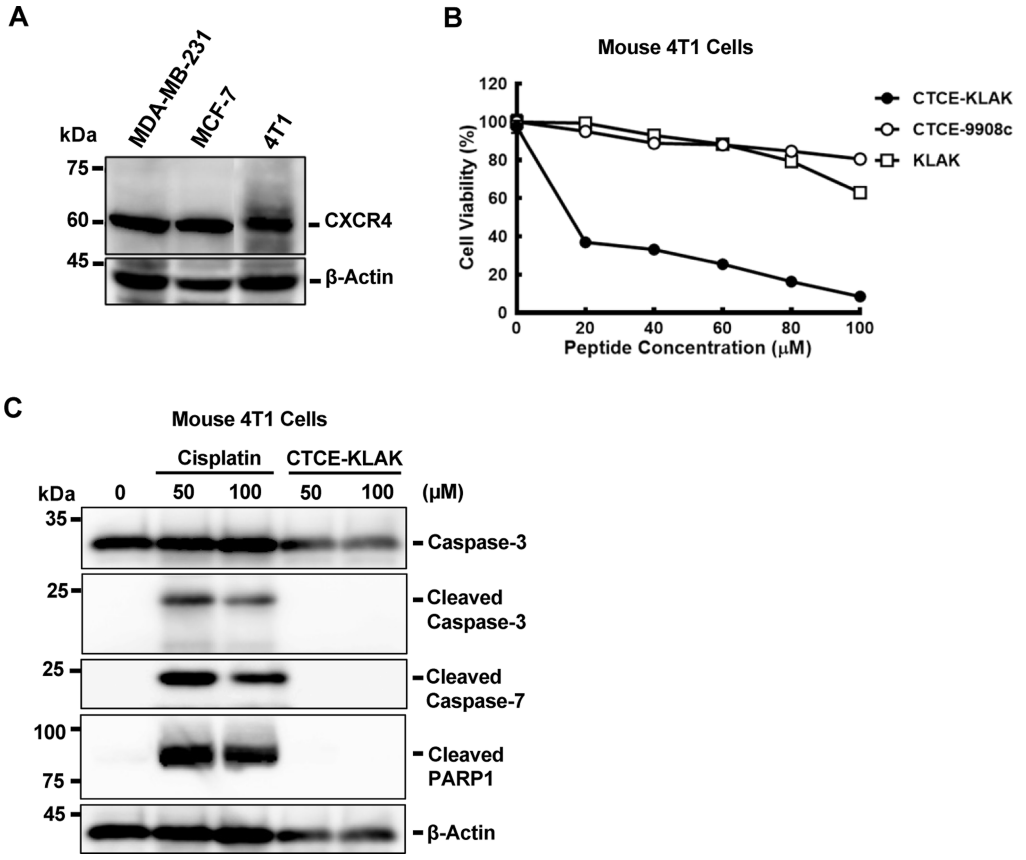

Figure 6. Cytotoxic activity of the CTCE-KLAK conjugate and CTCE-KLAK-induced cell death in mouse breast cancer 4T1 cells. (A) CXCR4 protein expression in human (MDA-MB-231 and MCF-7) and mouse (4T1) breast cancer cells. Protein was separated on 10% SDS-PAGE gel, and the blot was probed with an anti-CXCR4 monoclonal antibody. (B) Cytotoxicity of the CTCE-KLAK conjugate in the 4T1 cells. Cells were incubated with CTCE-KLAK or cisplatin for 24 h, and cell viability was measured by WST-8 assay. (C) Non-apoptotic cell death in the CTCE-KLAK-treated 4T1 cells. Cells were treated with 50 and 100 μM of cisplatin or CTCE-KLAK for 24 h, and cell lysates were subjected to Western blot with anti-caspase-3, anti-caspase-7, and anti-PARP-1 antibodies.

