

C Terminus of *Clostridium perfringens* Enterotoxin Downregulates CLDN4 and Sensitizes Ovarian Cancer Cells to Taxol and CarboplatinZhijian Gao¹, Xiaoyin Xu², Bruce McClane³, Qing Zeng², Babak Litkouhi¹, William R. Welch⁴, Ross S. Berkowitz^{1,5}, Samuel C. Mok⁶, and Elizabeth I.O. Garner^{1,5}**Abstract**

Purpose: We have previously shown that CLDN4 (encoding claudin-4), a cell tight junction (TJ) protein, is highly expressed in human epithelial ovarian carcinomas (EOC) but undetectable in normal ovaries. CLDN4 has been identified as a specific receptor for C terminus of *Clostridium perfringens* enterotoxin (C-CPE), a nontoxic molecule that may disrupt TJ barrier function and enhance cellular absorption. The purpose of this study was to determine the potential clinical applications of C-CPE and its effects on CLDN4 expression in EOC.

Experimental Design: Using a 3-dimensional culture model and monolayer culture of EOC cells, we examined the effects of C-CPE on CLDN4 expression by quantitative real-time PCR, immunofluorescence, and Western blot. The synergistic effect of C-CPE to clinically relevant chemotherapies (Taxol and Carboplatin) was observed in EOC culture and xenograft mice. Furthermore, we determined through oligonucleotide microarray analysis that the transcript profile alterations dysregulated as a consequence of C-CPE treatment.

Results: C-CPE treatment decreased protein expression and relocated CLDN4 from cell–cell contact regions to the cytoplasm. Particularly, C-CPE sensitized EOC cells to chemotherapeutic administration at low dosages and significantly inhibited tumor growth in a nontoxic manner. Furthermore, we provided genome-wide molecular evidence that C-CPE treatment is involved in the stimulation of the ubiquitin–proteasome pathway and the inhibition of cell metabolism in EOC cells.

Conclusions: The addition of C-CPE can enhance the effectiveness of Taxol or Carboplatin and significantly inhibited EOC cell growth in a CLDN4-dependent manner, suggesting that C-CPE may have promising therapeutic potential for EOC. *Clin Cancer Res*; 17(5); 1065–74. ©2010 AACR.

Introduction

Epithelial ovarian carcinoma (EOC) is the second most common gynecologic malignancy in the United States with 21,550 new cases diagnosed and 14,600 deaths from the

disease in 2009 (1). The most common primary chemotherapy regimen as a first-line therapy for patients with advanced-stage ovarian cancer is to use Taxol and Carboplatin. Although many patients initially respond to this chemotherapy, nearly 90% of patients with advanced-stage EOC have a recurrence and inevitably die after they develop chemotherapy-resistant disease (2). Furthermore, because these chemotherapy drugs do not differentiate between normal and tumor cells, toxicities caused by chemotherapy severely damage normal cells and eventually limit the tolerable dosage strength. Therefore, the development of effective therapies that alleviate toxic side effects and increase the therapeutic efficacy of chemotherapy is of immediate clinical importance.

Our group has found an ideal therapeutic target molecule, CLDN4 (encoding claudin-4), that is aberrantly overexpressed in human EOC (3–6) but low or even undetectable in normal ovaries (7, 8). Claudin proteins are a major constituent of cell tight junctions (TJ) and essential for the control of paracellular transport in the epithelium and endothelium (9, 10). Interestingly, CLDN4 has been described as a high-affinity receptor for the cytotoxic *Clostridium perfringens* enterotoxin (CPE), a 319-amino acid single polypeptide that may rapidly and

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Translational Relevance

Epithelial ovarian carcinoma (EOC) is the most aggressive gynecological tumor, and the development of effective therapies is of immediate clinical importance. In this work, we report a unique molecule, C terminus of *Clostridium perfringens* enterotoxin (C-CPE). We show that C-CPE disrupted the cellular tight junction barrier function by selectively decreasing and relocating the CLDN4 tight junction protein, and hence improved drug penetration into the CLDN4-expressing EOC cells in a 3-dimensional culture model and monolayer culture. More importantly, in a human EOC xenograft model, repeated intraperitoneal injections of C-CPE sensitize EOC xenografts to low-dose Taxol and significantly suppressed tumor growth without inducing overt toxicity throughout the treatment. Oligonucleotide microarray indicates that C-CPE was involved in the activation of the ubiquitin-proteasome pathway and inhibition of cell metabolism via the regulation of correlated molecules. The C-CPE-mediated therapy may thus represent a promising strategy for the treatment of EOC with fewer side effects and more therapeutic efficacies.

specifically trigger the lysis of cells expressing CLDN4 (11, 12). Prior structure-function analysis has revealed that CPE is functionally separated into N- and C-terminal domains (N- and C-CPE). N-CPE is responsible for its cytotoxic activity; whereas C-CPE, a small 30-amino acid fragment, has been shown to retain high affinity binding to its receptors CLDN3 and CLDN4 in a nontoxic manner (13). Despite its history as a food poisoning protein (14), the ability to specifically lyse cells expressing CLDN4 has led to CPE to be considered as a treatment for cancers. Santin and colleagues reported on the effective treatment of ovarian cancer with intraperitoneal (i.p.) CPE in severe combined immunodeficient (SCID) mice (15). Moreover, Kominsky and colleagues reported that intracranial administration of CPE significantly inhibited brain tumor growth in mice and increased their survival time (16).

However, because CLDN4 is expressed in certain normal tissues (17), the utility of CPE has been limited by its potentially significant toxic side effects (18, 19). C-CPE, the binding domain of CPE, overcomes the drawback of CPE and has recently emerged as a promising cancer therapeutic agent because of its unique properties: C-CPE can specifically target CLDN4, disrupt TJ barrier function (10, 22), and, hence, improve drug delivery in the epithelium and endothelium in a noncytotoxic manner (23, 24, 13). By far, the agent uptake via the paracellular pathway in the epithelia has been considered an attractive route for the absorption of chemotherapies, and cell TJs have been considered the primary paracellular barrier to the transport of solutes from the apical surface to the core of cells (23). Encouragingly, the work from our lab using the electric cell-substrate impedance sensing system has

shown that C-CPE decreased TJ barrier function in ovarian cancer cells in a dose- and CLDN4-dependent fashion, providing direct evidence that CLDN4 contributes to the formation of functional TJs in EOC (3). Given that CLDN4 is overexpressed in EOC and the main regulator of the barrier function of the cell TJ, the downregulation and disruption of CLDN4 by C-CPE treatment may thus provide an attractive potential for enhancing chemotherapeutic drug delivery in EOC.

