

# Folate-appended cyclodextrin carrier targets ovarian cancer cells expressing the proton-coupled folate transporter

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## Abstract

Folate receptor alpha (FR $\alpha$ ) is overexpressed in >80% of epithelial ovarian cancer (EOC). Accordingly, folate is attracting attention as a targeting ligand for EOC. For EOC patients, paclitaxel (PTX) is generally used as a first-line chemotherapeutic agent in combination with platinum-based drugs. Cyclodextrin (CyD) is a potential new formulation vehicle for PTX that could replace Cremophor-EL, a traditional formulation vehicle that causes significant side effects, including neutropenia. Several years ago, folate-appended  $\beta$ -CyD (Fol- $c_1$ - $\beta$ -CyD) was developed as an FR $\alpha$ -targeting drug carrier, but its efficacy as a treatment for EOC remains to be determined. In this study, we assessed the antitumor activity of PTX in Fol- $c_1$ - $\beta$ -CyD (PTX/Fol- $c_1$ - $\beta$ -CyD) in EOC-derived cell lines. We found that PTX/Fol- $c_1$ - $\beta$ -CyD killed not only FR $\alpha$ -expressing cells but also FR $\alpha$ -negative cells. In the FR $\alpha$ -negative A2780 cells, knockdown of proton-coupled folate transporter (PCFT) significantly decreased the cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD, whereas knockdown of FR $\alpha$  did not. By contrast, knockdown of either FR $\alpha$  or proton-coupled folate transporter (PCFT) decreased the cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD in FR $\alpha$ -expressing SK-OV-3 cells. Furthermore, the cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD in A2780 cells was increased at acidic pH, and this increase was suppressed by PCFT inhibitor. In mice intraperitoneally inoculated with FR $\alpha$ -expressing or PCFT-expressing EOC cells, intraperitoneal administration of PTX/Fol- $c_1$ - $\beta$ -CyD significantly suppressed the growth of both types of EOC cells relative to PTX alone, without inducing a significant change in the neutrophil/white blood cell ratio. Our data suggest that Fol- $c_1$ - $\beta$ -CyD targets not only FR $\alpha$  but also PCFT, and can efficiently deliver anticancer drugs to EOC cells in the peritoneal cavity.

## KEYWORDS

cyclodextrin, drug carrier, folate receptor alpha, ovarian cancer, proton-coupled folate transporter

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## 1 | INTRODUCTION

Ovarian cancer is the leading cause of deaths from gynecologic malignancies worldwide.<sup>1</sup> Most ovarian cancer patients are diagnosed at an advanced stage with widely metastatic disease within the peritoneal cavity and are treated with chemotherapy with platinum-based drugs after cytoreductive surgery.<sup>2</sup> For patients with epithelial ovarian cancer (EOC), paclitaxel (PTX) is generally used as a first-line chemotherapeutic agent in combination with platinum-based drugs. Due to its poor water solubility, PTX is formulated in a mixture of Cremophor-EL and ethanol, resulting in adverse reactions such as neutropenia.<sup>3</sup> Therefore, a safe formulation of PTX must be developed to improve anticancer treatment.

Cyclodextrin (CyD), a natural cyclic oligosaccharide produced from starch, is readily soluble in water and can increase the water solubility of various hydrophobic molecules by inclusion in their hydrophobic cavity.<sup>4,5</sup> Alcaro *et al* reported that PTX in CyD not only exhibited higher water solubility but was also more thermodynamically stable,<sup>6</sup> suggesting that CyD would be a suitable replacement for Cremophor-EL. In addition, when modified with tumor-targeting ligands such as folate, CyD can be used as agents for molecularly target therapies.<sup>7</sup>

