

## ORIGINAL ARTICLE

# Claudin-4 binder C-CPE 194 enhances effects of anticancer agents on pancreatic cancer cell lines via a MAPK pathway

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## Keywords

C-CPE, claudins, human pancreatic duct epithelial cells, MAPK, pancreatic cancer, tight junctions

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## Abstract

The C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) modulates the tight junction protein claudin and disrupts the tight junctional barrier. It also can enhance the effectiveness of anticancer agents. However, the detailed mechanisms of the effects of C-CPE remain unclear in both normal and cancerous cells. The C-CPE mutant called C-CPE 194 binds only to claudin-4, but the C-CPE 194 mutant called C-CPE m19 binds not only to claudin-4 but also to claudin-1. In the present study, to investigate the mechanisms of the effects of C-CPE on claudin expression, the tight junctional functions and the cytotoxicity of anticancer agents, human pancreatic cancer cells, and normal human pancreatic duct epithelial cells (HPDEs) were treated with C-CPE 194 and C-CPE m19. In well-differentiated cells of the pancreatic cancer cell line HPAC, C-CPE 194 and C-CPE m19 disrupted both the barrier and fence functions without changes in expression of claudin-1 and -4, together with an increase of MAPK phosphorylation. C-CPE 194, but not C-CPE m19, enhanced the cytotoxicity of the anticancer agents gemcitabine and S-1. In poorly differentiated pancreatic cancer cell line PANC-1, C-CPE 194, but not C-CPE m19, decreased claudin-4 expression and enhanced MAPK activity and the cytotoxicity of the anticancer agents. In normal HPDEs, C-CPE 194 and C-CPE m19 decreased claudin-4 expression and enhanced the MAPK activity, whereas they did not affect the cytotoxicity of the anticancer agents. Our findings suggest that the claudin-4 binder C-CPE 194 enhances effects of anticancer agents on pancreatic cancer cell lines via a MAPK pathway.

## Abbreviations

BEBM, bronchial epithelial cell basal medium; C-CPE, *Clostridium perfringens* enterotoxin; CLDN-1, claudin-1; CLDN-4, claudin-4; DMEM, Dulbecco's modified Eagle's medium; DTA, diphtheria toxin fragment A; FBS, fetal bovine serum; GEM, gemcitabine; HPDEs, human pancreatic duct epithelial cells; JNK, c-Jun N-terminal kinase; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PSIF, protein synthesis inhibitory factor; TBS, Tris-buffered saline; TEER, transepithelial electrical resistance.

## Introduction

Pancreatic cancer is known to be one of the most malignant cancers and is the fourth leading cause of

cancer-related death in Western countries, with a median survival of 6–7 months and a 5-year survival rate of 6% (Siegel et al. 2013). Surgical resection is the only potentially curative therapy for pancreatic cancer, which is

highly resistant to conventional chemotherapy regimens (Vincent et al. 2011). Thus, new molecular targets for therapeutic approaches must be developed to improve the poor conventional outcome of the disease.

Tight junctions are the most apical components of intercellular junctional complexes and they have both fence and barrier functions in normal epithelial cells (van Meer et al. 1986; Schneeberger and Lynch 1992; Gumbiner 1993; Cerejido et al. 1998). In some human cancers, including pancreatic cancer, tight junction protein claudins are abnormally regulated and are thus promising molecular targets for diagnosis and therapy (Morin 2005; Tsukita et al. 2008; Kojima and Sawada 2012). The claudin family, which consists of at least 27 members, is solely responsible for forming tight junction strands and has four transmembrane domains and two extracellular loops (Tsukita et al. 2001). The second extracellular loop is the receptor of *Clostridium perfringens* enterotoxin (CPE) (Fujita et al. 2000). *Clostridium perfringens* enterotoxin bound to its receptor causes changes in the membrane permeability via complex formation on the plasma membrane followed by the induction of apoptosis (McClane and Chakrabarti 2004). Claudin-3, -4, -6, -7, -8, and -14, but not claudin-1, -2, -5, and -10, are sensitive to CPE (Fujita et al. 2000). In pancreatic cancer, claudin-4, a high-affinity receptor of CPE, is frequently overexpressed (Michl et al. 2001; Karanjawala et al. 2008). In well-differentiated human pancreatic cancer cell line HPAC, CPE has a dose-dependent cytotoxic effect and the sensitivity to it is significantly decreased by knockdown of claudin-4 expression, using siRNA (Yamaguchi et al. 2011).

On the other hand, the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE; amino acids 184-319) binds to claudin-4 and disrupts the tight junctional barrier without a cytotoxic effect (Sonoda et al. 1999). C-CPE (amino acids 168-319) downregulates claudin-4 expression and sensitizes ovarian cancer cells to antitumor agents such as paclitaxel, and carboplatin (Gao et al. 2011). Claudin-4-targeting antitumor molecules that consist of C-CPE fused to protein synthesis inhibitory factor (PSIF) derived from *Pseudomonas aeruginosa* exotoxin or diphtheria toxin fragment A (DTA), is especially toxic to claudin-4-positive cancer cells in vivo and in vitro (Kakutani et al. 2010; Saeki et al. 2010). Furthermore, nontoxic C-CPE labeled with a fluorochrome shows high binding affinity specifically to claudin-4 positive pancreatic cancer cells (Neesse et al. 2013). It is thought that, in pancreatic cancer, C-CPE can enhance the effectiveness of clinically relevant chemotherapies.

Recently, it was found that a C-CPE mutant with 10 amino acids deleted at the N-terminal of C-CPE (C-CPE 194) had highly solubility in phosphate-buffered saline (PBS) and binding ability with claudin-4 (Uchida et al.

2010; Takahashi et al. 2011). Furthermore, the C-CPE mutant called C-CPE m19, which has the ability to bind not only with claudin-4 but also with claudin-1, was found after screening claudin binders from a C-CPE mutant-displaying library by using claudin-displaying budded baculovirus (Takahashi et al. 2012). However, the detailed effects of C-CPE 194 and C-CPE m19 on both normal and cancerous cells remain unknown.

In the treatment of advanced or metastatic pancreatic cancer, gemcitabine (GEM) has been widely used worldwide (Burris et al. 1997). In Japan, S-1 is widely used as one of the key drugs in the treatment of pancreatic cancer (Sudo et al. 2014). Recently, a randomized phase III trial (GEST [Gemcitabine and S-1 Trial] study) for advanced pancreatic cancer demonstrated the non-inferiority of S-1 to GEM (Ueno et al. 2013).

In the present study, we investigated the effects of C-CPE 194 and C-CPE m19 on claudin expression, tight junctional functions and the cytotoxicity of the anticancer agents GEM and S-1 in human pancreatic cancer cells in vitro compared to normal human pancreatic duct epithelial cells (HPDEs). The investigation of the effects in normal HPDEs is essential to develop safer and more effective diagnostic and therapeutic methods targeting claudins in pancreatic cancer. C-CPE 194 and C-CPE m19 disrupted both the barrier and fence functions without changes of claudins in the well-differentiated pancreatic cancer cell line HPAC. C-CPE 194, but not C-CPE m19, enhanced the cytotoxicity of GEM and S-1 in both HPAC cells and in the poorly differentiated pancreatic cancer cell line PANC-1. Their changes were controlled via the MAPK signaling pathway. In normal HPDEs, C-CPE 194 and C-CPE m19 decreased claudin-4 expression and enhanced the phosphorylation of MAPK, whereas they did not affect the cytotoxicity of the anticancer agents.

## Materials and Methods

### Reagents and inhibitors

Rabbit polyclonal anti-claudin-1, anti-claudin-4, anti-occludin and anti-tricellulin antibodies were obtained from Zymed Laboratories (San Francisco, CA). A rabbit polyclonal anti-actin antibody was purchased from Sigma-Aldrich, Inc. (St. Louis, MO). A rabbit polyclonal anti-phospho-ERK1/2 antibody was obtained from Cell Signaling (Beverly, MA). A rabbit polyclonal anti-ERK1/2 antibody was purchased from Promega Corporation (Madison, WI). A mouse monoclonal anti-his-tag antibody was purchased from Medical Biological Laboratories Co., Ltd. (Nagoya, Japan). Alexa Fluor 488 (green)-conjugated anti-rabbit IgG and Alexa Fluor 594 (red)-conju-

gated anti-mouse IgG antibodies were purchased from Molecular Probes, Inc. (Eugene, OR). Inhibitors of panPKC (GF109203X), mitogen-activated protein kinase kinase (MAPKK) (U0126), p38 MAPK (SB203580), phosphatidylinositol 3-kinase (PI3K) (LY294002), and c-Jun N-terminal kinase (JNK) (SP600125) were purchased from Calbiochem-Novabiochem Corporation (San Diego, CA). Gemcitabine (GEM) was purchased from Eli Lilly and Company (Indianapolis, IN). S-1 was purchased from Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan).