In this study, we showed that C-CPE treatment can disrupt TJ function through downregulation and relocation of CLDN4 and enhance the therapeutic efficacy of Taxol and Carboplatin *in vitro* and *in vivo* in EOC. Our study strongly suggests that C-CPE treatment may lead to a more effective and safer therapeutic strategy for EOC.

Materials and Methods

Cell lines and reagents

The human EOC cell lines of SKOV-3, OVCA429, TOV112D, and RMUG-L were purchased from American Type Culture Collection. SKOV-3, TOV112D, and RMUG-L cells were maintained in a 1:1 mixture of MM199 and MCDB 105 (Sigma) medium, and OVCA429 cells were maintained in the RPMI-1649 (Sigma) medium supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products). Normal human ovarian surface epithelium (HOSE) cells were obtained at the time of surgery from fresh ovarian scrapings for benign nonovarian conditions. A stable cell line of SKOV-3-IP-luciferase (SKOV-3-IP-Luc) was kindly provided by Dr. Anil Sood at M.D. Anderson Cancer Center and maintained in RPMI-1640 medium supplemented with 15% FBS.

Three-dimensional culture model

To establish an ovarian cancer cell three-dimensional (3D) culture model that mimics ovarian cancer cell growth feature in the human body (25, 26), SKOV3 or RMUG-L ovarian cancer cells were seeded at a density of 1×10^4 cells/well on a solidified layer of growth factor-reduced Matrigel (BD Biosciences) in culture medium supplemented with 2% Matrigel in an 8-well chamber slide. On the third day, the spheroid of the EOC cells formed. On day 10, the spheroids were treated with or without C-CPE (5 μ g/mL) for 72 hours. Subsequently, the morphologic changes and CLDN4 expression between C-CPE-treated and control spheroids were examined by immunofluorescent staining.

Cloning and purification of COOH terminus *Clostridium perfringens* enterotoxin

C-CPE was prepared as described previously (3). Briefly, C-CPE was attained using a 3' fragment of the CPE gene cloned into the pTrcHis B expression vector. Sonic lysates from *Escherichia coli* transformants expressing C-CPE were filtered with a MILLEXHV 0.45-mm polyvinylidene difluoride filter (Millipore) and then eluted from the column with an imidazole gradient. C-CPE-containing fractions were then pooled. The amount of C-CPE was estimated by the

Lowry method, and the final enriched C-CPE product (90%–95% purity) was stored frozen at -80°C until use. The Micro BCA Protein Assay Kit (Pierce Chemical Co.) was used to determine protein concentrations.

Western blot analysis

Equal amounts of proteins were run on 12% SDS-PAGE under reducing conditions and transferred electrophoretically onto nitrocellulose membranes as described previously (27). The membranes were blocked with 5% milk in Tris-buffered saline Tween-20 and probed with the primary antibody for anti-CLDN4 (Invitrogen) at 1:1,000. The blots were then incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich Inc.). Protein signals were detected using Super-Signal West Pico Chemiluminescent Substrate (Pierce Chemical Co.). Subsequently, blots were stripped and reprobed with anti- β -actin antibody (Sigma-Aldrich Inc.) as a protein-loading control. The CLDN4 bands from 3 independent Western blot experiments were quantified by densitometry analysis with the ImageJ software (the National Institutes of Health).

Immunofluorescence

The 3D culture of SKOV-3 or RMUG-L spheroids was fixed with 4% formaldehyde, permeabilized by 0.5% triton X-100, and blocked with 10% normal goat serum for 1 hour. The spheroids were incubated overnight with the primary antibody of CLDN4 (1:400) followed by the Cy5-conjugated secondary antibody. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI; Invitrogen) for 15 minutes. For monolayer culture, the confluent monolayer culture of OVCA429 cells were fixed with cold methanol, incubated overnight with the anti-CLDN4 primary antibody and subsequently followed with Cy5-conjugated secondary antibody. Using an inverted Leica fluorescence microscope, images were observed.

Immunohistochemical staining and TUNEL assay

Formalin-fixed, paraffin-embedded EOC xenograft tissue sections (10 μm) were deparaffinized and then heated at 95°C for 20 minutes in citrate buffer for antigen retrieval (27). The sections were then incubated with anti-Ki-67 antibody (Zymed) at 1:500 and immunohistochemical staining was done as described previously (28). Apoptotic cells in EOC xenografts were detected using a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining kit (R&D Systems, Inc.) according to the manufacturer's instructions. To evaluate proliferation activity and apoptosis of EOC cells in xenografts, Ki-67-positive nuclei and apoptotic nuclei were counted in high-power fields and defined as labeling index; the percentage of Ki-67-positive nuclei or apoptotic nuclei versus total number of cells per high field from pooled tumors from 5 mice in each treatment group was determined.

In vitro cell viability assay

The cytotoxic effects of C-CPE, chemotherapeutic agents, or C-CPE in combination with the chemotherapeutic agents were evaluated in SKOV-3, OVCA429, and TOV112D ovarian cancer cell lines by MTT assay as described previously (28). Briefly, the day before treatment, 3×10^3 to 6×10^3 of cells (depending on the growth rate and size of the cells) were plated per well in 96-well plates and then treated with C-CPE alone, Taxol (Sigma-Aldrich) alone, Carboplatin (Sigma-Aldrich) alone, or C-CPE in combination with Taxol or Carboplatin at the indicated concentrations and time length. Cell growth and viability was assessed by the determination of the dissolved formazan product of MTT (Chemicon International Inc.) with a plate reader at 570-nm wavelength.