Folate, an essential water-soluble vitamin that is abundant in dark leafy vegetables, is required by proliferating cells as a one-carbon source for DNA biosynthesis, repair and methylation.<sup>8</sup> Rapidly dividing cancer cells require an abundant supply of folate to support their metabolic needs, including accelerated DNA biosynthesis.<sup>9</sup> Folate receptor alpha (FR $\alpha$ ), a glycosylphosphatidylinositol (GPI)-anchored cell surface protein that binds folate with high affinity and coordinates its transport, is overexpressed in cancers of the ovary, breast, pleura, lung, cervix, endometrium, kidney, bladder and brain.<sup>10</sup> Importantly, FR $\alpha$  expression in normal tissues is restricted to the luminal side of tissues such as kidney and lung, and, except in kidneys, FR $\alpha$  is not exposed to folate in the circulatory system.<sup>11,12</sup> Therefore, to target FR $\alpha$ -positive cancers, many types of folate-conjugated drugs and antifolates have been developed in addition to CyD.<sup>7,13-16</sup> However, few studies have examined the dependence of those drugs on folate transporters other than FR $\alpha$ .<sup>16</sup>

In this study, we sought to validate the antitumor activity and specificity of PTX/Fol-c<sub>1</sub>- $\beta$ -CyD in EOC cell lines. We found that PTX/Fol-c<sub>1</sub>- $\beta$ -CyD targeted not only FR $\alpha$ -expressing cells but also cells that did not express FR $\alpha$ . PCFT was identified as a second target of PTX/Fol-c<sub>1</sub>- $\beta$ -CyD. Furthermore, the antitumor activity of PTX/Fol-c<sub>1</sub>- $\beta$ -CyD in A2780 cells, but not SK-OV-3 cells, was elevated under acidic pH. In mice intraperitoneally inoculated SK-OV-3 or A2780 cells, intraperitoneal administration of PTX/Fol-c<sub>1</sub>- $\beta$ -CyD treatment significantly suppressed tumor growth, without significantly changing in the neutrophil ratio. Our results demonstrate that Fol-c<sub>1</sub>- $\beta$ -CyD is a dual-targeted and effective drug carrier for treatment of FR $\alpha$ - and PCFT-positive cancer cells.

## 2 | MATERIALS AND METHODS

### 2.1 | Reagents

Fol-c<sub>1</sub>- $\beta$ -CyD (Figure 1A), as the commercial product ND201,17 was purchased from CyDing. Standard DMEM, PTX hydrochloride and folate were purchased from Wako Pure Chemical Industries. Folate-free DMEM was purchased from Merck. FBS was purchased from Bio West. Penicillin and streptomycin solutions were purchased from Nacalai Tesque. 4,4'-Diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) was purchased from Santa Cruz Biotechnology.

Standard DMEM was supplemented with 10% FBS, 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin. Folate-free DMEM containing 10% FBS, 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin was supplemented with 4.4 mg/L glucose, 584 mg/L L-glutamine and 3.7 mg/L NaHCO<sub>3</sub>, so that the concentrations of those components were the same as in standard DMEM.

The PTX/Fol-c<sub>1</sub>- $\beta$ -CyD complex was prepared as follows. Fol-c<sub>1</sub>- $\beta$ -CyD was dissolved in sodium bicarbonate buffer (pH 10.6), and PTX hydrochloride was added to make a solution containing 0.2 mM Fol-c<sub>1</sub>- $\beta$ -CyD and 0.2 mM PTX. The tube containing the solution was mixed overnight with inversion at 4°C, and then the mixture was applied to an Amicon Ultra centrifugal filters, 3000 NMWL (Millipore). Because the molecular weights of Fol-c<sub>1</sub>- $\beta$ -CyD and PTX are 4884 and 854 g/mol, respectively, unbound PTX was removed at this step. Tenfold concentration by centrifugation and tenfold dilution with the carbonate buffer (pH 10.6) were repeated four times to ultimately yield a 2-mM PTX/Fol-c<sub>1</sub>- $\beta$ -CyD solution. The same ultrafiltration treatment was performed for the control solution (Fol-c<sub>1</sub>- $\beta$ -CyD), to which PTX was not added. The solutions of 2 mM Fol-c<sub>1</sub>- $\beta$ -CyD and 2 mM PTX/Fol-c<sub>1</sub>- $\beta$ -CyD were used for the antitumor activity assays immediately after preparation.