cisplatin and paclitaxel are mainly used for the treatment. To develop molecular-targeted agents for treating this intractable cancer, we focused on the chemokine receptor CXCR4 as a potential target because CXCR4 is involved in metastasis and is highly expressed in breast cancer cells, especially metastatic cancer cells.[4] In this study, we synthesized the CXCR4-targeted antitumor peptidomimetic CTCE-KLAK using the CXCR4 antagonist CTCE-9908. The CTCE-KLAK conjugate exhibits tumor-selective and potent cytotoxicity against breast cancer cells, whereas the unconjugated peptides CTCE-9908c lack cytotoxic activity (Figure 3A). Previous studies showed that CTCE-9908 does not induce apoptosis but inhibits tumor cell growth through mitotic catastrophe with long-term exposure.[10,11] Our result is consistent with these data. CTCE-9908c did not show any cytotoxicity to breast cancer cells after 48 h treatment (Figure 4C), whereas the CTCE-KLAK conjugate killed almost all the cancer cells within 6 h (Figure 4B). These results suggest that the cytotoxic activity is primarily due to the KLAK motif of the conjugate. Furthermore, CTCE-KLAK significantly suppresses tumor growth and lung metastasis in a 4T1-bearing mouse. Systemic administration of CTCE-KLAK significantly suppressed the growth of 4T1 tumors. In contrast, the two unconjugated peptides (CTCE-9908c and the KLAK peptides) did not suppress, implying that the low dose of CTCE-9908 used in this study showed no antitumor effect *in vivo* (Figures 7B−D and S3A−C). In this study, intravenous administration of CTCE-KLAK (10 mg/kg/mouse) significantly inhibited lung metastasis in 4T1-bearing mice. However, the unconjugated CTCE-9908c peptide (10 mg/kg/mouse) did not (Figures 7E,F and S3D,E) inhibit. CTCE-9908 shows a potent inhibitory activity in metastasis.[22] Higher doses (25 mg/kg/mouse or more) than those used in these studies[8−10,23] may be necessary to inhibit metastasis. These findings strongly suggest that the antimetastatic effect of CTCE-KLAK is due to the cytotoxic ability of the KLAK motif. In addition, an intratumoral injection of CTCE-KLAK was ineffective for metastasis. Systemically administered peptides appear to act on metastatic cells at the tumor site and in the circulation, eventually reducing their attraction to organs that express high levels of SDF-1α ligand. The intratumoral injection only allows a minimal amount of the peptide to enter the systemic circulation. In summary, the *in vivo* antitumor activity is attributed to the cytotoxic activity of CTCE-KLAK rather than its CXCR4 antagonism.

In this study, we found that the expression level of CXCR4 does not correlate with the internalization of CTCE-KLAK (Figures 2A and 3B). CTCE-KLAK effectively killed the two breast cancer cells (MDA-MB-231 and MCF-7) but did not kill the other cells (MDA-MB-361 and MCF-10A) (Figure 3A). Since the latter cells express CXCR4 protein, they could take up CTCE-9908c effectively but not the conjugated CTCE-KLAK (Figures 2B and 3B). The differences in the cellular uptake of these two peptides are essential to