Animal studies

The Animal Experiment Committee of Harvard Medical School approved the experimental protocol with animal studies. Female SCID Nu/Nu strain mice at 6 to 8 weeks old were purchased from Charles River Laboratories and inoculated subcutaneously (s.c.) into the flank with 5×10^6 human SKOV-3 EOC cells in 100 μL of PBS. Four weeks later, the mice bearing a large ovarian tumor burden were randomly assigned into 3 treatment groups (5–7 mice per group) and administered i.p. with Taxol alone (20 mg/kg), Taxol combined with C-CPE (0.1 mg/kg), or vehicle (PBS) twice a week for 4 weeks. Tumor diameters were measured with a caliper weekly, and tumor volumes (V) were calculated using the formula: $V = (\text{length} \times \text{width}^2)/2$. Growth curves of tumors are presented as the mean volume relative to the start volume on the first day of the administration. After 4 weeks of treatment, the mice were sacrificed, and tumor xenografts and organs were fixed in 4% paraformaldehyde for histologic examination. Pathologists blinded to the experimental conditions classified the tumors.

Oligonucleotide microarray analysis and gene selection

A microarray analysis was done to compare the global gene expression profile between C-CPE-treated and -untreated SKOV-3 cells. The Human Release 3.0 oligonucleotide library (Sigma), containing 60-mer oligonucleotides representing 44,000 probe sets, was used for this microarray analysis. The oligonucleotides were dissolved at 10 $\mu\text{mol/L}$ concentration in 50 mmol/L sodium phosphate buffer (pH 8.5) and single spotted onto CodeLink slides, using an OmniGrid 100 microarrayer (Genomic Solutions) at the Advanced Technology Center (Rockville).

Total RNAs were extracted from the confluent SKOV-3 cells treated with or without C-CPE (5 $\mu\text{g/mL}$) for 72 hours using the RNeasy Mini kit (Qiagen). Dye-labeled aRNA was prepared as described in the Amino Alkyl MessageAmp II aRNA Amplification kit Manual (Ambion Inc.). Briefly, 1 μg of purified total RNA was reverse transcribed to generate double-stranded cDNA using T7 Oligo primer and reverse transcriptase. Following second-strand cDNA synthesis and purification, biotin-labeled antisense RNA

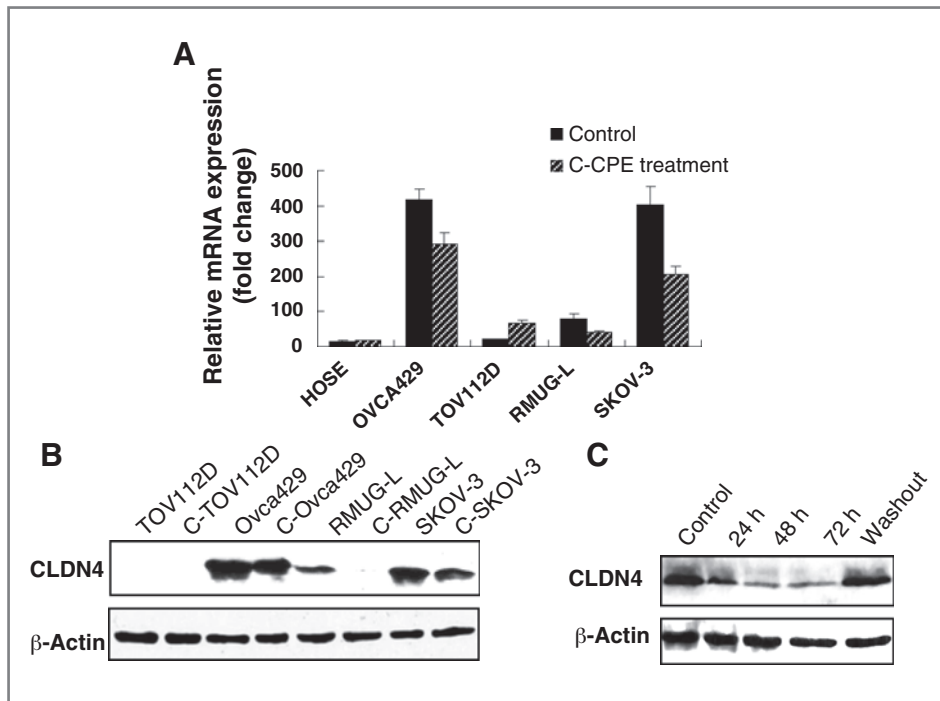


Figure 1. The regulation of CLDN4 by C-CPE treatment in EOC cells. **A**, qRT-PCR analysis of CLDN4 mRNA expression levels in various EOC and normal HOSE cells treated with or without C-CPE (5 µg/mL) for 48 hours. β -Actin was used as an endogenous control to standardize the amount of RNA in each reaction. Data, mean \pm SD from triplicate experiments. **B**, effect of C-CPE on CLDN4 protein expression in EOC cell lines. Whole lysates from the above EOC cell cultures were subjected to Western blot analysis with antibodies to CLDN4 and β -actin (loading control). **C**, C-CPE downregulated CLDN4 protein expression in a time-dependent manner. SKOV-3 cells were cultured with or without C-CPE (5 µg/mL) for 24, 48, and 72 hours and another 24 hours after removal of C-CPE. Whole cell lysates were subjected to Western blot analysis. The experiments were done in triplicate.

(aRNA) was modified with amino allyl UTP by *in vitro* transcription and coupled with Cy3 and Cy5. In all, 1.5 µg of dye-labeled aRNA of each RNA preparation was fragmented and combined with hybridization controls using *in situ* Hybridization Kit Plus (Agilent Technologies). The GeneChips were hybridized overnight at 45°C in a Gene-Machine Hyb4 slide hybridization machine (Genomic Solutions) and subsequently washed as recommended by the manufacturer. To improve the accuracy of the results, samples were run in triplicate.

Scanning was done on a ScanArray 4000XL Microarray Analysis System and quantified using ScanArray Express (Perkin Elmer). Normalized sample to control ratios were used for analysis. Data were analyzed using ArrayAssist 5.0 array analysis software. Genes were selected on the basis of fold change and *P* value cutoffs of 3.0 and less than 0.001, respectively. Dye swap comparisons were done to assure that no bias was introduced by the hybridization process.