### Preparation of C-CPE

C-CPE 194 and C-CPE m19 were kind gifts from the Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan as part of our joint research (Uchida et al. 2010; Takahashi et al. 2012). The plasmid encoding C-CPE 194 or C-CPE m19 was transfected into *Escherichia coli* BL-21 (DE 3) and protein expression was stimulated by the addition of isopropyl-D-thiogalactopyranoside. The cells were then harvested, lysed in buffer A (10 mmol/L Tris-HCl, pH 8.0, 400 mmol/L NaCl, 5 mmol/L MgCl<sub>2</sub>, 0.1 mmol/L phenylmethanesulfonyl fluoride, 1 mmol/L 2-mercaptoethanol, and 10% glycerol). The fusion protein was isolated from the cell lysates by affinity chromatography with HisTrap™ HP (GE Healthcare, Little Chalfont, UK). The buffer was changed to PBS by gel filtration and then the purified protein was stored at -80°C until use. The C-CPE protein was quantified with a BCA protein assay kit in which BSA served as the standard (Pierce Chemical, Rockford, IL).

### Cultures of cell lines and treatment

Human pancreatic cancer cell lines PANC-1 and HPAC were purchased from ATCC (Manassas, VA). PANC-1 cells and HPAC cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 10% dialyzed fetal bovine serum (FBS; Invitrogen, Carlsbad, CA). The media for all cell lines contained 100 U/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin-B. All cells were plated on 60-mm culture dishes (Corning Glass Works, Corning, NY) that were coated with rat tail collagen (500 µg of dried tendon/mL in 0.1% acetic acid), and incubated in a humidified 5% CO<sub>2</sub> incubator at 37°C.

The cells were treated with C-CPE 194 and C-CPE m19 at 2 or 4 µg/mL until 24 h and pretreated with the inhibitors of signal transduction pathways at 10 µmol/L 30 min before treatment with C-CPE 194 or C-CPE m19 at 2 µg/mL for 24 h. Some cells were treated with GEM

and S-1 at 1–100 nmol/L for 96 h with or without C-CPE 194 or C-CPE m19.

### Isolation and culture of human pancreatic duct epithelial cells and treatment

Human pancreatic tissues were obtained from patients with pancreatic or biliary tract diseases who underwent pancreatic resection in the Sapporo Medical University hospital. Informed consent was obtained from all patients, and the study was approved by the ethics committee of Sapporo Medical University (reference No.19-2-13).

The procedures for primary culture of human pancreatic duct epithelial cells (HPDEs) were as reported previously (Yamaguchi et al. 2010; Ito et al. 2011; Kyuno et al. 2013). Some primary cultured HPDEs were transfected with the catalytic component of telomerase, the human catalytic subunit of the telomerase reverse transcriptase (hTERT) gene (kindly provided by Dr. Robert Weinberg; Yamaguchi et al. 2010). The hTERT-HPDEs were plated on 60-mm culture dishes (Corning Glass Works, Corning, NY) that were coated with rat tail collagen (500 µg of dried tendon/mL 0.1% acetic acid). The cells were cultured in serum-free bronchial epithelial cell basal medium (BEBM; Lonza Walkersville, Inc., Walkersville, MD) supplemented with bovine pituitary extract (1% v/v), 5 µg/mL insulin, 0.5 µg/mL hydrocortisone, 50 µg/mL gentamicin, 50 µg/mL amphotericin-B, 0.1 ng/mL retinoic acid, 10 µg/mL transferrin, 6.5 µg/mL triiodothyronine, 0.5 µg/mL epinephrine, 0.5 ng/mL epidermal growth factor (Lonza Walkersville, Inc.), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich), and 2.5 µg/mL amphotericin-B and incubated in a humidified, 5% CO<sub>2</sub>:95% air incubator at 37°C. In this experiment, 2nd and 3rd passaged cells were used.

The cells were treated with 2 µg/mL C-CPE 194 or C-CPE m19 until 24 h. Some cells were treated with GEM and S-1 at 1–100 nmol/L for 96 h with or without C-CPE 194 or C-CPE m19.

### Immunohistochemical analysis

Immunohistochemical analysis was performed to evaluate the expression and distribution of claudin-1 and -4 in well-differentiated and poorly differentiated pancreatic cancer tissues. Deparaffinized tissue sections were immersed in 10 mmol/L sodium citrate (pH 6.0) and boiled for antigen retrieval by microwave (95°C, 30 min). Endogenous peroxidase activity was blocked, using 3% hydrogen peroxidase for 10 min. The sections were incubated with rabbit polyclonal claudin-1 and -4 antibodies (1:100 dilution) overnight at 4°C. The sections were incubated with a Dako REAL™ EnVision™/HRP, Rabbit/

Mouse (Dako REAL™ EnVision™ Detection System; Dako, code K5007, (Glostrup, Denmark)) for 1 h at room temperature. After washing with PBS, the labeled secondary antibody was visualized by adding Dako REAL™ Substrate Buffer (Dako REAL™ EnVision™ Detection System: Dako, code K5007) containing Dako REAL™ DAB+ Chromogen (Dako REAL™ EnVision™ Detection System: Dako, code K5007). The sections were counterstained with hematoxylin.

### Western blot analysis

For Western blotting of total cell lysates, the dishes were washed with PBS and 300  $\mu$ L of sample buffer (1 mmol/L NaHCO<sub>3</sub> and 2 mmol/L phenylmethylsulfonyl fluoride) was added to 60- or 35-mm culture dishes. The cells were scraped and collected in microcentrifuge tubes and then sonicated for 10 sec. The protein concentrations of the samples were determined, using a BCA Protein Assay Reagent Kit (Pierce Chemical Co., Rockford, IL). Aliquots of 15  $\mu$ g of protein/lane for each sample were separated by electrophoresis in 4/20% SDS polyacrylamide gels (Cosmo Bio Co., Tokyo, Japan). After electrophoretic transfer to nitrocellulose membranes (Immobilon; Millipore, Billerica, MA), the membranes were saturated with blocking buffer (Tris-buffered saline [TBS] with 0.1% Tween 20 and 4% skim milk) for 30 min at room temperature and incubated with polyclonal anti-claudin-1, anti-claudin-4, anti-phospho-ERK1/2, anti-ERK1/2 and anti-actin antibodies (1:1000) for 1 h at room temperature. The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Dako A/S, Copenhagen, Denmark) at room temperature for 1 h. The immunoreactive bands were detected, using an ECL Western blotting analysis system (GE Healthcare). Signals were quantified using Scion Image Beta 4.02 Win (Scion Co., Frederick, MA). Each set of results shown is representative of at least three separate experiments.

### Immunoprecipitation

The dishes were washed with PBS twice and 300  $\mu$ L of NP-40 lysis buffer (50 mmol/L Tris-HCl, 2% NP-40, 0.25 mmol/L Na-deoxycholate, 150 mmol/L NaCl, 2 mmol/L EGTA, 0.1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 10 mmol/L NaF, 2 mmol/L PMSF) was added to the 60-mm dishes. The cells were scraped off, collected in microcentrifuge tubes, and then sonicated for 10 sec. Cell lysates were incubated with protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology, Inc., Uppsala, Sweden) for 1 h at 4°C and then clarified by centrifugation at 15,000 *g* for 10 min. The supernatants were incubated with the anti-his-tag antibody bound to protein A-Sepharose CL-4B overnight

at 4°C. After incubation, immunoprecipitates were washed extensively with the same lysis buffer and subjected to Western blot analysis with anti-claudin-4 antibodies. Signals were quantified using Scion Image Beta 4.02 Win (Scion Co., Frederick, MA). Each set of results shown is representative of at least three separate experiments.