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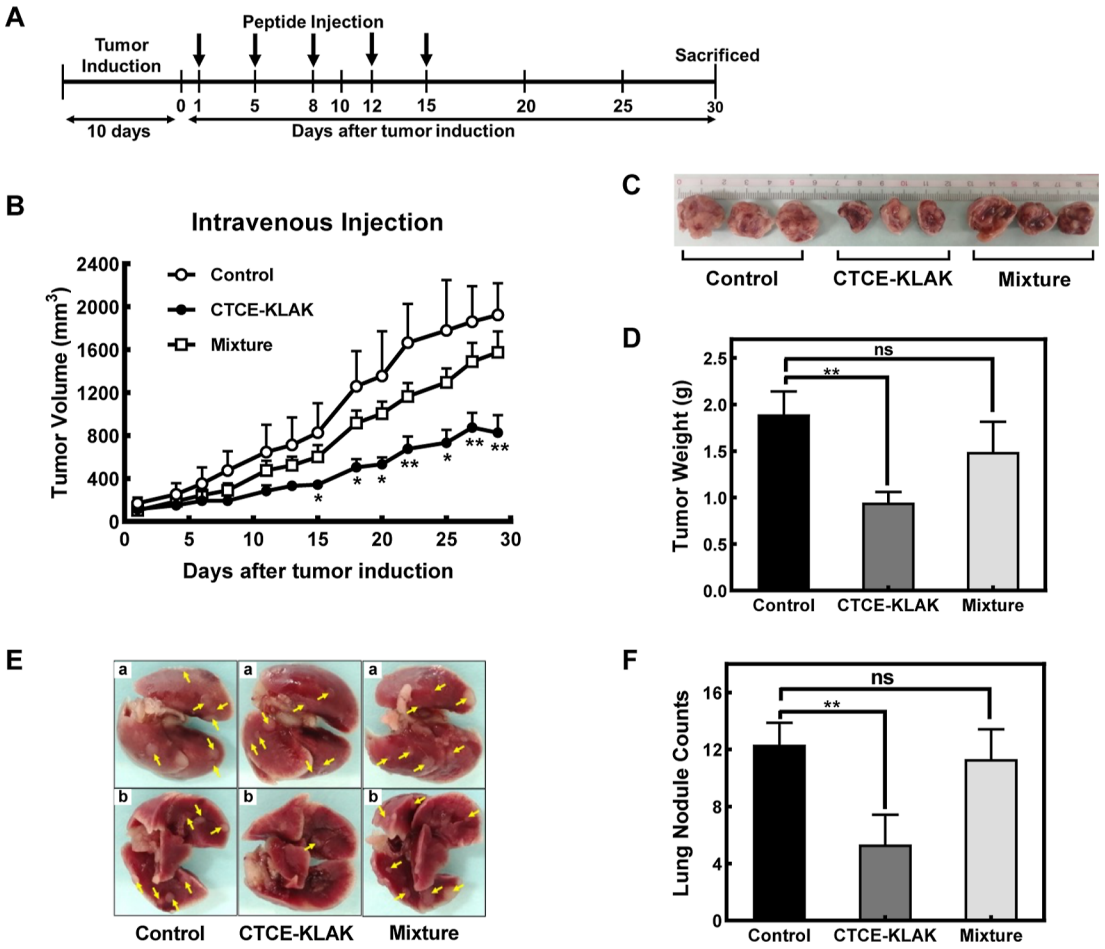

Figure 7. Inhibitory effect of the CTCE-KLAK conjugate on tumor growth and pulmonary tumor metastasis in mice. (A) Scheme of tumor induction with mouse 4T1 cells and treatment protocol. 4T1 cells were transplanted subcutaneously into mammary fat pads of female BALB/c mice. 4T1-bearing mice were administered intravenously 5 times with saline (control), CTCE-KLAK, or a mix of each unconjugated peptide (mixture, CTCE-9908c and KLAK = 1:1) at a dose of 10 mg/kg body weight. (B) Tumor volumes (n = 5 per group). (C) Comparison of size of the isolated tumor. (D) Isolated tumor weights (n = 5 per group). (E) Photograph of 4T1 metastasized lung. Yellow arrows indicate surface lung nodules, a and b represent dorsal and ventral lung views, respectively. (F) Number of lung nodules (n = 3 per group). Values are presented as mean ± SD. p values were determined using one-way ANOVA, $^{*}p < 0.05$; $^{**}p < 0.01$; and ns, not significant.

understanding the tumor cell selectivity of CTCE-KLAK. Two possibilities might explain why the CTCE-KLAK conjugate was not internalized into MDA-MB-361 and MCF-10A cells. One possibility is that CTCE-KLAK does not bind to the CXCR4 due to the involvement of specific molecules that interfere with these interactions. Alternatively, the KLAK motif of the conjugate binds to a secondary receptor, inhibiting its internalization. Besides these two possibilities, a CXCR4-independent pathway may be involved in the uptake of the CTCE-KLAK conjugate. Conjugation can alter the bioactive properties of CTCE-9908, allowing it to bind to other molecules distinct from CXCR4. These possibilities would be investigated in the future.

