Title: A claudin-targeting molecule as an inhibitor of tumor metastasis

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d) **Abbreviations**: C-CPE, C-terminal fragment of *Clostridium perfringens* enterotoxin from 194 to 319 amino acids; PSIF, protein synthesis inhibitory factor derived from *Pseudomonas* exotoxin; CL4-B16, claudin-4-expressing B16 cells; TJ, tight junction; CPE, *Clostridium perfringens* enterotoxin; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; C-CPE-PSIF, C-CPE-fused PSIF; PBS, phosphate buffered saline; ADR, adriamycin
Abstract

Tumor metastasis of epithelium-derived tumors is the major cause of death from malignant tumors. Overexpression of claudin is frequently observed in malignant tumors. However, claudin-targeting anti-metastasis therapy has never been investigated. We previously prepared a claudin-4-targeting anti-tumor molecule that consisted of the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) fused to protein synthesis inhibitory factor (PSIF) derived from *Pseudomonas* exotoxin. In the present study, we investigated whether claudin CPE receptors can be a target for tumor metastasis by using the C-CPE-fused PSIF as a claudin-targeting agent. One of the most popular murine metastasis models is the lung metastasis of intravenously injected B16 cells. Therefore, we first investigated the effects of the C-CPE-fused PSIF on lung metastasis of claudin-4-expressing B16 cells (CL4-B16 cells). Intravenous administration of the C-CPE-fused PSIF suppressed lung metastasis of CL4-B16 cells but not B16 cells. Injection of C-CPE-fused PSIF also inhibited tumor growth and spontaneous lung metastasis of murine breast cancer 4T1 cells inoculated into the subcutis. Treatment with C-CPE-fused PSIF did not show apparent side effects in mice. These findings indicate that claudin-targeting may be a novel strategy for inhibiting some tumor metastases.
Introduction

Metastasis is the primary cause of death for most cancer patients (Gupta and Massague, 2006; Steeg, 2006). Metastasis occurs during tumor growth and even during the surgical excision of the primary tumor. A great deal of effort has been made to overcome tumor metastasis, including the development of several potent methods for irradiation therapy, chemotherapy and immunotherapy. However, 7 million patients worldwide die from malignant tumors each year, and the majority of malignant tumors are derived from the epithelium (Jemal et al., 2008). Thus, the development of a novel anti-tumor strategy against epithelium-derived cancer metastasis is needed.

The epithelium is located at the border between the outer and inner body and tissue. Spaces between the adjacent cells in epithelium are sealed by tight junctions (TJs). TJs prevent free movement of solutes across epithelium through the paracellular spaces, and TJs also maintain cellular polarity by regulating the localization of cellular membrane proteins, such as transporters, ion channels and receptors, between the apical and basal sides of epithelial cells (Mitic and Anderson, 1998; Vermeer et al., 2003). Moreover, TJs control cell proliferation by regulating the localization of receptors on the cellular membrane and regulating the intracellular signal transduction for cellular proliferation and differentiation (Matter et al., 2005; Vermeer et al., 2003). These TJ functions are frequently deregulated during tumorigenesis, and tumor cells often exhibit abnormalities in cellular polarity and differentiation (Martin and Jiang, 2001; Wodarz and Nathke, 2007). The loss of the integrity of the TJ seal in tumors may contribute to the supply of nutrition critical for tumor growth and to the detachment of cancer cells from the primary tumor tissues, leading to the malignancy of tumors (Martin and Jiang, 2001; Mullin, 1997).

Freeze-fracture replica microscopy analysis revealed that TJs form a series of continuous strands within plasma membranes (Staehelin, 1973). TJ strands from the membranes of adjacent cells form a paired strand, and the paired strands seal the intercellular space between the adjacent cells. The TJ strands are composed of integral membrane proteins, such as occludin and claudin, and among them, claudin is a key structural and functional component of TJ seals (Furuse and Tsukita, 2006). Claudin, a tetra-transmembrane protein, comprises a family consisting of more than 20 members. The expression profiles and sealing functions of claudins differ among tissues. Claudin expression is often deregulated in human cancers such as breast, prostate, ovarian, gastric and pancreatic cancers (Kominsky, 2006; Morin, 2005). There is a relationship between dysregulated claudin and metastasis (Agarwal et al., 2005; Dhawan et al., 2005). These findings
indicate that claudin can be a potent target for cancer therapy.