Quantitative real-time PCR

For quantitative real-time PCR (qRT-PCR), total RNAs were isolated and purified from the confluent cultures of HOSE, SKOV-3, OVCA429, TOV112d, and RMUG-L cells. The RISM 7300 Sequence Detector (PE Applied Biosystems) was used for detecting the PCR products in real-time with the SYBR Green assay. cDNA obtained from 1 µg of total RNA was amplified in a 20-µL PCR reaction following the manufacturer's protocol. The comparative threshold cycle was used for the calculation of amplification fold, and the housekeeping gene β -actin was used as an endogenous control to standardize the amount of RNA in each reaction. The primers used are listed in the Supplementary Data.

Statistical analysis

The values in this study were expressed as mean \pm SD from at least 3 independent experiments. Statistical differences between groups were analyzed using Student's *t* test and *P* < 0.05 was considered statistically significant.

Results

C-CPE treatment decreased CLDN4 expression in EOC cells

As shown in Figure 1A, qRT-PCR analysis revealed that the CLDN4 mRNA levels were highest in the untreated OVCA429 and SKOV-3 cell lines, low in RMUG-L and TOV112D cell lines, and even undetectable in normal HOSE cells. After treatment with C-CPE (5 µg/mL) for 48 hours, the CLDN4 transcriptional levels were down-regulated in OVCA429, SKOV-3, and RMUG-L cells. However, C-CPE had no effect in HOSE or TOV112D cells. We further performed Western blot analysis to determine the CLDN4 protein levels in the above four EOC cell cultures. Similar to the qRT-PCR analysis results, CLDN4 protein levels were highest in OVCA429 cells, higher in SKOV-3 cells, low in RMUG-L, and even undetectable TOV112D cells. The treatment of C-CPE reduced the CLDN4 protein levels in OVCAR429, RMUG-L, and SKOV-3 cells, whereas CLDN4 protein was undetectable in both C-CPE-treated and -untreated TOV112D cells (Fig. 1B). Additionally, we found that the prolonged treatment with C-CPE (5 µg/mL) decreased CLDN4 protein levels in a time-dependent manner in SKOV-3 cells, which was reversible on removal of C-CPE for 24 hours (Fig. 1C).

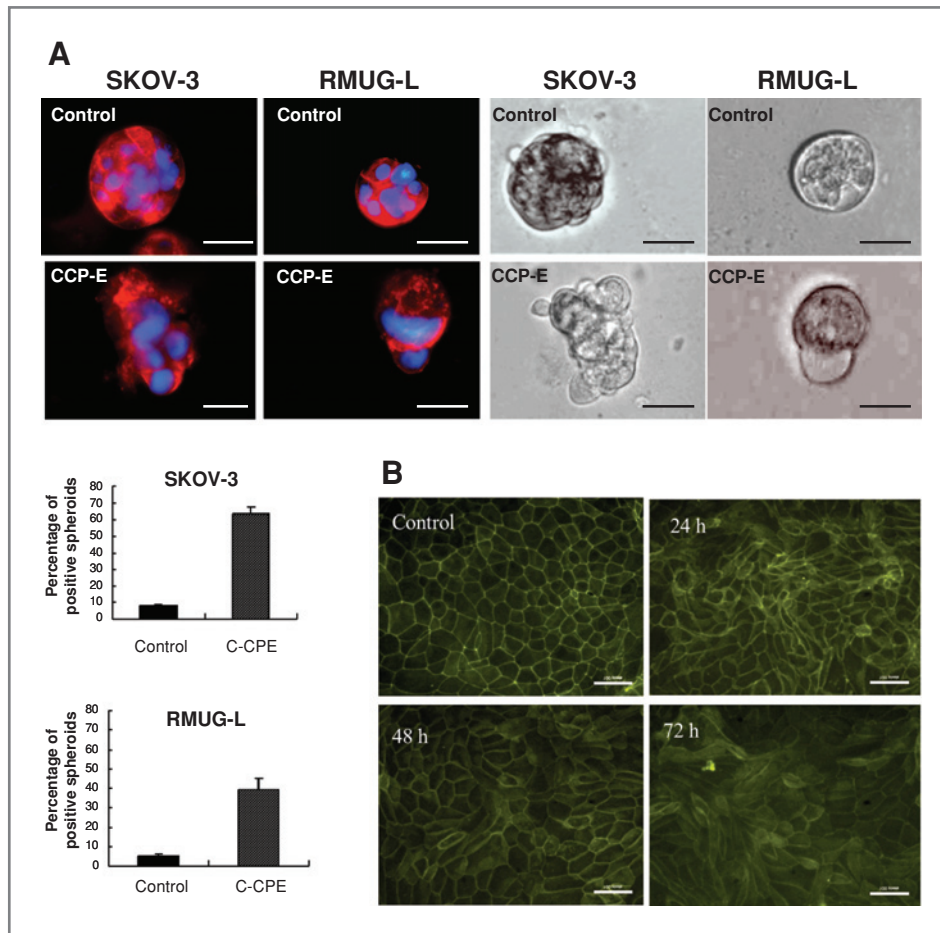


Figure 2. A, immunofluorescent staining of CLDN4 in SKOV-3 and RMUG-L spheroids in 3D culture. SKOV-3 and RMUG-L spheroids were cultured in growth factor–reduced Matrigel for 10 days and then treated with or without C-CPE (5 $\mu\text{g}/\text{mL}$) for 72 hours. Top, left, confocal fluorescence microscopy images show redistribution of CLDN4 in a diffused staining pattern and morphologic changes in C-CPE–treated spheroids. In contrast, CLDN4 was retained at the cell–cell border with strong staining and on membrane with weak cytoplasmic reactivity in control spheroids. Nuclei were counterstained with DAPI. Scale bars, 20 μm . Top, right, the phase-contrast images show the representative 3D structures of SKOV-3 and RMUG-L cells. Bottom, the percentage of positive spheroids was determined by counting 30 spheroids in each of 3 independent experiments. Spheroids were scored positive when the cells exhibited apparent CLDN4 relocalization and morphologic changes. *, $P < 0.001$ versus control. B, immunofluorescent staining of CLDN4 in monolayer culture of OVCA429 cells treated in the presence or absence of C-CPE (5 $\mu\text{g}/\text{mL}$) for indicated time. Scale bars, 40 μm .