Earlier studies have shown that tumor-targeting peptides fused with the KLAK domain induce apoptosis, as evidenced by plasma membrane blebbing, phosphatidylserine externalization, and caspase-3 activation.[13,14,16,17] The proapoptotic KLAK motif preferentially translocates to mitochondria and causes disruption of the mitochondrial membrane and releases cytochrome c, resulting in mitochondrial-dependent apoptosis.[13] Rather, we observed that the CTCE-KLAK conjugate containing the KLAK triggered necrotic cell death in the breast cancer cells. KLAK conjugate of aminopeptidases N/CD13-targeting peptide (NGR-peptide-1) induces caspase-independent cell death in human CD13-positive leukemic cells.[24] Cell death triggered by NGR-peptide-1 is thought to be related to the influx of calcium and the production of superoxide anions. Thus, pre-treatment of antioxidant *N*-acetylcysteine prevents cell death. However, the CTCE-KLAK-induced cell death was not inhibited by treating the *N*-acetylcysteine (data not shown). CTCE-KLAK-induced cell death may be mediated by a different mechanism than that of NGR-peptide-1. Recently, CXCR4 has been identified as a primary receptor for arginine-rich peptides such as octa-arginine.[25] Law *et al.* demonstrated that a fusion peptide r7-kla, which consists of the KLAK and an hepta-arginine, as a cell-penetrating peptide, induces rapid apoptosis on various cancer cell lines.[15] Combined with these results, the KLAK conjugate of CTCE-9908 must induce apoptosis upon binding to CXCR4, similar to r7-kla. It remains to be determined where the CTCE-KLAK conjugate translocates after the intracellular uptake and why the caspases do not activate. Further investigations are needed to understand the mechanism of CTCE-KLAK-induced cell death.

Necroptosis is one form of regulated necrotic cell death.[26] Recently, necroptosis-based cancer therapy has been proposed

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as a novel antitumor treatment strategy.[27,28] Apoptosis-based therapy using cisplatin, carboplatin, or paclitaxel is the principal strategy for cancer treatment, but its effectiveness is limited by drug resistance. Thus, triggering necrosis has emerged as a novel approach for bypassing apoptosis resistance in cancer therapy.[28] The present study demonstrates that CTCE-KLAK can induce necrosis in a tumor cell-selective manner. Therefore, CTCE-KLAK, as a necrosis inducer, could be a promising therapeutic to overcome apoptosis resistance in cancer cells. Conversely, chronic inflammatory responses to necrosis induction can lead to immunosuppression and promote tumor metastasis, implying that triggering necrosis might be unfavorable for cancer treatment. While controversy remains, further investigation of the necrotic pathway of CTCE-KLAK will lead to a better understanding of necrosis-based cancer therapy.

■ CONCLUSIONS

In the present study, we demonstrated that CXCR4-targeted CTCE-KLAK conjugate shows tumor-selective cytotoxic activity on the breast cancer cells and suppresses both tumor growth and pulmonary metastasis in the mouse model. Moreover, CTCE-KLAK induces necrotic cell death in breast cancer cells. Our findings shed light on new therapeutic strategies to treat refractory and apoptosis-resistant cancer diseases. The CXCR4-targeted intracellular delivery of cytotoxic agents may be a promising platform for treating triple-negative breast cancer.

■ EXPERIMENTAL SECTION

Cell Culture. Human breast cancer cell lines (MDA-MB-231-Luc, MCF-7, and MDA-MB-361-Luc) and mouse 4T1-Luc cells were obtained from the Japanese Collection of Research Bioresource Cell Bank (Osaka, Japan). Human mammary epithelial cell line MCF-10A was purchased from the American Tissue Type Culture Collection (Manassas, USA). Except for MCF-10A, all the cell lines were maintained in complete growth medium (10% fetal calf serum, RPMI-1640 medium, 100 U/mL penicillin, and 100 μg/mL streptomycin) and incubated at 37 °C with 5% CO_2. MCF-10A cells were cultured using the mammary epithelial cell growth medium kit (PromoCell GmbH, Heidelberg, Germany) as the complete growth medium.

Peptide Synthesis. All peptides were synthesized by the standard Fmoc solid-phase strategy using Initiator Alstra peptide synthesizer (Biotage, Uppsala, Sweden). After cleavage from the resin, the peptides were purified by reverse-phase high-performance liquid chromatography (RP-HPLC). The purity of the peptides was above 95% determined by RP-HPLC. The molecular mass of the peptides was measured by AutoFlex II MALDI-TOF MS (Bruker Daltonics, MA). The abbreviations or assigned names, the sequences, molecular masses, purity, and chromatographic spectrums for each of the peptides are shown in the Supporting Information in Table S1 and Figure S1.