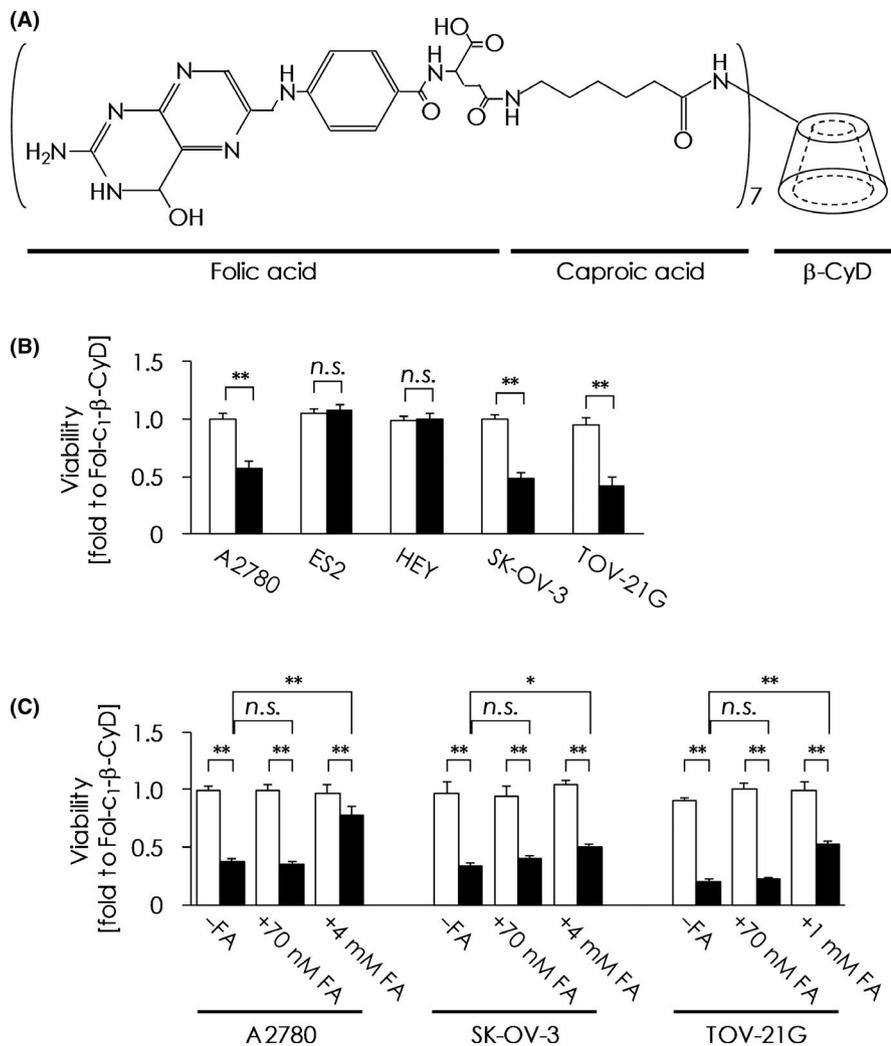
Antibodies were as follows: anti-FR $\alpha$  antibody (R&D Systems), anti-FR $\beta$ , anti-reduced folate carrier (RFC) and anti-PCFT antibodies (Santa Cruz Biotechnology), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Cell Signaling Technology), HRP-linked anti-rabbit IgG antibody (GE Healthcare) and HRP-linked anti-mouse IgG (GE Healthcare).

### 2.2 | Cell culture

The human ovarian cancer cell lines A2780, ES2, HEY, SK-OV-3 and TOV-21G were kind gifts from Dr Fumitaka Kikkawa (Nagoya University, Nagoya, Japan). All cells were maintained in standard DMEM at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. SK-OV-3-Luc.ip1 and A2780-Luc.ip1 cells were established as described by Yu *et al*<sup>18</sup>, except that BALB/c-*nu* mice were used rather than homozygous *nu/nu* mice.