Exposure to C-CPE decreased and relocated CLDN4 in 3D and monolayer cultures

Although the exact mechanism leading to decreased TJ function by C-CPE in EOC cells remained unclear, herein, our immunofluorescence data from 3D and monolayer cultures provided some insight. As shown in Figure 2A, CLDN4 was retained at the cell–cell border with strong staining and on membrane with weak cytoplasmic reactivity in SKOV-3 and RMUG-L control spheroids. In contrast, after incubation with C-CPE (5 $\mu\text{g}/\text{mL}$) for 72 hours, CLDN4 distribution was removed from cell–cell contacts to cytoplasm in a diffused pattern of staining. Moreover, disorganized morphologic changes were observed in these spheroids, which were more apparent in the SKOV-3 cells than in the RMUG-L cells; however, the nuclei did not exhibit the distinct features of apoptosis, such as nuclear shrinkage,

condensation or fragmentation. In monolayer culture, C-CPE treatment led to a gradually decreased staining of CLDN4 between cell–cell contact and more diffused staining in cytoplasm in a time-dependent manner (Fig. 2B). Thus, our findings directly evidence that C-CPE may diminish cell TJ barrier function by specifically disintegrating and relocating CLDN4 expressions in EOC cells. This result is supported by a previous report that treatment of MDCK cells with C-CPE removed CLDN4 to cytoplasm and disrupted TJ in a time- and dose-dependent manner (29).

C-CPE treatment sensitized CLDN4-expressing EOC cells to chemotherapies

Because C-CPE treatment decreases CLDN4 levels and disrupts TJs between adjacent cells, we hypothesized that the combination of C-CPE with chemotherapeutic agents

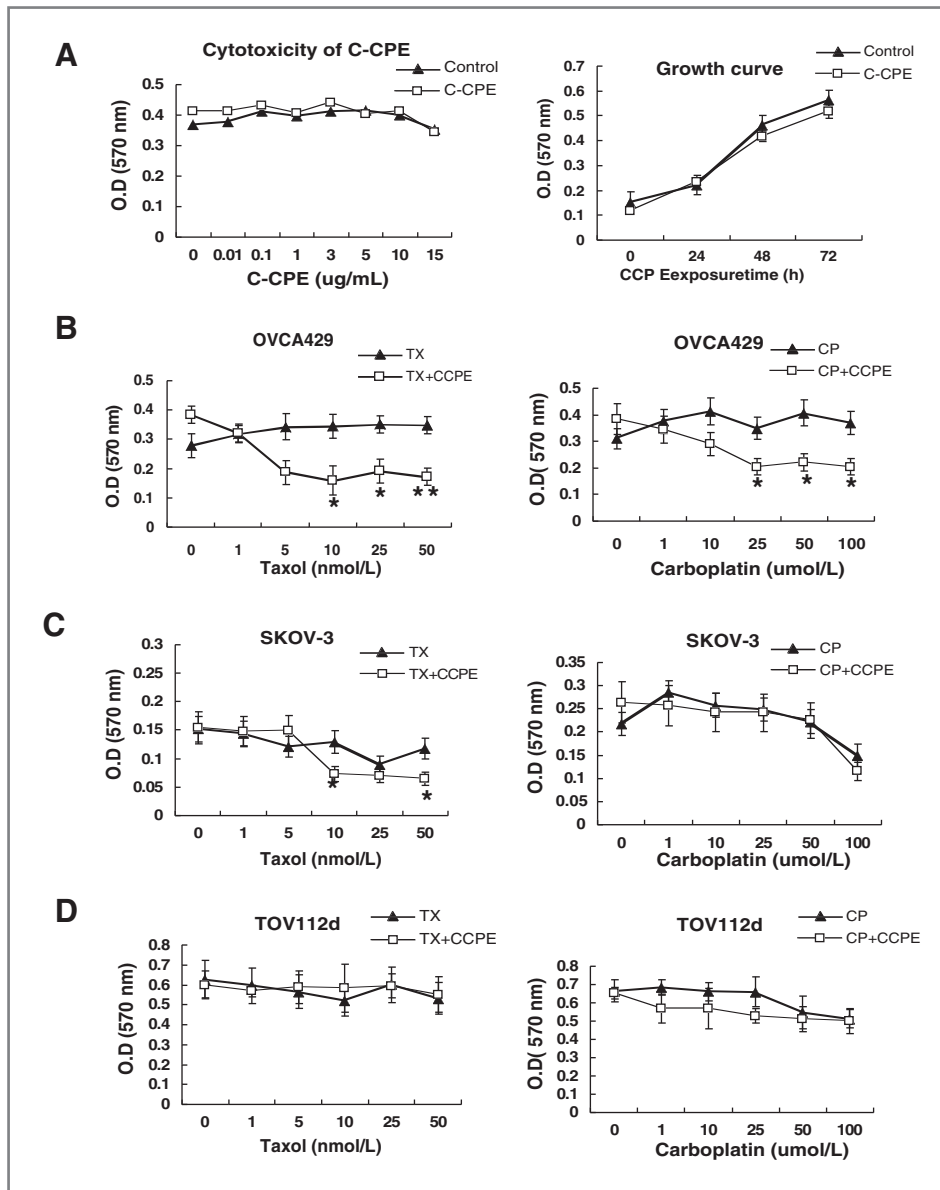


Figure 3. C-CPE enhanced chemosensitivities of EOC cells in a CLDN4-dependent manner *in vitro*. **A**, cytotoxicity of C-CPE. Left, after exposure to increasing concentrations of C-CPE (0.01–15 µg/mL) or medium alone (control) for 48 hours, cell growth and viability of SKOV-3 cell was evaluated by MTT assay. Right, after treatment with or without C-CPE (5 µg/mL) for indicated time, growth curve of SKOV-3 cells was measured by MTT assay. **B–D**, C-CPE effect on chemosensitivity of EOC cells to low-dose chemotherapy agents. On the basis of the result of Figure 1B, three EOC cell lines expressing CLDN4 protein at varying levels were selected: OVCA429 (highest), SKOV-3 (higher), and TOV112d (deficient) cells. Left, after exposure to Taxol alone at the indicated concentrations or the combination of Taxol and C-CPE (5 µg/mL) for 24 hours, cell viability was compared in each cell lines by MTT assay, respectively. Right, after exposure to Carboplatin alone at the indicated concentrations or the combination of Carboplatin and C-CPE (5 µg/mL) for 24 hours, cell viabilities were compared in each cell lines by MTT assay, respectively. Data, mean ± SD from triplicate experiments. *, $P < 0.05$; **, $P < 0.001$. TX, Taxol; CP, Carboplatin.