***In Vitro* Cytotoxicity Assay.** Cells (2×10^4 cells/100 μL) were seeded in a 96-well plate and then incubated for 24 h with various concentrations of peptides (CTCE-KLAK, CTCE-9908c, and KLAK) or cisplatin for the indicated time. Cytotoxicity was assessed by measuring the absorbance at 450 nm with a microplate reader for 1−2 h after adding WST-8 reagent solution (Dojindo, Kumamoto, Japan) according to the manufacturer's guidelines. Cell viability of 100% was calculated by adding phosphate-buffered saline (PBS) as a control. For apoptosis inhibition assay, cells were pre-treated with 50 μM z-VAD-fmk, a pan-caspase inhibitor, for 45 min and then treated with 30, 60, and 100 μM of CTCE-KLAK or cisplatin for 48 h. Plasma membrane integrity was assessed based on LDH leakage into the culture medium from cells. LDH leakage was determined using the LDH-Cytotoxic Test Wako kit (Wako, Osaka, Japan) by measuring the absorbance at 560 nm. LDH leakage 100% was assessed in 0.2% (w/v) Tween 20.

Fluorescence Microscopic Analysis. Cells (3×10^4 cells/well) in an eight-well chamber were incubated with 20 μM of fluorescent-labeled peptides (Fam-CTCE-9908c and Fam-CECE-KLAK) for 3 h in a complete medium. After treatment, cells were fixed with 4% paraformaldehyde (PFA) and incubated with or without Hoechst 33342 at room temperature for 10 min. Cells were observed under a FSX100 fluorescence microscope (Olympus, Tokyo, Japan).

Western Blot Analysis. Cells (3×10^5) were seeded overnight in a six-well plate and treated with 50 and 100 μM of CTCE-KLAK or cisplatin in a complete growth medium and incubated for the indicated time. Cells were washed with ice-cold PBS and lysed in the RIPA buffer (Fujifilm, Tokyo, Japan) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/mL pepstatin A, 10 μg/mL leupeptin, and 10 μg/mL aprotinin). Cell lysates (25 μg) were separated on a 12% SDS-polyacrylamide gel and electroblotted to a polyvinylidene difluoride membrane. The blots were blocked with 5% skim milk in PBS for 30 min and probed overnight at 4 °C with following primary antibodies: CXCR4 (1:1000; 60042-1-Ig; Proteintech, Rosemont, IL), caspase-3 (1:1000; #9662; Cell Signaling Technology, Danvers, MA), cleaved caspase-3 (Asp175) (1:1000; #9664; Cell Signaling Technology), caspase-7 (1:1000; #12827; Cell Signaling Technology), PARP1 (1:1000; #9542; Cell Signaling Technology), and β-actin (1:10,000; 60042-1-Ig; Proteintech). Then, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h, at 4 °C, and specific protein bands were detected using the Luminata Crescendo Western HRP substrate (Millipore, MA).

***In Vivo* Tumor Growth and Pulmonary Metastasis.** BALB/c mice were obtained from the Japan SLC (Shizuoka, Japan). All experimental procedures were performed according to guidelines for the Institutional Animal Care and Use Committee of Sojo University. To develop the tumor mouse model, 4T1 cells (5×10^5 cells/100 μL PBS) were injected into the mammary fat pads just beneath the fourth nipple of 5 week old female BALB/c mice under anesthesia.[29] When the tumors reached 120 mm^3, the mice were divided into three groups. For the treatment groups, the mice were administered with each peptide (3 mg/mL in saline) at 10 mg/kg *via* intravenous tail injection or *in situ* intratumoral injection five times for 15 days. For the control group, the mice were treated with saline. Tumor volume (V) was calculated using the following formula: $V = 0.524 \times L \times W^2$, where L and W were the longest and widest diameter (mm) of the tumor, respectively.[30] All mice were sacrificed at the end of the experiments, and the tumors were incised and weighed. The lung metastasis was assessed by counting the number of nodules grown on the lung surface, as described by Hill *et al.*[31] In brief, the lungs of the sacrificed mice were removed and fixed in 4% PFA overnight. Tumor nodules on the lung were

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counted using digital images of the dorsal and ventral lung views.