*Clostridium perfringens* enterotoxin (CPE), a 35-kDa polypeptide, causes food poisoning in humans. CPE binds to its receptor, and then CPE causes changes in the membrane permeability by complex formation on the plasma membrane followed by the induction of oncosis and apoptosis (McClane and Chakrabarti, 2004). The local administration of CPE suppresses solid tumor growth (Kominsky et al., 2004; Michl et al., 2001; Santin et al., 2005); however, whole CPE had never been applied into a ligand for claudin CPE receptors because of its strong cytotoxicity. The receptor-binding region of CPE (C-CPE) can be used for claudin-targeted cancer therapy (Saeki et al., 2009). Immunotoxins, consisting of a protein toxin connected to a binding ligand, such as an antibody or growth factor, have been developed and used for clinical therapy. Protein synthesis inhibitory factor (PSIF) derived from *Pseudomonas* exotoxin is a widely used protein toxin (Kreitman and Pastan, 2006), and intratumoral administration of the claudin-4-targeting PSIF has been shown to attenuate solid tumor growth (Saeki et al., 2009). In the present study, we investigated whether claudin CPE receptors can be a target for tumor metastasis by using the claudin-4-targeting PSIF as a claudin-targeting agent.
Materials and methods

Cell culture

Mouse melanoma cell line B16-BL6 and mouse breast cancer cell line 4T1 were cultured in modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) and Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS and 10 mmol/ml HEPES, respectively. The cells were maintained in a 5% CO₂ atmosphere at 37°C.

Preparation of B16 cells stably expressing claudin-4

Mouse claudin-4 cDNA was subcloned into pcDNA3.1 plasmid coding a neomycin resistance gene. The claudin-4 expression vector was transfected into B16 cells, and B16 cells stably expressing claudin-4 (CL4-B16) were isolated by geneticin selection.

Immunoblot analysis

Cells were lysed in lysis buffer (50 mM Tris (pH 7.4), 8.25 mg/ml NaCl, 1% NP-40, 2 mM SDS and protease inhibitor cocktail (Sigma-Aldrich St. Louis, MO)). The cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred onto a polyvinylidene difluoride membrane, followed by immunoblotting with anti-claudin-4 (Zymed Laboratories, San Francisco, CA) or anti-β-actin Ab (Sigma-Aldrich). After incubating with a peroxidase-labeled secondary Ab (Chemicon, Temecula, CA), the immunoreactive band was visualized by chemiluminescence reagents (Amersham Biosciences, Little Chalfont, UK).

Cell proliferation assay

B16 or CL4-B16 cells (2 × 10⁴ cells) were seeded into a 24-well plate. At the indicated time points, the cells were stained with trypan blue, and the number of viable cells was counted by using a hemocytometer under a microscope.

In vitro metastasis analysis

A cell culture insert with an 8-µm pore size membrane (BD Biosciences Discovery Labware, Bedford, MA) was used for the invasion assay. The upper surface of the chamber was coated with 50 µl of 0.1 mg/ml of Matrigel (BD Biosciences), and the lower chamber was filled with DMEM.
containing 10% FBS. Cells were cultured to about 80% confluency and serum-starved overnight (0.5% FBS). Then, cells (1 × 10^5 cells) suspended in DMEM with 0.5% FBS were placed into the upper chamber and incubated at 37°C for 24 h. After incubation, non-invading cells were removed, and the membrane was stained with Diff-Quick reagent. The stained cells in five randomly selected fields were counted under a microscope.

**Preparation of C-CPE-PSIF**

The C-terminal fragment of CPE (C-CPE)-fused PSIF (C-CPE-PSIF) was prepared as described previously (Saeki et al., 2009). Briefly, pET-C-CPE-PSIF was transduced into *Escherichia coli* BL21 (DE3) (Novagen, Darmstadt, Germany), and the production of C-CPE-PSIF was induced by the addition of 0.25 mM isopropyl-D-thiogalactopyranoside. The cells were harvested and then lysed in buffer A (10 mM Tris-HCl (pH 8.0), 400 mM NaCl, 5 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM 2-mercaptoethanol and 10% glycerol). The lysates were centrifuged, and the resultant supernatant was applied to HiTrap Chelating HP (GE Healthcare, Little Chalfont, UK). The proteins were eluted by imidazole in buffer A. The buffer was exchanged with phosphate-buffered saline (PBS) by using a PD-10 column (GE Healthcare), and the purified protein was stored at -80°C until use. Protein was quantified by using a BCA protein assay kit (Pierce Chemical, Rockford, IL) with BSA as a standard.