### Immunocytochemistry

The cells were grown on 35-mm glass-base dishes (Iwaki, Chiba, Japan) coated with rat tail collagen. They were fixed with cold acetone and ethanol (1:1) at 20°C for 10 min. After rinsing in PBS, the sections and cells were incubated with polyclonal anti-claudin-1 (1:100) and monoclonal anti-claudin-4 (1:100) antibodies, and polyclonal anti-claudin-4 (1:100), and monoclonal anti-his-tag (1:1000) antibodies at room temperature for 1 h, and then with Alexa Fluor 488 (green)-conjugated anti-rabbit IgG (1:200) and Alexa Fluor 594 (red)-conjugated anti-mouse IgG (1:200) at room temperature for 1 h. DAPI (Sigma-Aldrich) was used for counterstaining of nuclei in the cells. The specimens were examined, using a confocal laser scanning microscope (LSM510; Carl Zeiss, Jena, Germany).

### Cell viability assay

The cytotoxic effects of C-CPE (194 and m19) and chemotherapeutic agents with or without C-CPE on each pancreatic cancer cell line and hTERT-HPDEs were assessed using Cell Counting Kit-8 (DOJINDO, Komamoto, Japan) in accordance with the manufacturer's instructions. Cells were seeded at 3–5  $\times$  10<sup>3</sup> cells/well (depending on the growth rate and size of cells) in 96-well plates (Corning, NY) and precultured for 48 h in a CO<sub>2</sub> incubator at 37°C. They were then exposed to various concentrations of C-CPE and gemcitabine or S-1 alone and in combination. When evaluating the cytotoxic effects of C-CPE, cells were treated with 2 or 4  $\mu$ g/mL C-CPE 194 or C-CPE m19 for 24 h. When evaluating the cytotoxic effects of the chemotherapeutic agents, various concentrations of gemcitabine (1–100 nmol/L) or S-1 (1–100  $\mu$ g/mL) were added to the wells and the cells were incubated for 96 h. In combination experiments, cells were treated with 0.01–2  $\mu$ g/mL C-CPE 194 or 2  $\mu$ g/mL C-CPE m19 8 h before being treated with gemcitabine or S-1. Then 10  $\mu$ L of Cell Counting Kit-8 solution was added to each well, followed by incubation for 2 h at 37°C. The optical density of each well was measured at 450 nm with an iMark microplate reader (Bio-Rad, Hercules, CA). The ratio of absorbance was calculated and then presented as the mean  $\pm$  SD of than triplicate or more experiments.



## Measurement of transepithelial electrical resistance

The cells were cultured to confluence in the inner chambers of 12-mm Transwell 0.4- $\mu\text{m}$  pore-size filters (Corning Life Science). Transepithelial electrical resistance (TEER) was measured using an EVOM voltmeter with an ENDOHM-12 (World Precision Instruments, Sarasota, FL) on a heating plate (Fine, Tokyo, Japan) and was monitored using a cellZscope (nanoAnalytics, Münster Germany), a computer controlled automated multiwell device (12 wells). The values were expressed in standard units of ohms per square centimeter and presented as the mean  $\pm$  SD of triplicate experiments. For calculation, the resistance of blank filters was subtracted from that of filters covered with cells.

## Diffusion of BODIPY-sphingomyelin

For measurement of the tight junctional fence function, we used diffusion of BODIPY-sphingomyelin (Balda *et al.* 1996) with some modification (Kojima *et al.* 2011). Sphingomyelin/BSA complexes (5 nmol/L) were prepared in P buffer (10 nmol/L HEPES, pH 7.4, 1 mmol/L sodium pyruvate, 10 mmol/L glucose, 3 mmol/L  $\text{CaCl}_2$ , and 145 mmol/L NaCl), using BODIPY-FL-sphingomyelin (Molecular Probes) and defatted BSA. Cells plated on glass-bottom microwell plates (Mat Tek Corp., Ashland, MA) were loaded with BODIPY-sphingomyelin/BSA complex for 1 min on ice, after which they were rinsed with cold DMEM and mounted in DMEM on a glass slide. The samples were analyzed by confocal laser scanning microscopy (LSM5; Carl Zeiss, Jena, Germany). All pictures shown were generated within the first 5 min of the analysis.

## Data analysis

Results are given as means  $\pm$  SEM. Differences between groups were tested by ANOVA followed by a post hoc test and an unpaired two-tailed Student's *t* test.

## Results

### Expression and distribution of C-CPE targeting claudin-1 and claudin-4 in well- and poorly differentiated pancreatic duct carcinomas

We examined the expression and distribution of claudin-1 and claudin-4 in well- and poorly differentiated pancreatic duct carcinomas (Fig. 1). In well-differentiated pancreatic carcinoma, claudin-1 was in part detected in gland

duct-like cancer cells, and claudin-4 was highly expressed in all cancer cells (Fig. 1). In poorly differentiated pancreatic carcinoma, claudin-1 and claudin-4 were detected at the membranes among several cancer cells (Fig. 1).

### Cytotoxic effect of C-CPE in HPAC cells

To investigate the cytotoxic effect of C-CPE in well-differentiated pancreatic cancer cell line HPAC, the cells were treated with C-CPE 194 or C-CPE m19 at 2 or 4  $\mu\text{g}/\text{mL}$  for 24 h, and cell viability assay was performed. Cytotoxic effects were not observed at any concentration of C-CPE 194 or C-CPE m19 (Fig. 2A).

### Effects of C-CPE on barrier function and fence function in HPAC cells

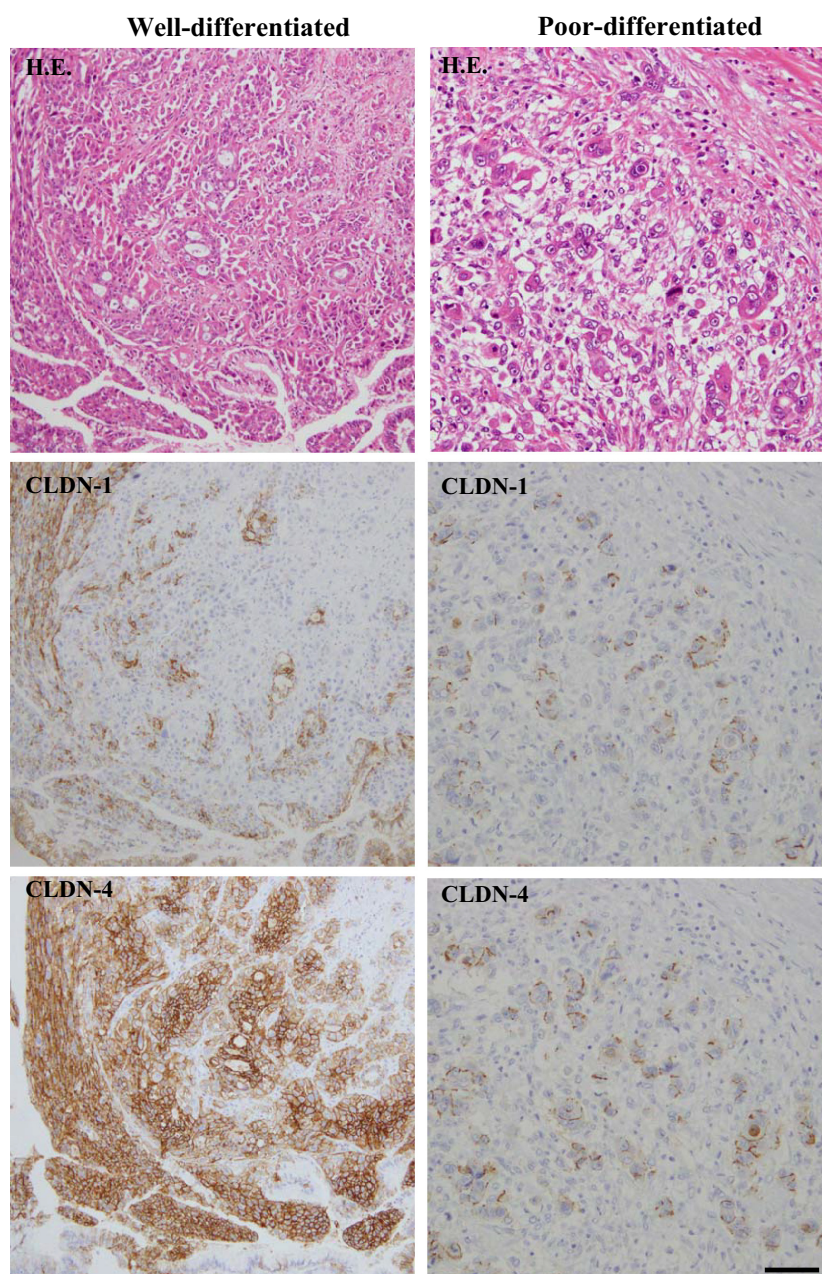
To investigate the effect of C-CPE on the barrier function in HPAC cells, the cells were treated with 2  $\mu\text{g}/\text{mL}$  C-CPE 194, or C-CPE m19 for 24 h and TEER values were measured. Significant decreases of TEER values were observed in both C-CPE 194- and C-CPE m19-treated cells (Fig. 2B). To investigate the effect of C-CPE on the fence function in HPAC cells, BODIPY-sphingomyelin diffusion in the membrane was measured in HPAC cells 24 h after treatment with 2  $\mu\text{g}/\text{mL}$  C-CPE 194, or C-CPE m19. In the control, the BODIPY-sphingomyelin was effectively retained in the apical domain. In HPAC cells after treatment with C-CPE 194 or C-CPE m19, the probe diffused through the tight junctions, strongly labeled the basolateral surfaces and appeared to penetrate the cells (Fig. 2C).