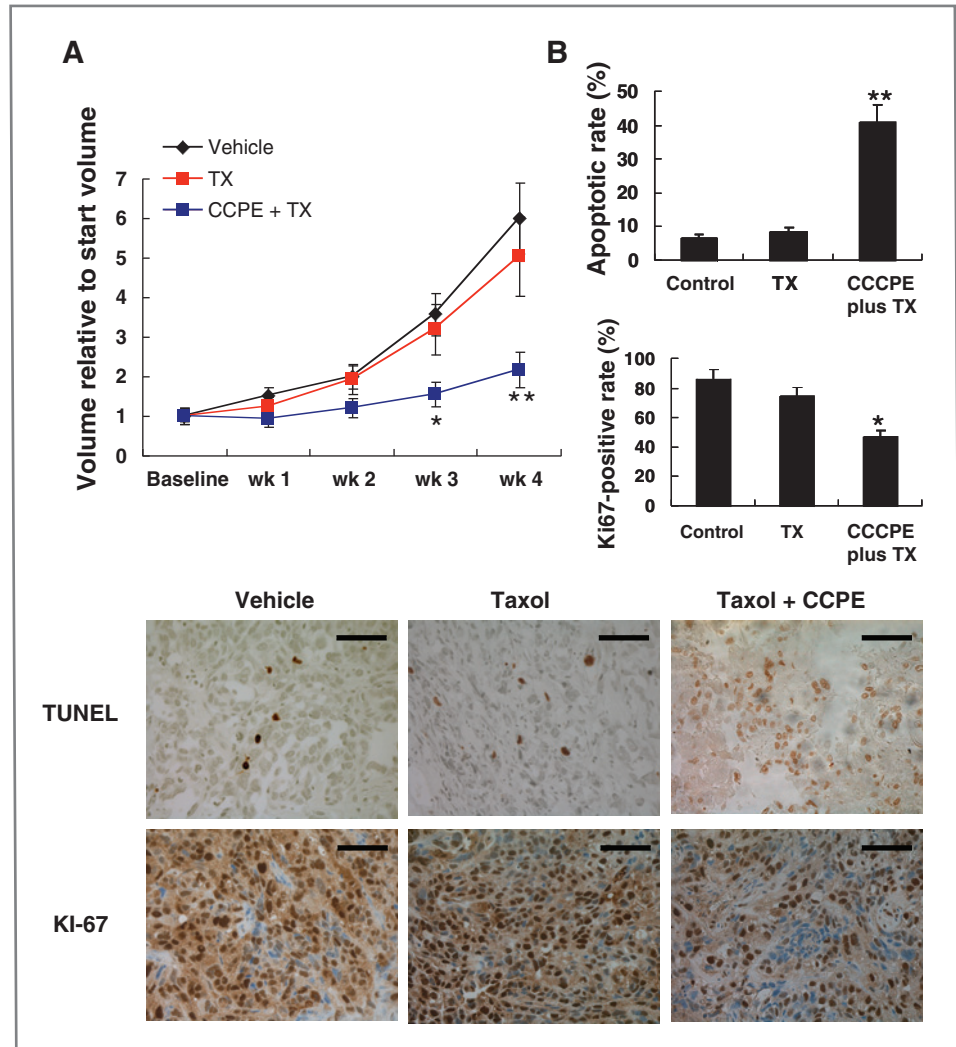
may increase drug delivery to the core of tumors and sensitize EOC cells to chemotherapies.

Before testing this hypothesis, we verified whether C-CPE can be used in a noncytotoxic manner. Consistent with previous studies in other cell types (14, 30), the treatment with increasing concentrations of C-CPE (0.01–15 µg/mL) for 48 hours did not show any cytotoxicity to SKOV-3 cells (Fig. 3A). Meanwhile, growth curve analyses show that prolonging the exposure time to C-CPE (5 µg/mL) from 24 hours to 72 hours did not affect SKOV-3 cell viability and growth either, confirming the noncytotoxicity of C-CPE.

We next determined whether C-CPE can enhance the chemosensitivity of EOC cells to the low-dose Taxol or Carboplatin. To determine the correlation between the levels of CLDN4 protein expression and the chemosensi-

tivity of EOC cell lines, based on the result in Figure 1B, three EOC cell lines expressing CLDN4 protein at varying levels—OVCA429 (highest), SKOV-3 (higher), and TOV112d (deficient)—were selected and treated with Taxol (1–50 nmol/L) alone, Carboplatin (1–100 µmol/L) alone, or C-CPE (5 µg/mL) in combination with Taxol or Carboplatin for 24 hours. As shown in Figure 3B, compared with the single agent of Taxol or Carboplatin at low concentrations that did not strongly inhibit tumor cell growth, the combination therapies significantly inhibited tumor cell growth and sensitized the OVCA429 cells to the cytotoxicity of the chemotherapies. In the SKOV3 cell line, C-CPE treatment significantly enhanced the cell chemosensitivities to Taxol, but, interestingly, it did not enhance the antitumor effect of Carboplatin at the indicated concentrations (Fig. 3C). This result is likely explained by the lower

Figure 4. The combination therapy of C-CPE and Taxol attenuates EOC xenograft growth *in vivo*. Female SCID mice were inoculated s.c. with 5×10^6 SKOV-3 cells. Four weeks later, the mice harboring large tumor burden were divided randomly into 3 groups and administered i.p. with Taxol alone (20 mg/kg), Taxol combined with C-CPE (0.1 mg/kg), or vehicle (PBS) twice a week for 4 weeks. **A**, growth curves of tumors were presented as the mean volume normalized to the start volume. *, the combination of C-CPE and Taxol led to a significant tumor suppression compared with vehicle or Taxol alone ($P < 0.05$). Data, mean \pm SD from the mice in each group. **B**, immunostaining of Ki67 and TUNEL to evaluate cell proliferation and apoptosis in EOC xenografts. After 4 weeks of treatment, the combination therapy markedly attenuates tumor cell proliferation and accelerates apoptosis relative to the use of single-agent Taxol. The graph represents the percentage of apoptotic or Ki-67 positive nuclei per high field from pooled tumors from 5 mice per group. *, $P < 0.05$; **, $P < 0.001$. Scale bar, 50 μ m.