**In vitro cytotoxic analysis**

In the cytotoxic assay, B16 cells and CL4-B16 cells were seeded onto a 96-well culture dish at 1 × 10^4 cells per well. After 24 h, the cells were treated with C-CPE-PSIF for 24 h at the indicated concentration. The cytotoxicity was determined by a WST-8 kit, according to the manufacturer’s instructions (Nacalai, Kyoto, Japan).

**In vivo anti-tumor activity**

Female BALB/c mice (7-8 weeks old) and C57/BL6 mice (7-8 weeks old) were obtained from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan). The mice were housed in an environmentally controlled room at 23 ± 1.5°C with a 12-h light/dark cycle. The animal experiments were performed according to the guidelines of Osaka University.

For an experimental metastasis model, B16 cells or CL4-B16 cells (5 × 10^5 or 1 × 10^6 cells) in 100 μl PBS were injected intravenously into the tails of C57/BL6 mice. Then, mice received
intravenously administered PBS or C-CPE-PSIF three times per week. Two weeks after the inoculation of the cells, mice were sacrificed, and the number of lung metastasis colonies was counted under a microscope. For anti-tumor activity in B16 cells, $1 \times 10^5$ B16 or CL4-B16 cells were injected subcutaneously into the right flanks of C57BL/6 mice. Vehicle or C-CPE-PSIF was intravenously administered three times per week. Calipers were used to measure the minimum and maximum tumor diameters, and the tumor volume was calculated as $a \times b \times b/2$, where $a$ represents the minimum tumor diameter, and $b$ represents the maximum tumor diameter. The body weight of mice was also monitored.

For anti-tumor activity in 4T1 cells, 4T1 cells ($1 \times 10^5$ cells) in 50 μl PBS were injected subcutaneously into the right flanks of BALB/c mice. PBS, C-CPE-PSIF or adriamycin (ADR) was intravenously administered, and the tumor size and body weight of mice were monitored. Mice were sacrificed 35 days after tumor inoculation, and lung metastasis was evaluated by staining with India ink.

Statistical analysis

Data were analyzed by using Dunnet’s method. The statistical significance for all comparisons was set at $p < 0.05$. 
Results

Preparation of claudin-4-expressing B16 BL6 cells

To investigate the effects of C-CPE-PSIF on tumor metastasis, we selected murine B16 cells, which have a high propensity to metastasize to the lung (Saiki, 1997). Western blotting analysis revealed no expression of claudin-4 in B16 cells (Fig. 1A). B16 cells were not sensitive to C-CPE-PSIF (Fig. 2A). We transfected claudin-4 cDNA into B16 cells, and we established stable claudin-4-expressing B16 cells (CL4-B16, Fig. 1A). The proliferation rate was not affected by exogenously expressed claudin-4 (Fig. 1B). Metastasis has multiple processes, including motility and invasion (Steeg, 2006). To investigate the invasion of CL4-B16 cells, we performed a Boyden chamber migration assay. Cells were seeded onto the cell culture insert of matrigel-coated membrane with an 8-μm pore size, and the cells that invaded the apical membrane and reached the basal membrane were counted. As shown in Fig. 1C, the invasion activity was increased 17-fold in CL4-B16 cells as compared to that in parental B16 cells. Lung metastasis of CL4-B16 cells was observed when intravenously injected into mice; however, the number of lung metastasis colonies of CL4-B16 cells was smaller than that of the parental B16 cells (Fig. 1D). These findings indicate that CL4-B16 cells can be used as a metastasis model of claudin-4-expressing cancer cells. We discuss the elevation of migration activity and lower lung metastasis in CL4-B16 cells in the Discussion section.