### Effect of C-CPE on the expression of tight junction proteins in HPAC cells

To investigate whether C-CPE affected the expression of tight junction proteins in HPAC cells, the cells were treated with 2  $\mu\text{g}/\text{mL}$  C-CPE 194 or C-CPE m19 for 4, 8, and 24 h. In Western blots, no change of tight junction proteins was observed in the cells after treatment with C-CPE 194 or C-CPE m19 compared to the control (Fig. 2D).

### Effect of C-CPE on localization of claudin-1 and claudin-4 in HPAC cells

To investigate the effect of C-CPE on the localization of claudin-1 and claudin-4 proteins in HPAC cells, the cells were treated with 2  $\mu\text{g}/\text{mL}$  C-CPE 194 or C-CPE m19 for 24 h and immunocytochemistry was performed. Claudin-1 and claudin-4 were decreased at the membranes and claudin-4 was detected in perinuclear regions (Fig. 3A) after both treatments.

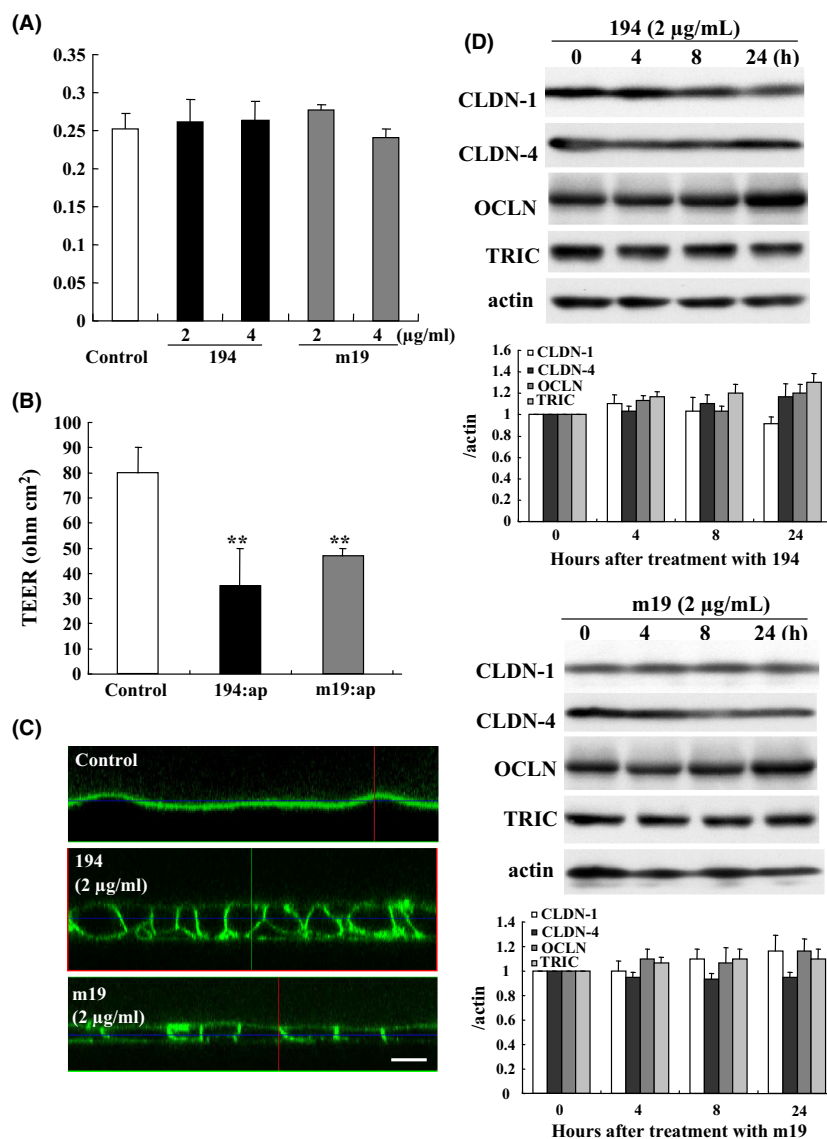


**Figure 1.** Hematoxylin-eosin (H.E.) staining and immunohistochemical staining for claudin-1 (CLDN-1) and claudin-4 (CLDN-4) in well- and poorly differentiated pancreatic carcinomas. Bar: 50  $\mu$ m.

### Behavior of C-CPE in HPAC cells after treatment

To investigate the behavior of C-CPE in HPAC cells after treatment, Western blotting was performed after coimmunoprecipitation and immunocytochemistry by using an anti-his-tag antibody. In the Western blots, claudin-4 binding C-CPE 194 or C-CPE m19 was detected at 4, 8, and 24 h after treatment (Fig. 3B).

After washout following treatment for 24 h, neither claudin-4-binding C-CPE 194 or C-CPE m19 was detected (Fig. 3B). In the present study, claudin-1 was not detected in Western blots after coimmunoprecipitation, using the anti-his-tag antibody (data not shown). In immunocytochemistry at 24 h after treatment, C-CPE 194, and C-CPE m19 were detected in cytoplasm and some dots were colocalized with claudin-4 (Fig. 3C).