Hemolysis Assay. The effect of CTCE-KLAK peptides on mouse RBCs was evaluated by a hemolysis assay.[32] In brief, fresh heparinized peripheral blood collected from BALB/c mice was centrifuged at 1000*g* for 5 min. RBCs were washed three times with PBS and prepared in 4% cell suspension in PBS. 50 μL of diluted RBCs were incubated in a 96-well plate with 50 μL of various concentrations of each peptide and incubated for 1 h at room temperature. After centrifugation, the absorbance of the supernatant was measured at 450 nm. PBS and 2% (w/v) Tween-20 were used as negative and positive control, respectively.

Statistical Analysis. Statistical analysis was performed in GraphPad Prism software 5. Results are presented as the mean ± standard deviation (SD) of *n* independent experiments. The statistical significance of results was analyzed using a multiple *t*-test and one-way analysis of variance (ANOVA). The statistical significance was set to $p < 0.05$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c02415.

Details of synthesized peptides; chromatogram of purified peptides; caspase activation and PARP1 cleavage in CTCE-KLAK- or cisplatin-treated MCF-7 cells; inhibition of tumor growth and metastases by intratumoral administration of each peptide; and hemolytic activity of the peptides (PDF)

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Author Contributions

A.A. and A.K. conceived the study. A.K. managed the research group. A.A., M.M., and A.K. designed the peptides and conducted the experiments. A.A. and A.K. wrote the manuscript. All authors checked and approved the experimental results and the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CXCR4, C-X-C chemokine receptor 4; ER, estrogen receptor; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; Gly, glycine; HER2, human epidermal growth factor receptor type 2; HRP, horseradish peroxidase; LDH, lactate dehydrogenase; MALDI-TOF-MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; PARP-1, poly-(ADP-ribose)polymerase-1; RBC, red blood cell; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PgR, progesterone receptor; RIPA, radio immunoprecipitation assay; RP-HPLC, reverse-phase high-performance liquid chromatography; Pro, proline; SDF-1α, stromal-derived factor-1 α; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WST-8, water-soluble tetrazolium salt

■ REFERENCES

(1) Bianchini, G.; Balko, J. M.; Mayer, I. A.; Sanders, M. E.; Gianni, L. TNBC: Challenges and Oppotunities of a Heterogenous Disease. *Nat. Rev. Clin. Oncol.* **2016**, *13*, 674−690.

(2) Yin, L.; Duan, J. J.; Bian, X. W.; Yu, S. C. Triple-Negative Breast Cancer Molecular Subtyping and Treatment Progress. *Breast Cancer Res.* **2020**, *22*, 61.

(3) Azari, M.; Bahreini, F.; Uversky, V. N.; Rezaei, N. Current Therapeutic Approaches and Promising Perspectives of Using Bioengineered Peptides in Fighting Chemoresistance in Triple-Negative Breast Cancer. *Biochem. Pharmacol.* **2023**, *210*, 115459.

(4) Müller, A.; Homey, B.; Soto, H.; Ge, N.; Catron, D.; Buchanan, M. E.; McClanahan, T.; Murphy, E.; Yuan, W.; Wagner, S. N.; Barrera, J. L.; Mohar, A.; Verástegui, E.; Zlotnik, A. Involvement of Chemokine Receptors in Breast Cancer Metastasis. *Nature* **2001**, *410*, 50−56.

(5) Fernandis, A. Z.; Prasad, A.; Band, H.; Klösel, R.; Ganju, R. K. Regulation of CXCR4-Mediated Chemotaxis and Chemoinvasion of Breast Cancer Cells. *Oncogene* **2004**, *23*, 157−167.

(6) Dewan, M. Z.; Ahmed, S.; Iwasaki, Y.; Ohba, K.; Toi, M.; Yamamoto, N. Stromal Cell-Derived Factor-1 and CXCR4 Receptor Interaction in Tumor Growth and Metastasis of Breast Cancer. *Biomed. Pharmacother.* **2006**, *60*, 273−276.

(7) Kang, H.; Watkins, G.; Douglas-Jones, A.; Mansel, R. E.; Jiang, W. G. The Elevated Level of CXCR4 Is Correlated with Nodal Metastasis of Human Breast Cancer. *Breast* **2005**, *14*, 360−367.