### 2.3 | In vitro antitumor activity assay

Several articles have reported the folate concentrations in sera from healthy individuals and patients of various diseases; the



**FIGURE 1** A, Chemical structure of Fol- $c_1$ - $\beta$ -CyD was adapted with permission from Dr Hirotsu. B, In vitro antitumor activity of PTX/Fol- $c_1$ - $\beta$ -CyD in epithelial ovarian cancer (EOC) cell lines. Each cell line was treated with 20  $\mu$ M Fol- $c_1$ - $\beta$ -CyD (white bars) or 20  $\mu$ M PTX/Fol- $c_1$ - $\beta$ -CyD complex (equivalent to PTX concentration, black bars) in folate-free DMEM for 24 h, and then CCK-8 assays were conducted. C, In vitro antitumor activity of PTX/Fol- $c_1$ - $\beta$ -CyD in A2780, SK-OV-3 and TOV-21G cells was decreased by the addition of folate as a competitor of folate receptor alpha (FR $\alpha$ ). Experimental conditions were as described in (B) except for incubation with indicated concentrations of folate. Data are shown as fold change relative to Fol- $c_1$ - $\beta$ -CyD-treated cells, expressed as means  $\pm$  SEM ( $n > 3$ ). \*\* $P < .01$ ; \* $P < .05$ ; n.s., no significant difference

concentrations were in the range of 1-30  $\mu$ g/L.<sup>19-23</sup> Because the folate content in standard DMEM is 4 mg/L, significantly higher than the physiological concentration, all in vitro antitumor activity assays were performed with folate-free DMEM. Folate was only supplied by FBS, which contains 7-25  $\mu$ g/L folate.<sup>24,25</sup> EOC cell lines were seeded onto 96-well microplates at  $1 \times 10^4$  cells/well (Iwaki) and incubated for 24 hours to allow the cells to attach. Culture was performed in standard DMEM at 37°C under a 5% CO<sub>2</sub> humidified atmosphere. The cells were then washed with PBS(-), and 100  $\mu$ L of folate-free DMEM containing 20  $\mu$ M Fol- $c_1$ - $\beta$ -CyD or 20  $\mu$ M PTX/Fol- $c_1$ - $\beta$ -CyD (equivalent to the PTX concentration) was added; the sample was then incubated for 24 h. In the competition assay, folate was added (final concentration, 4 mM) at the same time. In the PCFT inhibitor assay, DIDS (to 0.2 mM) or an equivalent volume of DMSO (vehicle) was added at the same time. After washing once with PBS (-), 100  $\mu$ L of the standard DMEM containing 10% Cell Counting Kit-8 reagent (Dojindo) was added to each well of the microplate and incubated for 1 hour at 37°C. The absorbance at 450 nm with the reference wavelength at 620 nm was read on a MultiScan FC system (Thermo Fisher Scientific).

## 2.4 | Immunoblotting

Cell lysates were fractionated by SDS-PAGE, and proteins were transferred to PVDF membranes (Millipore) and immunoblotted using respective antibodies. Note that detection of FR $\beta$ , RFC or PCFT required the use of Can Get Signal Immunoreaction Enhancer Solution (Toyobo) as the reaction solution. Moreover, incubation with both primary and secondary antibodies had to be performed overnight at 4°C. The protein bands were visualized using the enhanced ImageQuant LAS 4000 system (GE Healthcare). After antibody removal using Stripping Solution (Wako Pure Chemical Industry), detection with anti-GAPDH antibody was performed.