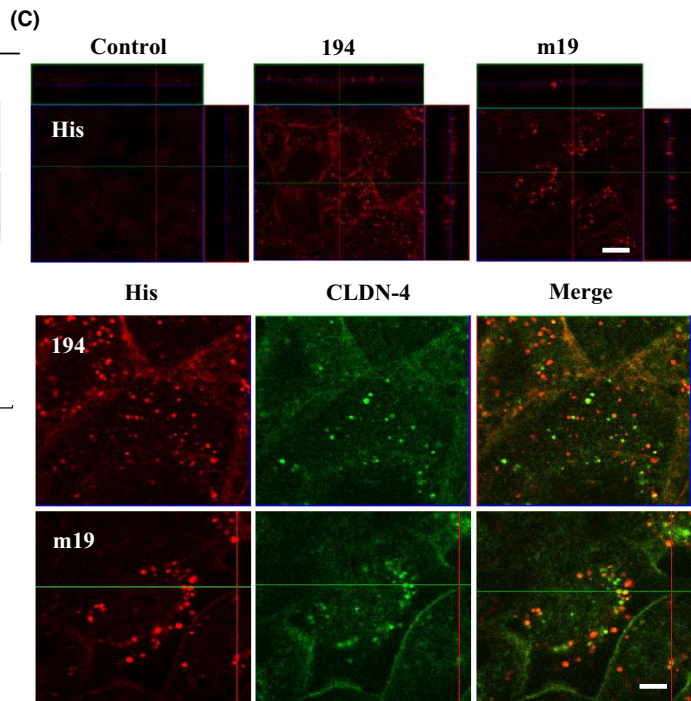
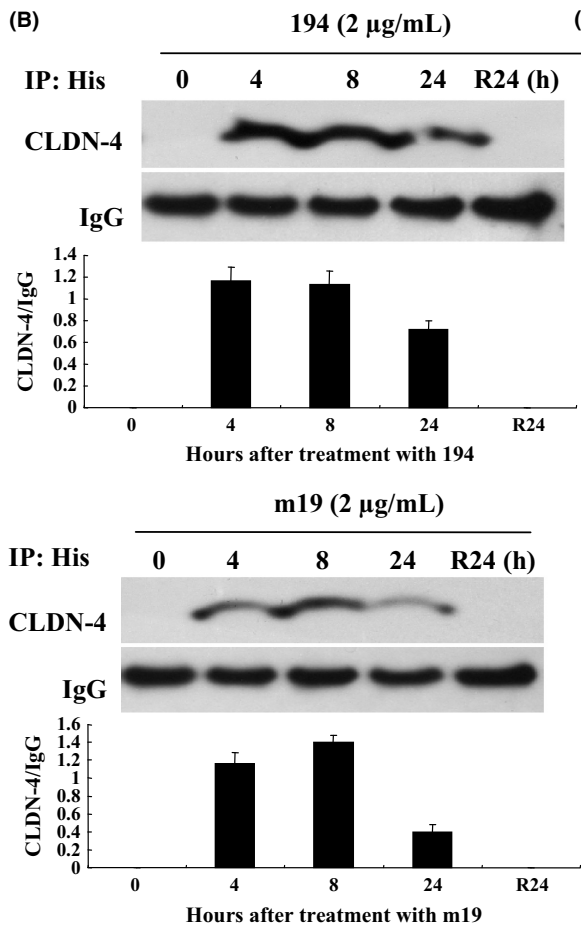
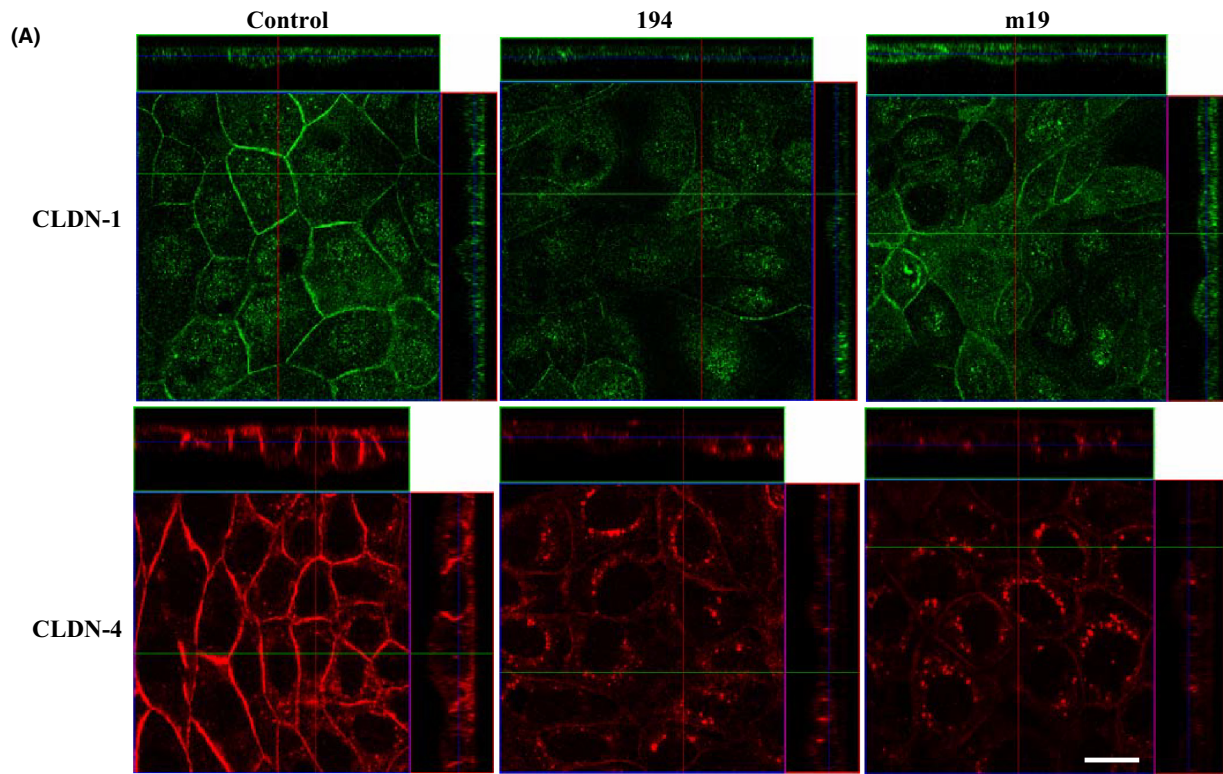
**Figure 2.** (A) Cell viability assay of HPAC cells after treatment with 2–4  $\mu\text{g}/\text{mL}$  C-CPE 194 or C-CPE m19 for 24 h. (B) Barrier function examined by TEER values in HPAC cells after treatment with 2  $\mu\text{g}/\text{mL}$  C-CPE 194 or C-CPE m19 for 24 h. \*\* $P < 0.01$  versus control. (C) Fence function examined by diffusion of labeled BODIPY-sphingomyelin in HPAC cells after treatment with 2  $\mu\text{g}/\text{mL}$  C-CPE 194 or C-CPE m19 for 24 h. Bar: 10  $\mu\text{m}$ . (D) Western blots for claudin (CLDN)-1, -4, occludin (OCLN) and tricellulin (TRIC) in HPAC cells after treatment with 2  $\mu\text{g}/\text{mL}$  C-CPE 194 or C-CPE m19 for 24 h. The corresponding expression levels of D are shown as bar graphs. 194: C-CPE 194. m19: C-CPE m19.

### C-CPE downregulates the barrier and fence functions of tight junctions via a MAPK signaling pathway in HPAC cells

To investigate which signaling pathways were associated with downregulation of the barrier and fence functions by C-CPE in HPAC cells, the cells were pretreated with MAPKK inhibitor U0126, panPKC inhibitor GF109203X, c-Jun N-terminal kinase (JNK) inhibitor SP600125, phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002, and

p38 MAPK inhibitor SB203580, each at 10  $\mu\text{mol}/\text{L}$ , before treatment with 2  $\mu\text{g}/\text{mL}$  C-CPE 194 or C-CPE m19. The TEER value was used to indicate the barrier function. The decrease of the TEER value induced by C-CPE 194 was completely prevented by U0126 and slightly restored by GF109203X and SP600125 (Fig. 4A). The decrease of the TEER value induced by C-CPE m19 was slightly restored by U0126, GF109203X and SP600125 (Fig. 4A). In the BODIPY-sphingomyelin diffusion was used to indicate the fence function, the probe diffusion to the basolateral







**Figure 3.** (A) Immunocytochemistry for claudin-1 and claudin-4 in HPAC cells after treatment with 2  $\mu\text{g}/\text{mL}$  C-CPE 194 or C-CPE m19 for 24 h. Bar: 10  $\mu\text{m}$ . (B) Western blots for claudin-4 after coimmunoprecipitation using an anti-his-tag antibody in HPAC cells after treatment with 2  $\mu\text{g}/\text{mL}$  C-CPE 194 or C-CPE m19 for 4, 8, 24 h and 24 h after washout (R24). The corresponding expression levels of B are shown as bar graphs. (C) Immunocytochemistry and double-immunostaining for his-tag and claudin-4 in HPAC cells after treatment with 2  $\mu\text{g}/\text{mL}$  C-CPE 194 or C-CPE m19 for 24 h. Bar: 10  $\mu\text{m}$ . CLDN-1: claudin-1, CLDN-4: claudin-4, 194: C-CPE 194, m19: C-CPE m19.

surfaces induced by C-CPE 194 was completely prevented by U0126 (Fig. 4B), whereas the diffusion to the basolateral surfaces induced by C-CPE m19 slightly remained (Fig. 4B). To investigate whether C-CPE induced changes of MAPK signaling in HPAC cells, the cells were treated with 0.01  $\mu\text{g}/\text{mL}$  C-CPE 194 or 2  $\mu\text{g}/\text{mL}$  C-CPE m19 with or without U0126 and Western blotting was performed. Increases of phospho-ERK1/2 were observed in the cells treated with C-CPE 194 and C-CPE m19 compared to the control and these increases were prevented by U0126 (Fig. 4C).

### C-CPE 194, but not C-CPE m19, enhances the effects of anticancer agents on HPAC cells via the MAPK signaling pathway

To investigate whether C-CPE enhanced the effects of anticancer agents on HPAC cells, the cells were treated with 1–100 nmol/L GEM or 1–100  $\mu\text{g}/\text{mL}$  S-1 with or without 0.01  $\mu\text{g}/\text{mL}$  C-CPE 194 or 2  $\mu\text{g}/\text{mL}$  C-CPE m19 and a cell viability assay was carried out using Cell Counting Kit-8. Both GEM and S-1 decreased cell viability in a dose-dependent manner. C-CPE 194 significantly enhanced the decreases of cell viability induced by GEM and S-1, whereas C-CPE m19 did not affect the cell viability (Fig. 5A and B).