CLDN4 protein levels in SKOV-3 cells than in OVCA429 cells (Fig. 1B); moreover, Carboplatin is less potent than Taxol, and it usually requires a higher dose and longer treatment time to achieve the same effectiveness of Taxol (31). We further examined the C-CPE effect in the TOV112D cell line. As expected, TOV112D cells were completely resistant to the monotherapy of Taxol or Carboplatin, or the combination therapy with C-CPE, corresponding with the lack of CLDN4 expression (Fig. 3D). These *in vitro* data indicated that C-CPE enhanced the chemosensitivity of EOC cells to Taxol or Carboplatin in a CLDN4-dependent manner, rendering the CLDN4-negative normal HOSE cells and other normal cells resistant to the cytotoxic effects of chemotherapies.

C-CPE enhanced the efficacy of Taxol in an EOC xenograft model

To test whether C-CPE is safe and effective in preclinical experiments, female nude mice bearing large SKOV-3 xenografts for 4 weeks were treated i.p. with Taxol (20 mg/kg)

alone, Taxol (20 mg/kg) combined with C-CPE (0.1 mg/kg), or vehicle (PBS) twice a week for a period of 4 weeks. At the end of treatment, the combination therapy of C-CPE and Taxol significantly suppressed the tumor growth by 59% compared with the vehicle, which was very different from the administration of Taxol alone that showed little antitumor effect on the large tumor burdens. After sacrifice, the therapeutic effects on EOC cell growth were further evaluated by examining Ki-67 positive and apoptotic cells in tumor sections. As shown in Figure 4B, the population of Ki67-positive cells was substantially decreased in the mice treated with the combination therapy compared with the mice receiving Taxol or vehicle alone. A greater percentage of TUNEL-positive cells were observed in tumor sections from the combination therapy-treated mice compared with the vehicle- or Taxol-treated mice, which suggests that the combination therapy not only inhibits tumor cell proliferation but also accelerates apoptosis to attenuate tumor growth. The combination therapy was well tolerated, and no significant weight loss or observable toxicities were

Table 1. Representative list of upregulated and downregulated genes in C-CPE-treated SKOV-3 cells versus control cells

Gene symbol	Name	Functions	Fold change
<i>NDUFB9</i>	NADH dehydrogenase (ubiquinone) 1 beta subcomplex	Metabolism	8
<i>TNFRSF21</i>	Tumor necrosis factor receptor superfamily, member 21	Tumor suppressor	8
<i>MMP23B</i>	Matrix metalloproteinase 23B	Metastasis/adhesion	7
<i>RAB27B</i>	RAB27B, member RAS oncogene family	Oncogene	7
<i>QPCTL</i>	Glutamyl-peptide cyclotransferase-like	Metabolism	7
<i>UBQLN1</i>	Ubiquilin 1	Ubiquitin pathway	6
<i>UBE2J1</i>	Ubiquitin-conjugating enzyme E2, J1	Ubiquitin pathway	6
<i>UBE4A</i>	Ubiquitination factor E4A	Ubiquitin pathway	6
<i>RAB18</i>	member RAS oncogene family	Oncogene	6
<i>UCHL1</i>	Ubiquitin carboxyl-terminal esterase L1	Ubiquitin pathway	5
<i>SFN</i>	Stratifin	Metastasis/growth inhibition	4
<i>CCNB1</i>	Cyclin B1	Cell cycle	4
<i>PGM1</i>	Phosphoglucomutase 1	Metabolism	-2
<i>MMP2</i>	Matrix metalloproteinase 2	Metastasis/adhesion	-3
<i>SYK</i>	Spleen tyrosine kinase	Metabolism	-4
<i>RAP2A</i>	RAP2A, member of RAS oncogene family	Oncogene	-4
<i>PGM1</i>	Phosphoglucomutase 1	Metabolism	-4
<i>MKNK2</i>	MAP kinase interacting serine/threonine kinase 2	Metabolism	-5
<i>CD47</i>	CD47 antigen	Metastasis/adhesion	-5
<i>SULT1C1</i>	Sulfotransferase family, cytosolic, 1C, member 1	Metabolism	-5
<i>ACHE</i>	Acetylcholinesterase	Metabolism	-5
<i>RIT1</i>	Ras-like without CAAX 1	Oncogene	-6
<i>WISP2</i>	Wnt1 inducible signaling pathway protein 2	Wnt signaling Pathway	-11

observed throughout the treatment period. Histologic examination revealed no toxicity in the kidney or liver (data not shown).

Genes correlated with C-CPE treatment in EOC cells

To investigate the molecular mechanisms underlying the role of C-CPE treatment in ovarian cancer cells, we carried out microarray analysis using the 3.0 oligonucleotide library to compare the gene profiling changes between C-CPE-treated cells and control SKOV-3 cells. Microarray data have been uploaded into the GEO system with the GEO accession number GSE22421. After filterization and statistical analyses, we identified 24 representative functional molecules on the basis of their known functions (Table 1). Among these molecules, 6 genes have been selected and validated by qRT-PCR (Supplementary Data 2). Interestingly, we noted that a group of the upregulated molecules (4 of 12) induced by C-CPE treatment are correlated with the ubiquitin-proteasome pathway that is responsible for most eukaryotic intracellular protein degradation and essential for many fundamental cellular processes, including the regulation of receptor signaling pathways, proliferation, angiogenesis, and apoptosis (32). So far, some drugs targeting the ubiquitin-proteasome pathway have been developed and have significant anti-tumor efficacy, such as in breast cancer (33). On the contrary, C-CPE treatment attenuated a group of molecules (6 of 11) linked with cell metabolism, an important

cellular process for generating the energy to meet the demands of cancer cell growth.