## 2.5 | Quantitative real-time PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen). First-strand-cDNA were synthesized from total RNA using the High Capacity-cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR (qRT-PCR) was performed using Fast SYBR Green Master Mix (Thermo Fisher Scientific) with the Step

One Plus PCR System (Applied Biosystems). Primer sequences were as follows: FR $\alpha$ , 5'-CAG GGA CAG ACA TGG CTC AGC G-3' (forward) and 5'-CTC CCC TAC AGC CAC CCA C-3' (reverse); FR $\beta$ , 5'-TGT AGC CAC CAT GTG CAG TGC C-3' (forward) and 5'-GGC ATT CTT CCA GGG ACT GC-3' (reverse); RCF, 5'-CAC CTC GTG TGC TAC CTT TGC TTC-3' (forward) and 5'-AGG TAG TCG GTG AGC AGG AAC AC-3' (reverse); PCFT, 5'-ACG CAG TAT CTG TGG CAC CGC TTC-3' (forward) and 5'-GTA GAG GGT CCA GTG GGA GGT AAG-3' (reverse); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-ATG GGG AAG GTG AAG GTC G-3' (forward) and 5'-GGG GTC ATT GAT GGC AAC AAT A-3' (reverse). mRNA levels were normalized against the corresponding levels of GAPDH mRNA.

## 2.6 | Small interfering RNA and expression vectors

Small interfering RNA specific for FR $\alpha$  and PCFT were designed in our laboratory and generated by Hokkaido System Science. The sense sequences of the siRNA were as follows: FR $\alpha$ -437, 5'-UAU AUA GGU AGG AAA CAU CCU-3'; PCFT-856, 5'-UUC AUA AAG GGU UAA GAU GUC-3'. For FR $\alpha$  knockdown, commercially available siRNA was also used (Santa Cruz Biotechnology). siRNA specific for RFC was purchased from Santa Cruz Biotechnology. AllStars Negative Control siRNA (siASN) were purchased from Qiagen. Lipofectamine RNAiMAX (Invitrogen) was used to transfect siRNA into the cells.

The FR $\alpha$  and PCFT coding sequences were amplified from cDNA of SK-OV-3 and inserted into pIRES2-ZsGreen1 (Takara Bio), and the resultant recombinant plasmids pIRES2-ZsGreen1-FR $\alpha$  and pIRES2-ZsGreen1-PCFT were used as FR $\alpha$  and PCFT expression vectors, respectively. Primer sequences were as follows: *Bgl*III-hFR $\alpha$ , 5'-atg cag atc tAT GGC TCA GCG GAT GAC AAC ACA G-3'; FR $\alpha$ -*Xma*I, 5'-agc tcc cgg gTC AGC TGA GCA GCC ACA GCA G-3'; *Bgl*III-hPCFT, 5'-atg cag atc tAT GGA GGG GAG CGC GAG-3'; hPCFT-*Xma*I, 5'-agc tcc cgg gTC AGG GGC TCT GGG GAA ACT-3'. Transfection of expression vectors was carried out using a NEPA-21 electroporator (Nepagene).

## 2.7 | Animal experiments

All study protocols were approved by the Animal Experiment Committee, Graduate School of Medicine, Nagoya University (approval no. 30257), and by the Recombination DNA Advisory Committee of Nagoya University (approval no. 18-34).

BALB/*c-nu* and ICR mice were purchased from Charles River Laboratories International. Mice were maintained in specific pathogen-free barrier facilities, with ad libitum access to food and water intake, at 23°C and 40-60% humidity under a 12-h light/dark cycle at the Division of Experimental Animals, Nagoya University.