We then investigated whether the enhancement of the effects of the anticancer agents on HPAC cells induced by C-CPE 194 was associated with the MAPK signaling pathway. MAPKK inhibitor U0126 prevented the decrease of cell viability induced by C-CPE 194 in both the treatments with GEM and S-1 (Fig. 5C).

### Effects of C-CPE on tight junction proteins and MAPK pathway in PANC-1 cells

The poorly differentiated pancreatic cancer cell line PANC-1 exhibits loss of the barrier and fence functions of tight junctions (Kojima *et al.* 2011; Yamaguchi *et al.* 2011). To investigate the effects of C-CPE on the expression and localization of claudin-1 and claudin-4 and the MAPK signaling pathway in PANC-1 cells, the cells were treated with 2  $\mu\text{g}/\text{mL}$  C-CPE 194, or C-CPE m19 for 24 h. Western blots revealed that an increase of phospho-ERK1/2 and a decrease of claudin-4 in PANC-1 cells were induced by C-CPE 194 but not C-CPE m19 in a time-dependent manner (Fig. 6A). Immunocytochemistry

showed that a decrease of claudin-4, but not claudin-1, at the membranes was induced by C-CPE 194 but not C-CPE m19 (Fig. 6B).

### C-CPE 194, but not C-CPE m19, enhances the effects of anticancer agents via the MAPK signaling pathway in PANC-1 cells

To investigate whether C-CPE enhanced the effects of anticancer agents on PANC-1 cells, the cells were treated with 1–100 nmol/L GEM or 1–100  $\mu\text{g}/\text{mL}$  S-1 with or without 0.01  $\mu\text{g}/\text{mL}$  C-CPE 194, or 2  $\mu\text{g}/\text{mL}$  C-CPE m19.

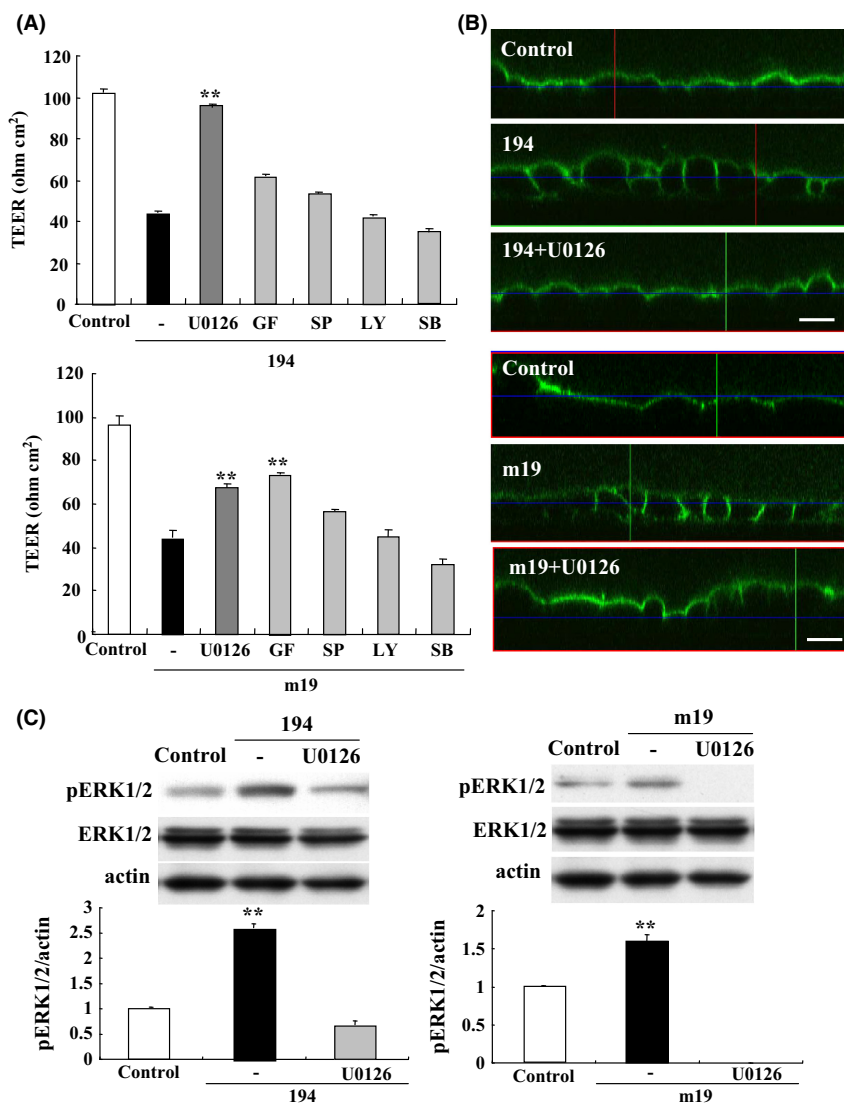
In the treatments with GEM and S-1, dose-dependent decreases of cell viability in PANC-1 cells were observed. C-CPE 194 significantly enhanced the decrease of cell viability induced by GEM or TS-1, whereas C-CPE m19 did not affect the cell viability (Fig. 6C and D). MAPKK inhibitor U0126 prevented the decrease of cell viability induced by C-CPE 194 in the treatments with GEM and S-1 (Fig. 6E).

### Effects of C-CPE on tight junction proteins and MAPK pathway in hTERT-HPDE cells

To investigate the effects of C-CPE in normal pancreatic duct epithelial cells, hTERT-HPDE cells, which we first established (Yamaguchi *et al.* 2010), were treated with 2  $\mu\text{g}/\text{mL}$  C-CPE 194 or C-CPE m19 for 24 h. Western blots revealed a time-dependent increase of phospho-ERK1/2 and decrease of claudin-4 in hTERT-HPDE cells induced by C-CPE 194 and C-CPE m19 (Fig. 7A). Immunocytochemistry showed that a decrease of claudin-4, but not claudin-1, at the membranes was induced by C-CPE 194 but not C-CPE m19 (Fig. 7B).

### C-CPE does not enhance the effects of anticancer agents on hTERT-HPDE cells

To investigate whether C-CPE enhanced the effects of anticancer agents on hTERT-HPDE cells, the cells were treated with 1–100 nmol/L GEM or 1–100  $\mu\text{g}/\text{mL}$  S-1 with or without 0.01  $\mu\text{g}/\text{mL}$  C-CPE 194 or 2  $\mu\text{g}/\text{mL}$  C-CPE m19. In the treatments with GEM and S-1, dose-dependent decreases of cell viability in hTERT-HPDE cells were observed but neither C-CPE 194 nor C-CPE m19 affected the cell viability (Fig. 7C and D).