(8) Huang, E. H.; Singh, B.; Cristofanilli, M.; Gelovani, J.; Wei, C.; Vincent, L.; Cook, K. R.; Lucci, A. A CXCR4 Antagonist CTCE-9908 Inhibits Primary Tumor Growth and Metastasis of Breast Cancer. *J. Surg. Res.* **2009**, *155*, 231−236.

(9) Richert, M. M.; Vaidya, K. S.; Mills, C. N.; Wong, D.; Korz, W.; Hurst, D. R.; Welch, D. R. Inhibition of CXCR4 by CTCE-9908 Inhibits Breast Cancer Metastasis to Lung and Bone. *Oncol. Rep.* **2009**, *21*, 761−767.

(10) Wong, D.; Kandagatla, P.; Korz, W.; Chinni, S. R. Targeting CXCR4 with CTCE-9908 Inhibits Prostate Tumor Metastasis. *BMC Urol.* **2014**, *14*, 12.

(11) Kwong, J.; Kulbe, H.; Wong, D.; Chakravarty, P.; Balkwill, F. An Antagonist of the Chemokine Receptor CXCR4 Induces Mitotic Catastrophe in Ovarian Cancer Cells. *Mol. Cancer Ther.* **2009**, *8*, 1893−1905.

(12) Javadpour, M. M.; Juban, M. M.; Lo, W. C. J.; Bishop, S. M.; Alberty, J. B.; Cowell, S. M.; Becker, C. L.; McLaughlin, M. L. De Novo Antimicrobial Peptides with Low Mammalian Cell Toxicity. *J. Med. Chem.* **1996**, *39*, 3107−3113.

(13) Ellerby, H. M.; Arap, W.; Ellerby, L. M.; Kain, R.; Andrusiak, R.; Rio, G. D.; Krajewski, S.; Lombardo, C. R.; Rao, R.; Ruoslahti, E.; Bredesen, D. E.; Pasqualini, R. Anti-Cancer Activity of Targeted pro-Apoptotic Peptides. *Nat. Med.* **1999**, *5*, 1032−1038.

(14) Karjalainen, K.; Jaalouk, D. E.; Bueso-Ramos, C. E.; Zurita, A. J.; Kuniyasu, A.; Eckhardt, B. L.; Marini, F. C.; Lichtiger, B.; O'Brien, S.; Kantarjian, H. M.; Cortes, J. E.; Koivunen, E.; Arap, W.;