Anti-tumor activity of C-CPE-PSIF in CL4-B16 cells

Prior to in vivo experiments, we investigated the in vitro cytotoxicity of C-CPE-PSIF in CL4-B16 cells. As shown in Fig. 2A, C-CPE-PSIF showed dose-dependent cytotoxicity in CL4-B16 cells, decreasing their viability to 35% at 100 ng/ml. In contrast, parental B16 cells were not sensitive to C-CPE-PSIF even at 1 μg/ml, indicating that C-CPE-PSIF may target claudin-4. Claudin-4 is expressed in the intestines, liver and kidney (Morita et al., 1999). To determine a safe dose of C-CPE-PSIF, we checked serum biochemical markers of liver (alanine aminotransferase) and kidney (blood urea nitrogen) injury in mice injected with C-CPE-PSIF. After intravenous administration of C-CPE-PSIF (5 μg/kg), the mice showed no sign of injury (data not shown). In the following in vivo experiments, the doses of C-CPE-PSIF were less than or equal to 5 μg/kg. B16 or CL4-B16 cells were intravenously injected into mice, and then C-CPE-PSIF was intravenously administered every 2 days. Two weeks after the tumor cell injection, the number of
lung metastasis colonies was counted. As shown in Fig. 2B, C-CPE-PSIF treatment decreased the number of lung metastasis colonies from 39 ± 17 to 10 ± 4 at 5 μg/kg. In contrast, C-CPE-PSIF treatment did not affect the lung metastasis of B16 cells (Fig. 2B). C-CPE-PSIF suppressed the growth of CL4-B16 cells but not B16 cells inoculated into the right flank of mice (Fig. 2C). These data suggest that claudin-4-targeting may be a potent strategy to suppress tumor metastasis and growth.

Suppression of the primary tumors and metastasis of 4T1 cells

To clarify the potency of a claudin-4-targeting strategy, we investigated the effect of C-CPE-PSIF on a spontaneous metastasis model. 4T1 cells are murine cancer cells that spontaneously metastasize to the lung after being subcutaneously inoculated (Wong et al., 2002). We investigated whether C-CPE-PSIF suppresses the spontaneous lung metastasis of 4T1 cells. On day 33, the tumor volume was 1801.2 ± 848.5 mm$^3$ in the vehicle-treated group and 740.5 ± 94.6 mm$^3$ in the group treated with 5 μg/kg of C-CPE-PSIF (Fig. 3A). The number of lung metastasis colonies was decreased to 2 ± 1 colonies at 5 μg/kg of C-CPE-PSIF (Fig. 3B). A dose of 2 μg/kg of C-CPE-PSIF did not suppress tumor growth but did prevent lung metastasis. The circulating tumor cells might be more sensitive to C-CPE-PSIF than tumor cells in the solid tumor tissue. C-CPE-PSIF treatments did not cause a decrease in body weight (Fig. 3C), and there were no apparent biochemical side effects (Suppl. Fig. 1). ADR, which is frequently used in clinical chemotherapy, suppressed the tumor growth from 970.3 ± 278.4 mm$^3$ to 458.6 ± 51.4 mm$^3$ at 4 mg/kg (Fig. 3D). As shown in Fig. 3E, 4 mg/kg of ADR decreased the number of lung metastasis colonies (24 ± 13 colonies in the vehicle-treated group, 6 ± 4 colonies in the ADR-treated group). However, the ADR-treated mice experienced a 26% loss of body weight, which is a sign of side effects (Fig. 3F). Thus, the anti-tumor activity of C-CPE-PSIF may be more potent than that of ADR. These results indicate that the claudin-4-targeting therapy may be a potent strategy for tumor therapy with a low level of side effects and a high level of anti-tumor activity.
Discussion

Most malignant tumors are derived from the epithelium, and metastasis is the major cause of death from cancers. In the present study, we found that systemic administration of a claudin-targeting molecule suppressed cancer metastasis, indicating that claudin-targeting may be an effective therapy against cancer metastasis.

Although the in vitro metastasis activity of CL4-B16 cells was higher than that of parental B16 cells, the in vivo lung metastasis of CL4-B16 cells was lower than that of B16 cells. As shown in Suppl. Fig. 2, claudin-4 increased the invasiveness and migration activity of B16 cells in vitro and decreased lung metastasis in vivo. A possible explanation for this discrepancy may be the experimental model, which evaluates a different stage of metastasis. The migration and invasion activity involved in the early stage of metastasis was investigated in the in vitro analysis, while extravasation and colonization to an organ involved in the late stage of metastasis was evaluated by the in vivo experiment. The altered expression of claudin-4 changed the metastasis of 4T1 cells to the heart and liver, suggesting that claudin affects organ-specific metastasis (Erin et al., 2009). Claudin-4 may suppress the lung metastasis of B16 cells.