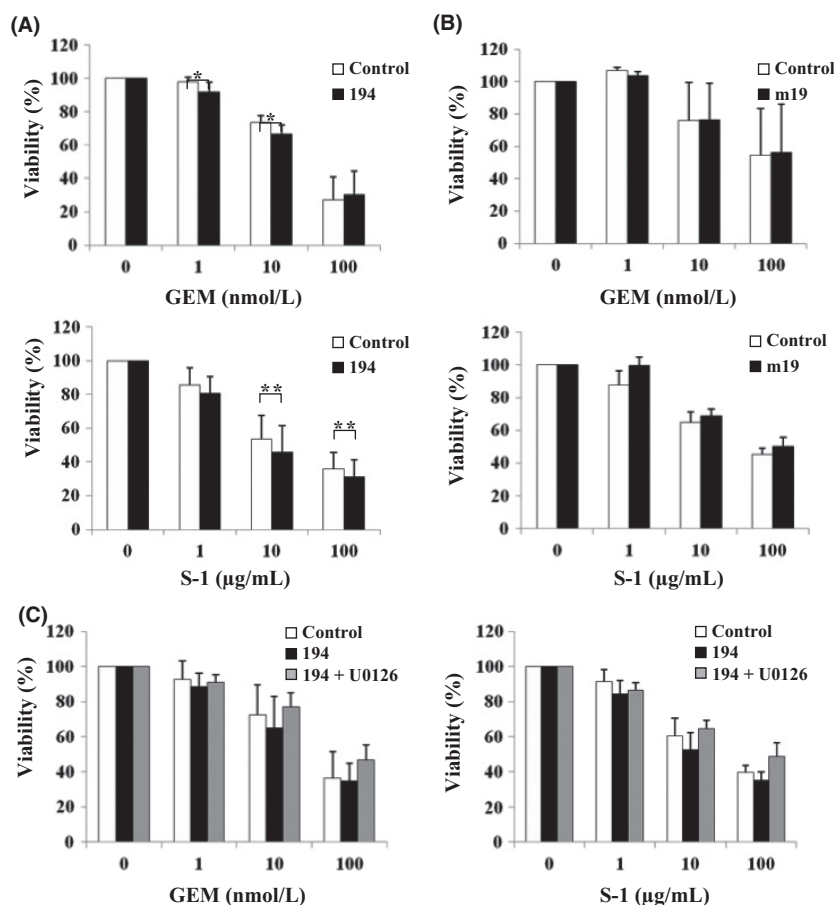
**Figure 4.** (A) TEER values in HPAC cells pretreated with MAPKK inhibitor U0126, panPKC inhibitor GF109203X (GF), c-Jun N-terminal kinase (JNK) inhibitor SP600125 (SP), phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (LY) and p38 MAPK inhibitor SB203580 (SB), each at 10 μmol/L, before treatment with 2 μg/mL C-CPE 194 or C-CPE m19 for 24 h. \*\**P* < 0.01 versus without inhibitors. (B) Fence function examined by diffusion of labeled BODIPY-sphingomyelin in HPAC cells pretreated with 10 μmol/L MAPKK inhibitor U0126 before treatment with 2 μg/mL C-CPE 194 or C-CPE m19 for 24 h. Bar: 10 μm. (C) Western blots for phospho-ERK1/2 and ERK 1/2 in HPAC cells after treatment with 0.01 μg/mL C-CPE 194 or 2 μg/mL C-CPE m19 for 8 h with or without 10 μmol/L MAPKK inhibitor U0126. The corresponding expression levels of C are shown as bar graphs. \*\**P* < 0.01 versus control. 194: C-CPE 194. m19: C-CPE m19.

## Discussion

In the present study, we demonstrated that claudin-4 binder C-CPE 194 enhanced the cytotoxicity of the anti-cancer agents GEM and S-1 on human pancreatic cancer cells but not normal HPDE cells via the MAPK signaling pathway.

It is reported that C-CPE does not have a cytotoxic effect on MDCK I cells (Sonoda et al. 1999). In the present study, the C-CPE mutants C-CPE 194 and C-CPE

m19 did not have cytotoxicity in pancreatic cancer cells and normal HPDEs. C-CPE disrupts the tight junctional barrier function in various types of cells (Sonoda et al. 1999; Masuyama et al. 2005; Gao et al. 2011; Matsuhisa et al. 2012; Takahashi et al. 2012). In the present study, when well-differentiated HPAC cells were treated with C-CPE mutants C-CPE 194 or C-CPE m19, they disrupted both the barrier and fence functions of tight junctions without changes in expression of claudin-1 and -4 in Western blots. In immunocytochemistry, claudin-1 and



**Figure 5.** Cell viability assay in HPAC cells treated with 1–100 nmol/L gemcitabine (GEM) or 1–100 µg/mL S-1 for 96 h with or without C-CPE 194 (A) or C-CPE m19 (B). (C) Cell viability assay in HPAC cells treated with 1–100 nmol/L gemcitabine (GEM) or 1–100 µg/mL S-1 with or without C-CPE 194 or C-CPE 194 and 10 µmol/L MAPKK inhibitor U0126. 194: C-CPE 194. m19: C-CPE m19. \* $P < 0.05$  and \*\* $P < 0.01$  versus control.

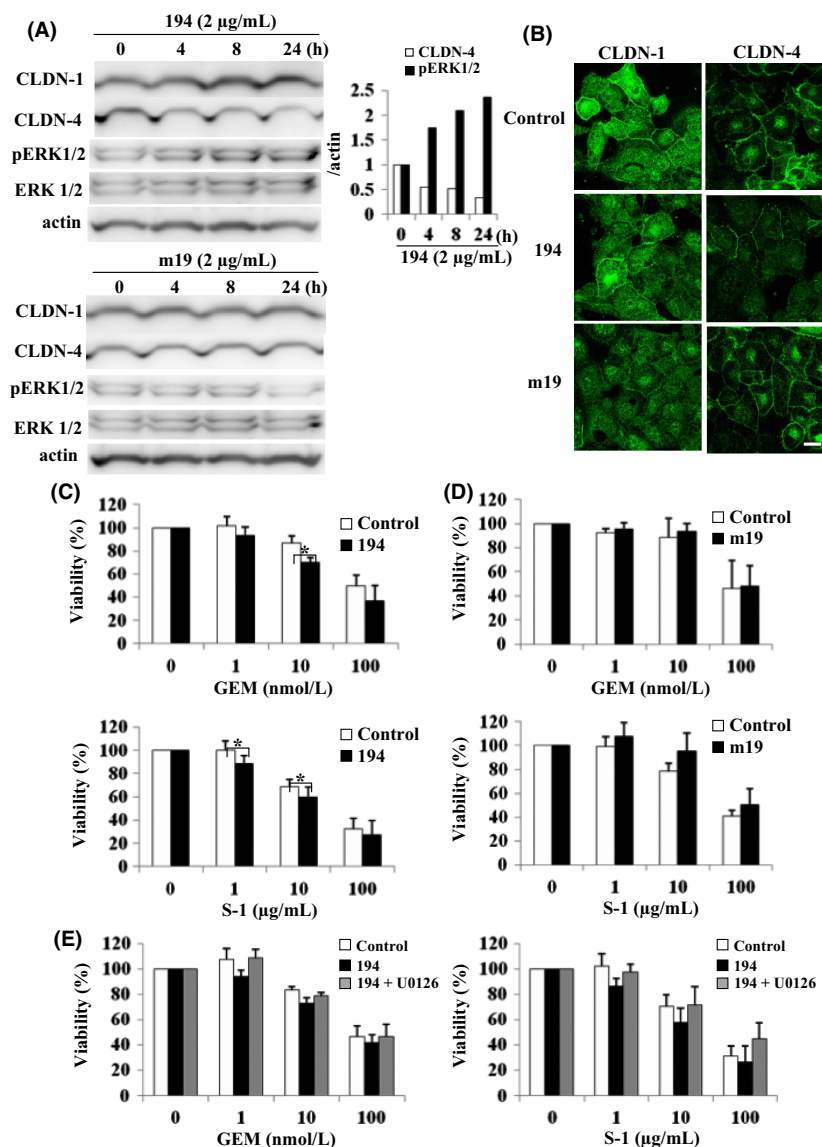
claudin-4 were found to be decreased at the membranes, and claudin-4 was in part colocalized with C-CPE 194 or C-CPE m19 in cytoplasm. Claudin-4 binding of C-CPE 194 or C-CPE m19 was detected in Western blots after coimmunoprecipitation.

The detailed mechanism of the disruption of the tight junction barrier by C-CPE and C-CPE mutants remains unclear. It is thought that the tight junctional functions are regulated via distinct signal transduction pathways (Stuart and Nigam 1995; González-Mariscal et al. 2008; Kojima et al. 2009). When pretreatment was done with inhibitors of the various signal transduction pathways, MAPKK inhibitor U0126, panPKC inhibitor GF109203X, JNK inhibitor SP600125, PI3K inhibitor LY294002, and p38 MAPK inhibitor SB203580, before treatment with C-CPE 194 and C-CPE m19, the MAPKK inhibitor U0126 prevented the disruption of the barrier and fence functions. Furthermore, C-CPE 194 and C-CPE m19 enhanced the activity of MAPK phosphorylation, which

can be prevented by U0126. These results suggested that C-CPE in part regulated the functions of tight junctions via the MAPK signaling pathway.