BALB/*c-nu* mice (female, age 7 weeks) were intraperitoneally injected with a suspension of SK-OV-3-Luc.ip1 or A2780-Luc.ip1

cells ( $1 \times 10^5$  cells/500  $\mu$ L). Approximately 1 week later, saline (negative control), PTX (5 mg/kg) or PTX/Fol- $c_1$ - $\beta$ -CyD complex (<5 mg PTX/kg and 25 mg/kg of PTX/Fol- $c_1$ - $\beta$ -CyD) was administered to tumor-bearing mice by single intraperitoneal injection. The starting amount of PTX used in preparation of the Fol- $c_1$ - $\beta$ -CyD solution was 5 mg/kg, but unbound PTX was removed during the ultracentrifugation process; thus, the concentration is indicated as "<5 mg PTX/kg." Tumor volume was monitored weekly using an IVIS Spectrum (Perkin Elmer). The body weight was monitored every 3-4 days. In the experiment, the time when the diameter of the tumor protruding outside the abdomen exceeded 20 mm was regarded as a humanitarian end point, and the mouse was killed with CO<sub>2</sub> inhalation followed by cervical dislocation. ICR mice (female; age 7 weeks) were intraperitoneally administered PTX/Fol- $c_1$ - $\beta$ -CyD (5 mg/kg of PTX conjugated with 25 mg/kg of Fol- $c_1$ - $\beta$ -CyD, molar ratio 1:1.14) or saline. Blood samples were taken from tail arteries of the mice 3, 7 and 10 days after the drug injection and collected into Micro Hematocrit Tubes K2EDTA (Vitrex Medical A/S, Herlev, Denmark). Neutrophil to total white blood cell ratio was analyzed on a VetScan HM5 (Abaxis).

## 2.8 | Statistical analyses

Data are given as means  $\pm$  SEM. The unpaired *t*-test was used to compare the means between two groups. ANOVA and Fisher's protected least significance difference test were used for multiple comparisons. A *P*-value < 0.05 was interpreted as significant.