Claudin is a structural and functional component of TJs (Furuse and Tsukita, 2006). What is the role of claudin in metastasis? Metastasis is composed of three steps: leaving the primary site, entering the blood flow and invading the distant site. In the first step, the combination of claudin members in the TJ strands may be important. The claudin family contains at least 24 members. Claudin is believed to form homo- and hetero-type claudin-strands, and the pattern of the strands differs among tissues and determines the properties of TJ seals (Furuse and Tsukita, 2006). For example, rigid TJ seals were formed when claudin-11 or -15 was exogenously expressed in MDCK cells, whereas the expression of claudin-11 or -15 reduced the TJ integrity in LLC-PK1 cells by its dominant negative effects on TJ sealing (Van Itallie et al., 2003). Dominant negative effects of claudin-4 on the TJ barrier might contribute to an acceleration in the detachment of cancer cells from the primary tumor tissue. In the second and third steps of metastasis, cancer cells must move through the extracellular matrix at the primary site and the distant site. Cancer cells must degrade the extracellular matrix by the expression of matrix metalloproteinase and increase their motility. Claudin expression enhanced invasion with increased matrix metalloproteinase activity (Agarwal et al., 2005). There is a relationship between the levels of claudin-1/-4 and the metastasis of human cancers, including hepatic, colon, ovarian and gastric cancers (Agarwal et al., 2005; Halder et al.,...
The overexpression of claudin suppressed cancer metastasis in human pancreatic and gastric cancers (Michl et al., 2003; Mima et al., 2005; Ohtani et al., 2009). Claudin-4 suppressed or accelerated in vitro and in vivo metastasis of human cancer cells (Agarwal et al., 2005; Ohtani et al., 2009). Cell-cell interaction through TJs regulates cell growth signaling (Matter et al., 2005). Taken together, these findings indicate that claudin family members might control several steps of cancer metastasis. The precise molecular mechanism and role of claudin in cancer metastasis remain to be determined.

Whether a claudin-4-targeting method causes severe side effects is critical for its clinical application in cancer therapy. Claudins play pivotal roles in TJ barrier and fence functions by maintaining cellular polarity in normal epithelium (Furuse and Tsukita, 2006). Claudins are believed to be more accessible in tumors than in normal epithelium. Claudins form TJ seals in lateral membranes between adjacent cells in normal epithelium, whereas claudins are exposed on the cell surface during tumorigenesis (Kominsky, 2006; Soler et al., 1999). Indeed, no local or systemic side effects have been observed after the intratumoral administration of CPE (Kominsky et al., 2007; Santin et al., 2007). Here, we also found that the systemic administration of C-CPE-PSIF causes no significant increase in biochemical markers (AST, ALT and BUN) for toxicity at a therapeutic dose of 5 μg/kg (Suppl. Fig. 2). Thus, a claudin-targeting strategy might have weak side effects.

It is difficult to prepare recombinant claudin protein due to its hydrophobic property, and claudin has low antigenicity. Until recently, an antibody against the extracellular loop domain of claudin had never been successfully prepared, and C-CPE was the only known claudin binder. Recently, Romani et al. prepared a single-chain antibody fragment against claudin-3 by using phage display technology (Romani et al., 2009). They found that the antibody fragment binds to ovarian and uterine carcinoma cells in vitro. More importantly, a therapeutic monoclonal antibody against claudin-4 was developed. Suzuki et al. successfully prepared anti-claudin-4 antibody by immunizing claudin-4-expressing tumor cells into a mouse with autoimmune disease (Suzuki et al., 2009). The antibody mediates antibody-dependent cellular cytotoxicity and both in vitro and in vivo anti-tumor activity. Although the preparation of anti-claudin antibody may lead to a breakthrough in cancer therapy, the immunogenicity associated with immunotoxin clinical therapies is a future problem (Kreitman and Pastan, 2006). The C-terminal 30 amino acids are the minimum functional domain of C-CPE to bind to claudin-4 (Hanna et al., 1991). The C-terminal 30 amino acid fragment was used to deliver a cytokine to claudin-4-expressing cells by genetic fusion (Yuan
et al., 2009). Humanized antibody and the claudin-4-targeting peptide may be useful for cancer therapy in the near future.