Pasqualini, R. Targeting Neuropilin-1 in Human Leukemia and Lymphoma. *Blood* **2011**, *117*, 920−927.
(15) Law, B.; Quinti, L.; Choi, Y.; Weissleder, R.; Tung, C. A Mitochondrial Targeted Fusion Peptide Exhibits Remarkable Cytotoxicity. *Mol. Cancer Ther.* **2006**, *5*, 1944−1949.
(16) Rege, K.; Patel, S. J.; Megeed, Z.; Yarmush, M. L. Amphipathic Peptide-Based Fusion Peptides and Immunoconjugates for the Targeted Ablation of Prostate Cancer Cells. *Cancer Res.* **2007**, *67*, 6368−6375.
(17) Sioud, M.; Mobergslien, A. Selective Killing of Cancer Cells by Peptide-Targeted Delivery of an Anti-Microbial Peptide. *Biochem. Pharmacol.* **2012**, *84*, 1123−1132.
(18) Nishimura, S.; Takahashi, S.; Kamikatahira, H.; Kuroki, Y.; Jaalouk, D. E.; O'Brien, S.; Koivunen, E.; Arap, W.; Pasqualini, R.; Nakayama, H.; Kuniyasu, A. Combinatorial Targeting of the Macropinocytotic Pathway in Leukemia and Lymphoma Cells. *J. Biol. Chem.* **2008**, *283*, 11752−11762.
(19) Shah, G. M.; Shah, R. G.; Poirier, G. G. Different Cleavage Pattern for Poly(ADP-Ribose) Polymerase during Necrosis and Apoptosis in HL-60 Cells. *Biochem. Biophys. Res. Commun.* **1996**, *229*, 838−844.
(20) Walsh, J. G.; Cullen, S. P.; Sheridan, C.; Lüthi, A. U.; Gerner, C.; Martin, S. J. Executioner Caspase-3 and Caspase-7 Are Functionally Distinct Proteases. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 12815−12819.
(21) Pulaski, B. A.; Ostrand-Rosenberg, S. Mouse 4T1 Breast Tumor Model. *Curr. Protoc. Immunol.* **2000**, *39*, 20.2.1−20.2.16.
(22) Shi, Y.; Riese, D. J.; Shen, J. The Role of the CXCL12/CXCR4/CXCR7 Chemokine Axis in Cancer. *Front. Pharmacol.* **2020**, *11*, 574667.
(23) Kim, S. Y.; Lee, C. H.; Midura, B. V.; Yeung, C.; Mendoza, A.; Hong, S. H.; Ren, L.; Wong, D.; Korz, W.; Merzouk, A.; Salari, H.; Zhang, H.; Hwang, S. T.; Khanna, C.; Helman, L. J. Inhibition of the CXCR4/CXCL12 Chemokine Pathway Reduces the Development of Murine Pulmonary Metastases. *Clin. Exp. Metastasis* **2008**, *25*, 201−211.
(24) Bouchet, S.; Tang, R.; Fava, F.; Legrand, O.; Bauvois, B. The CNGRC-GG-$_D$ $(KLAKLAK)_2$ Peptide Induces a Caspase-Independent, Ca^{2+}-Dependent Death in Human Leukemic Myeloid Cells by Targeting Surface Aminopeptidase N/CD13. *Oncotarget* **2016**, *7*, 19445−19467.
(25) Tanaka, G.; Nakase, I.; Fukuda, Y.; Masuda, R.; Oishi, S.; Shimura, K.; Kawaguchi, Y.; Takatani-Nakase, T.; Langel, U.; Graslund, A.; Okawa, K.; Matsuoka, M.; Fujii, N.; Hatanaka, Y.; Futaki, S. CXCR4 Stimulates Macropinocytosis: Implications for Cellular Uptake of Arginine-Rich Cell-Penetrating Peptides and HIV. *Chem. Biol.* **2012**, *19*, 1437−1446.
(26) Degterev, A.; Huang, Z.; Boyce, M.; Li, Y.; Jagtap, P.; Mizushima, N.; Cuny, G. D.; Mitchison, T. J.; Moskowitz, M. A.; Yuan, J. Chemical Inhibitor of Nonapoptotic Cell Death with Therapeutic Potential for Ischemic Brain Injury. *Nat. Chem. Biol.* **2005**, *1*, 112−119.
(27) Yan, J.; Wan, P.; Choksi, S.; Liu, Z. Necroptosis and Tumor Progression. *Trends Cancer* **2022**, *8*, 21−27.
(28) Zang, X.; Song, J.; Li, Y.; Han, Y. Targeting Necroptosis as an Alternative Strategy in Tumor Treatment: From Drugs to Nanoparticles. *J. Controlled Release* **2022**, *349*, 213−226.
(29) Zhang, G. L.; Zhang, Y.; Cao, K. X.; Wang, X. M. Orthotopic Injection of Breast Cancer Cells into the Mice Mammary Fat Pad. *J. Visualized Exp.* **2019**, *143*, No. e58604.
(30) Lai, L. H.; Fu, Q. H.; Liu, Y.; Jiang, K.; Guo, Q. M.; Chen, Q. Y.; Yan, B.; Wang, Q. Q.; Shen, J. G. Piperine Suppresses Tumor Growth and Metastasis *In Vitro* and *In Vivo* in a 4T1 Murine Breast Cancer Model. *Acta Pharmacol. Sin.* **2012**, *33*, 523−530.
(31) Hill, L. L.; Perussia, B.; McCue, P. A.; Korngold, R. Effect of Human Natural Killer Cells on the Metastatic Growth of Human Melanoma Xenografts in Mice with Severe Combined Immunodeficiency. *Cancer Res.* **1994**, *54*, 763−770.
(32) Solarte, V. A.; Rosas, J. E.; Rivera, Z. J.; Arango-Rodríguez, M. L.; García, J. E.; Vernot, J. P. A Tetrameric Peptide Derived from Bovine Lactoferricin Exhibits Specific Cytotoxic Effects against Oral Squamous-Cell Carcinoma Cell Lines. *BioMed Res. Int.* **2015**, *2015*, 630179.

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