*Clostridium perfringens* enterotoxin (amino acids 168–319) downregulates claudin-4 expression and sensitizes ovarian cancer cells to antitumor agents such as paclitaxel and carboplatin (Gao et al. 2011). In the present study, in pancreatic cancer cell lines HPAC and PANC-1, C-CPE 194 enhanced the cytotoxicity of the anticancer agents GEM and S-1 and the effect was prevented by the MAPKK inhibitor U0126. In the well-differentiated pancreatic cancer cell line HPAC, with barrier and fence functions of tight junctions, C-CPE 194 disrupted the functions and enhanced the MAPK activity without changing the claudin-4 expression. In the poorly differentiated pancreatic cancer cell line PANC-1, in which the tight junctional functions are lost (Yamaguchi et al. 2011), C-CPE 194 decreased claudin-4 expression and enhanced the MAPK activity. In pancreatic cancer cells



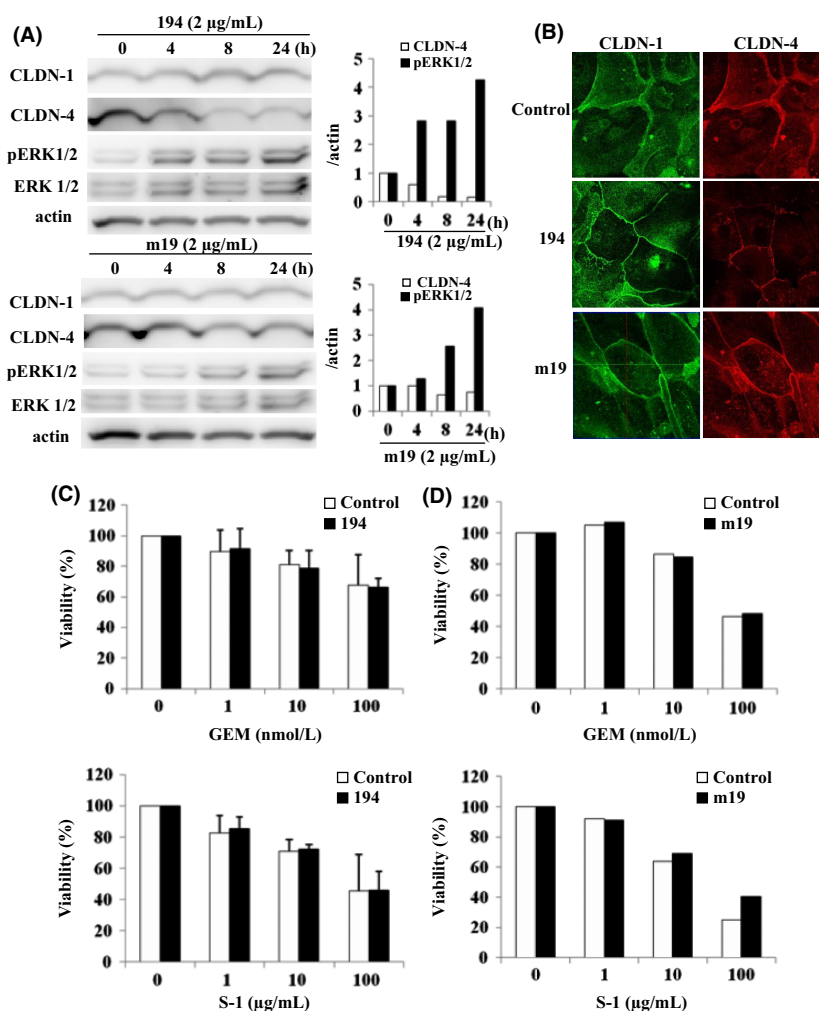


**Figure 6.** (A) Western blots for claudin-1, -4, phospho-ERK1/2 and ERK 1/2 in PANC-1 cells after treatment with 2  $\mu\text{g/mL}$  C-CPE 194 or C-CPE m19 for 4, 8 and 24 h. The corresponding expression levels of A are shown as bar graphs. (B) Immunocytochemistry for claudin-1 and claudin-4 in PANC-1 cells after treatment with 2  $\mu\text{g/mL}$  C-CPE 194 or C-CPE m19 for 24 h. Bar: 10  $\mu\text{m}$ . Cell viability assay in PANC-1 cells treated with 1–100 nmol/L gemcitabine (GEM) or 1–100  $\mu\text{g/mL}$  S-1 for 96 h with or without C-CPE 194 (C) or C-CPE m19 (D). (E) Cell viability assay in PANC-1 cells treated with 1–100 nmol/L gemcitabine (GEM) or 1–100  $\mu\text{g/mL}$  S-1 with or without C-CPE 194 or C-CPE 194 and 10  $\mu\text{mol/L}$  MAPKK inhibitor U0126. CLDN-1: claudin-1, CLDN-4: claudin-4, 194: C-CPE 194. m19: C-CPE m19. \* $P < 0.05$  versus control.

that have activating *KRAS* mutation, abnormality of the MAPK signaling pathway is frequently observed (Bardeesy and DePinho 2002; Hingorani et al. 2003; Jones et al. 2008).

In normal HPDE cells, C-CPE 194 decreased claudin-4 expression and enhanced the MAPK activity, whereas it did not affect the cytotoxicity of anticancer agents. In normal HPDE cells in vitro, claudin-4 is localized in the most apical regions, which are tight junctional areas,

whereas in pancreatic cancer HPAC cells and PANC-1 cells, claudin-4 is found not only in the most apical regions but also at basolateral membranes (Yamaguchi et al. 2011). It is thought that C-CPE binds to the free second extracellular loop of claudins outside tight junctions (Fujita et al. 2000). These findings suggest that, in pancreatic cancer cell lines, C-CPE 194 binds to the free second extracellular loop of claudin-4 outside tight junctions and that, in normal HPDEs, it did not bind to that



**Figure 7.** (A) Western blots for claudin-1, -4, phospho-ERK1/2 and ERK 1/2 in hTERT-HPDE cells after treatment with 2 μg/mL C-CPE 194 or C-CPE m19 for 4, 8 and 24 h. The corresponding expression levels of A are shown as bar graphs. (B) Immunocytochemistry for claudin-1 and claudin-4 in hTERT-HPDE cells after treatment with 2 μg/mL C-CPE 194 or C-CPE m19 for 24 h. Bar: 10 μm. Cell viability assay in hTERT-HPDE cells treated with 1–100 nmol/L gemcitabine (GEM) or 1–100 μg/mL S-1 for 96 h with or without C-CPE 194 (C) or C-CPE m19 (D). CLDN-1: claudin-1, CLDN-4: claudin-4, 194: C-CPE 194. m19: C-CPE m19.

of claudin-4 in tight junction areas. Thus, C-CPE 194 may be safe for normal HPDEs when used as a potential therapeutic agent against human pancreatic cancer cells.

C-CPE 194 mutant C-CPE m19 binds not only to claudin-4 but also to claudin-1. In the present study, in HPAC cells, it also disrupted barrier and fence functions without changes in expression of claudin-1 and -4. However, the disruption of the barrier and fence functions induced by C-CPE m19 was incompletely prevented by the MAPKK inhibitor U0126, whereas C-CPE m19 enhanced the MAPK activity. In normal HPDEs, C-CPE m19 decreased claudin-4 expression and enhanced the MAPK activity. However, it did not affect the cytotoxicity of the anticancer agents against pancreatic cancer cells or

normal HPDEs. In the present study, the reason for the latter phenomenon remains unclear.

In pancreatic cancer, C-CPE 194 not only downregulates the barrier and fence functions of tight junctions without cytotoxicity but also enhances the therapeutic efficiency of the anticancer agents GEM and S-1 via the MAPK signaling pathway. We previously reported that in normal human nasal epithelial cells, C-CPE 194 treatment disrupted the barrier and fence functions without changes in expression of claudin-4 or cytotoxicity, whereas they transiently increased the activity of ERK1/2 phosphorylation (Kojima *et al.* 2015). ERK1/2 phosphorylation is closely associated with the barrier and fence functions by C-CPE 194 treatment in HPAC cells. However, in the

present study, the detailed mechanisms of relationship between ERK1/2 phosphorylation and the effects of anti-cancer agents enhanced by C-CPE 194 were unclear.

In conclusion, C-CPE may be effective to facilitate drug delivery of anticancer agents in pancreatic cancer.

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## Author Contributions

Participated in research design: Kojima, Kondoh, Sawada, Hirata, Kimura, and Furahata, while experiments were conducted by Kono, Kojima, Konno, Kohno, Kyuno, Imamura and Ito. Kondoh and Kojima contributed new reagents or analytic tools, while Kono and Kojima performed data analysis and contributed to the writing of the manuscript.

## Disclosures

The authors declare no conflicts of interest.

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