## 3 | RESULTS

### 3.1 | Fol- $c_1$ - $\beta$ -CyD confers cell selectivity by binding to paclitaxel

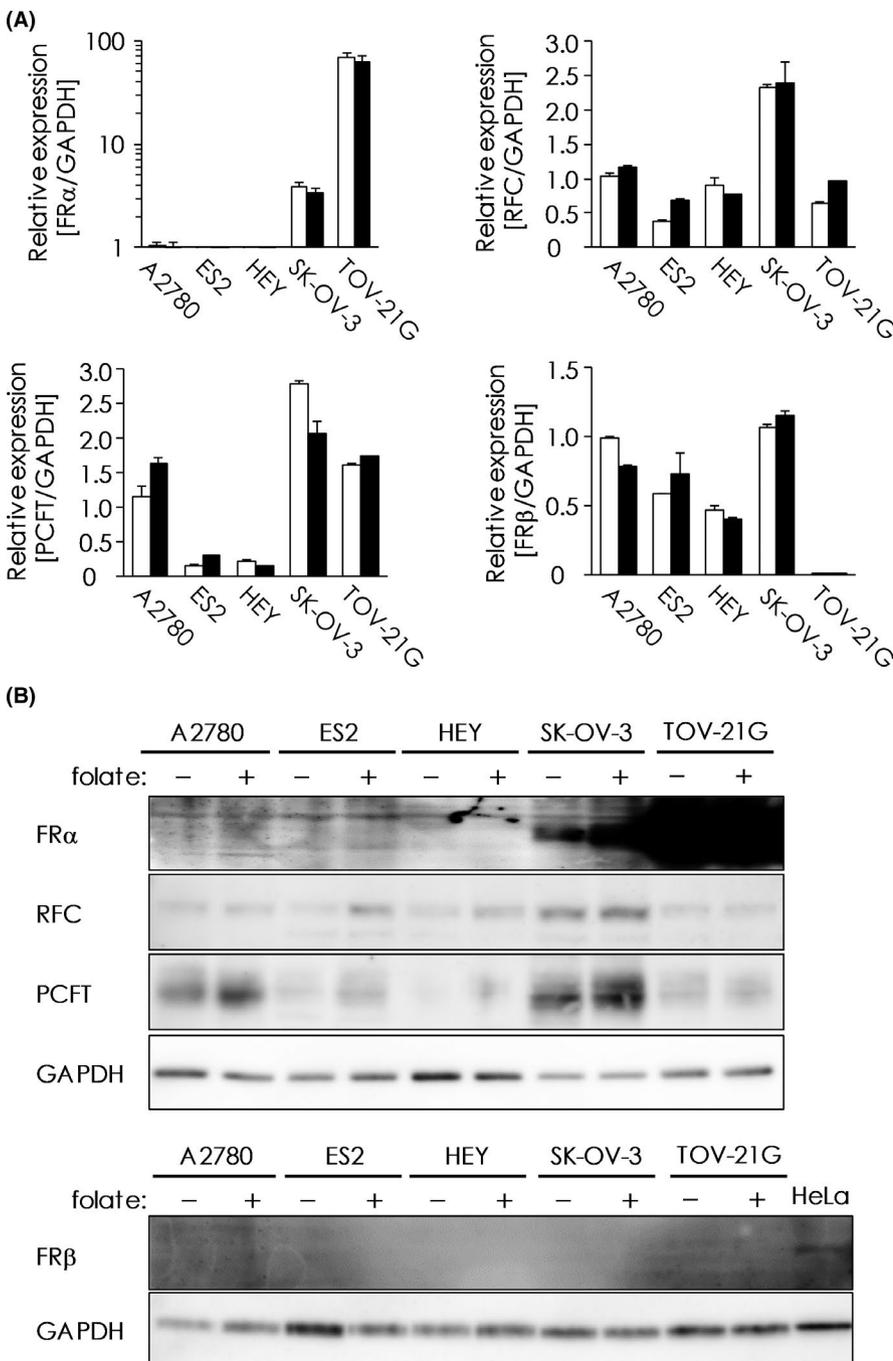
We first tested the cytotoxicity of PTX alone in EOC cell lines (Figure S1). PTX treatment was cytotoxic in all cell lines, with similar efficacy. In all cell lines tested, cytotoxicity reached a plateau at 100 nM. Based on these results, we conclude that PTX uptake efficiency is similar among the EOC cell lines, consistent with the idea that PTX is taken up by non-specific passive diffusion.<sup>26</sup> Next, we tested the cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD (Figure 1B). After incorporation of PTX into Fol- $c_1$ - $\beta$ -CyD, free PTX was removed by ultrafiltration (see Materials and Methods) because in the preliminary experiments performed without this purification step, no cell selectivity of PTX/Fol- $c_1$ - $\beta$ -CyD was observed (Figure S2). PTX/Fol- $c_1$ - $\beta$ -CyD exerted cytotoxicity in A2780, SK-OV-3 and TOV-21G cells, but not in ES2 or HEY cells, indicating that Fol- $c_1$ - $\beta$ -CyD inhibits cytotoxicity of PTX toward ES2 and HEY cells. In addition, because the cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD was somewhat selective after the ultrafiltration treatment, we conclude that free PTX had been efficiently removed. To further clarify the selectivity of PTX/Fol- $c_1$ - $\beta$ -CyD, we performed competition assays (Figure 1C). The cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD was inhibited by excess folate but not by physiological folate,

indicating that PTX/Fol- $c_1$ - $\beta$ -CyD was incorporated into those EOC cell lines through the folate transport system. Collectively, these data suggest that Fol- $c_1$ - $\beta$ -CyD binds to PTX and suppresses non-specific cytotoxicity while conferring specificity to the folate transport system.

### 3.2 | Proton-coupled folate transporter expression is higher in A2780 cells than ES2 and HEY cells

Next, we analyzed the mRNA and protein levels of folate receptors and transporters in the EOC cell lines (Figure 2A,B, respectively).