In summary, this is the first report to indicate that systemic injection of a claudin-targeting molecule suppresses tumor growth and metastasis. Hematologic cells do not develop TJs; therefore, a claudin-targeting therapy may have no hematologic toxicity. We anticipate that claudin targeting will be a potent strategy for cancer therapy.
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References


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Footnotes

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**Figure legends**

**Figure 1.** Claudin-4-expressing B16 melanoma (CL4-B16) cells.

A) Preparation of CL4-B16 cells. Cell lysates from B16 and CL4-B16 cells were subjected to SDS-PAGE, followed by western blotting with claudin-4 and β-actin. β-actin is a control for an endogenous protein. B) The effect of claudin-4 on cellular proliferation in B16 cells. B16 or CL4-B16 cells (2 × 10^4 cells) were seeded onto a 24-well plate. Then, the cell numbers were counted by trypan blue dye exclusion assay at the indicated periods. Data are shown as means ± SD (n=4). C) Effect of claudin-4 on invasion in B16 cells. B16 or CL4-B16 cells (1 × 10^5 cells) were seeded into the upper well of the cell-culture insert coated with matrigel. After 24 h, the cells that invaded the bottom membrane of the insert were stained with DiffQuick reagent and counted under a microscope. Data are shown as means ± SD (n=4). *Significantly different from B16 cells (p < 0.01). D) Lung metastasis of CL4-B16 cells. B16 or CL4-B16 cells (5 × 10^5 or 1 × 10^6 cells) were injected into the tail veins of mice. After 14 days, the mice were sacrificed, the lungs were fixed, and the colonies on the lung surface were counted. Data are shown as means ± SD (n=5). *Significantly different from B16 cells (p < 0.01).

**Figure 2.** Anti-tumor activity of C-CPE-PSIF for CL4-B16 cells.

A) Cytotoxicity of C-CPE-PSIF in CL4-B16 cells. B16 cells (upper panel) and CL4-B16 cells (lower panel) were treated with PSIF or C-CPE-PSIF at the indicated concentration for 24 h. The cell viability (%) was measured by a WST-8 kit, according to the manufacturer’s instructions (Nacalai). Data represent the mean ± SD (n=3). *Significantly different from the vehicle-treated group (p < 0.05). B) Anti-metastatic activity of C-CPE-PSIF on lung metastasis of B16 or CL4-B16 cells. B16 or CL4-B16 cells (1 × 10^6 cells) were injected into the tail veins of mice on day 0, and vehicle or C-CPE-PSIF (2 or 5 µg/kg) was intravenously injected on days 0, 2, 4, 7, 9, 11, and 13. On day 14, the mice were sacrificed, their lungs were fixed, and the colonies on the lung surface were counted. Data are shown as means ± SD (n=5). *Significantly different from the vehicle-treated group (p < 0.05). C) Antitumor activity of C-CPE-PSIF on CL4-B16 subcutaneously inoculated allograft. B16 (upper panel) or CL4-B16 (lower panel) cells (1 × 10^5 cells) were intradermally inoculated into the right flank of mice on day 0, and PBS or C-CPE-PSIF (2 or 5 µg/kg) was intravenously injected three times a week. Tumor volume was monitored. Each point is the mean ± SD (n=5). The data are representative of two independent experiments.
Figure 3. Anti-tumor activity of C-CPE-PSIF for murine breast cancer 4T1 cells.

4T1 cells (1 × 10^5 cells) were intradermally inoculated into the right flanks of mice on day 0, and C-CPE-PSIF (A-C) or ADR (D-F) was intravenously injected three times or two times a week at the indicated dose, respectively. Tumor volume (A, D) and body weight (B, E) were monitored. On day 35, the mice were sacrificed, their lungs were stained with India ink, and the number of spontaneous metastases were determined (C, F). Data are shown as means ± SD (n=5). *Significantly different from the vehicle-treated group (p < 0.01). The data are representative of two independent experiments.
Figure 2A
Figure 2C

**B16**

- **PBS**
- **C-CPE-PSIF 5 μg/kg**

**Tumor volume (mm³)**

**Days after tumor inoculation**

**CL4-B16**

- **PBS**
- **C-CPE-PSIF 2 μg/kg**
- **C-CPE-PSIF 5 μg/kg**

**Tumor volume (mm³)**

**Days after tumor inoculation**
A.

Tumor volume (mm$^3$)

- PBS
- C-CPE-PSIF 2 µg/kg
- C-CPE-PSIF 5 µg/kg

Days after tumor inoculation

B.

Number of lung metastasis

C-CPE-PSIF (µg/kg)

0 2 5

*
Figure 3CD

C. 

Body weight (g) over Days after tumor inoculation for different treatments: PBS, C-CPE-PSIF 2 μg/kg, and C-CPE-PSIF 5 μg/kg.

D. 

Tumor volume (mm³) over Days after tumor inoculation for PBS and ADR 4 mg/kg treatments.