Discussion

Currently, the primary treatment for advanced ovarian cancer is combination chemotherapy, using platinum-based drugs such as Taxol combined with Carboplatin. Although this treatment regimen is initially effective in a high percentage of cases, most patients will relapse due to the development of chemoresistance (2). In addition, toxicities caused by chemotherapy severely damage normal cells and limit the dosage strength tolerated by patients. Therefore, successful treatment of advanced ovarian cancer is challenged by the development of effective therapies that alleviate toxic side effects and increase the therapeutic efficacy of chemotherapy. Tantamount to achieving this goal is the identification of appropriate molecular targets for therapy that are differentially expressed between EOC and normal tissue.

In this study, we have selected CLDN4 as a clinical target because it is one of the most highly upregulated genes in 90% of ovarian cancer, including all 4 major subtypes (serous, mucinous, clear cell, and endometrioid), but at very low levels in normal tissues (3-6). Because claudin proteins are major components of TJs and play an important role in the regulation of paracellular transport of cells (9, 10), as the specific receptor of C-CPE, the

downregulation of CLDN4 by C-CPE may maintain impressive potential for enhancing chemotherapy absorption through improving paracellular permeability. We previously found that C-CPE disrupts TJ barrier function in ovarian cancer cells in a dose- and CLDN4-dependent fashion (3). In this study, we further directly evidenced that after exposure to C-CPE, CLDN4 was decreased and removed from cell TJs to cytoplasm in the 3D culture and the monolayer culture of EOC cells (Figs. 1 and 2). Consistent with our study, other groups also found that C-CPE diminishes TJ barrier function by specifically disintegrating and relocating CLDN4 expression in Madin–Darby canine kidney cells and Caco-2 cells (29, 34). Because CLDN4 is overexpressed in most EOCs (3–6), the findings that C-CPE dysregulates CLDN4 expression and disrupts TJ barrier in EOC cells led us to consider that C-CPE treatment may be used to facilitate subsequent drug delivery to ovarian cancer cells.

To test our hypotheses, we conducted an evaluation of the efficacy and toxicity of the combination therapy of C-CPE and chemotherapeutic agents *in vitro* and in EOC xenograft mice. We found that C-CPE sensitized EOC cells to the cytotoxicity of Taxol or Carboplatin at relatively lower dosages in a CLDN4-dependent manner (Fig. 3). More importantly, compared with the single-agent Taxol, the combination therapy of C-CPE and Taxol significantly suppressed the large tumor burdens in animals via inhibiting tumor cell proliferation and accelerating apoptosis (Fig. 4). Moreover, C-CPE effectively avoids the significant toxic drawback of CPE encountered in the previous studies (18, 19). Kondoh and colleagues also showed that C-CPE enhanced the absorption of dextran by more than 400 times compared with other clinically used enhancers in the rat jejunum (23), supporting our study on the clinical potential of C-CPE. Recently, Yuan and colleagues fused the nontoxic C-CPE_{290–319} domain to TNF to effectively treat ovarian cancer, opening a new perspective for a C-CPE–based specific drug delivery into CLDN4-overexpressing ovarian cancers (35).

How does C-CPE enhance the absorption of drugs? In this study, we showed that, after treatment with C-CPE, CLDN4 was removed from TJs and internalized. C-CPE may thus selectively inhibit the barrier function of CLDN4 with the result being improved drug penetration and accumulation in the core of the tumor. Moreover, our microarray showed that C-CPE treatment may be involved in stimulation of the ubiquitin–proteasome pathway and inhibition of cell metabolism via regulation of the correlated molecules in EOC cells (Table 1). On the basis of these results, we conclude that C-CPE may enhance the sensitivity of EOC cells to chemotherapies via (i) disrupting cell TJs, (ii) inhibiting cancer cell metabolism, and (iii) stimulating the activity of ubiquitin–proteasome pathway in ovarian cancer cells. Many studies have shown that TJ structures are maintained between mitotic cells and neighboring cells throughout the cell cycle, including during cell division (36–38). This allows C-CPE to improve the delivery of anticancer drugs into cancer cells at all times, which has clinical importance in treating cancers because many anticancer drugs, such as Taxol, act during the M phase.

Given the fact that CLDN4 is expressed in certain normal tissues (17), including the lining layer of the digestive track, urothelial layer of bladder, and fallopian, systemic administration of C-CPE and chemotherapeutic agents might affect these normal cells. In this context, we have chosen *i.p.* administration for the animal study because it may reduce the concern of adverse effects of medication in normal tissues that reside outside the peritoneum. Additionally, the efficacy enhancement by C-CPE allows chemotherapeutic agents to be administered at relatively low concentrations and, thus, alleviate the toxic effects of chemotherapies. In this study, the *i.p.* administration of C-CPE and Taxol was well tolerated, and no adverse events were observed throughout the complete treatment in animals. Recently, the National Cancer Institute clinical announcements also recommended *i.p.* chemotherapy to women with advanced ovarian cancer because it significantly improved the survival of patients and spared more normal tissue (39). Therefore, the *i.p.* administration of C-CPE and chemotherapy may represent an effective and applicable treatment for ovarian cancer.

Despite the potential of C-CPE in the treatment of EOC, its clinical application may face challenges common to that of all foreign proteins. For example, multiple administration of C-CPE may likely induce the development of neutralizing antibodies in patients that may reduce the efficacy of C-CPE administrations. Although C-CPE is a nontoxic small molecule and no significant adverse events were observed in this study, careful studies need to be done to accurately determine the immune response against C-CPE when administered by the *i.p.* route in humans.

Taken together, this study shows that the treatment of C-CPE may disrupt TJ by decreasing and redistributing CLDN4 and hence improving the penetration and efficacy of chemotherapy in EOC cells. We also report the first *in vivo* study that the repeated *i.p.* injections of C-CPE can sensitize EOC tumor to low-dose Taxol and significantly suppressed the large tumor burdens *in vivo*. The C-CPE–mediated therapy may thus provide a novel strategy to treat ovarian cancer and other CLDN4-overexpressing tumors (17, 40) with more efficacy and fewer side effects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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