SK-OV-3 and TOV-21G cells expressed FR $\alpha$ , as expected, but A2780 cells did not express FR $\alpha$ , despite being sensitive to PTX/Fol- $c_1$ - $\beta$ -CyD, suggesting that FR $\alpha$  is not necessary for the cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD in A2780 cells. Although several folate receptors and transporters are expressed in human cells, it remains unknown which receptor/transporter contributes to uptake of folate-conjugated cyclodextrin. Hence, we compared the expression levels of other folate receptor and transporter genes among EOC cell lines. RFC expression was slightly higher in SK-OV-3 cells but almost the same in all EOC cell lines. FR $\beta$  expression in EOC cell lines was detectable by real-time PCR but not by western blot, suggesting that FR $\beta$  is not involved in folate uptake in these EOC lines. PCFT



**FIGURE 2** Expression of folate-associated genes folate receptor alpha (FR $\alpha$ ), FR $\beta$ , reduced folate carrier (RFC) and proton-coupled folate transporter (PCFT) in epithelial ovarian cancer (EOC) cell lines. Each cell line was incubated in the folate-free DMEM (white bars) or standard DMEM (black bars) for 24 h, and quantitative real-time PCR (qRT-PCR) (A) or immunoblot (B) analyses were conducted. In the qRT-PCR analyses, values were normalized against the corresponding levels of GAPDH mRNA. The value for A2780 grown in folate-free DMEM was set to 1. Data are shown as means  $\pm$  SEM ( $n = 3$ ). In the immunoblot analyses, whole-cell lysate from HeLa cells was used as a positive control for FR $\beta$  expression

expression was higher in A2780 cells than in ES2 and HEY cells and comparable to that in SK-OV-3 and TOV-21G cells. Based on these results, we speculated that PTX/Fol- $c_1$ - $\beta$ -CyD is incorporated into A2780 cells via PCFT.

### 3.3 | Both FR $\alpha$ and PCFT are essential for cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD in SK-OV-3, but not in A2780

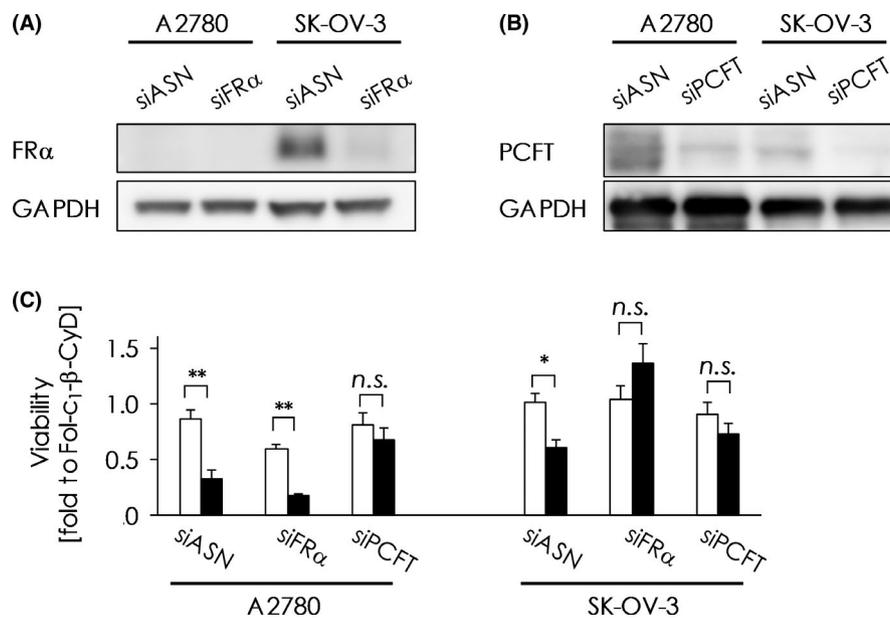
We tested the effect of FR $\alpha$  or PCFT depletion on the cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD. For this purpose, we transfected A2780 and SK-OV-3 cells with FR $\alpha$ -targeting or PCFT-targeting siRNA and then monitored the cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD (Figure 3). In SK-OV-3 cells, knockdown of either FR $\alpha$  or PCFT suppressed the protein levels of each gene (Figure 3A,B), and decreased the cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD (Figure 3C), suggesting that both proteins are involved in PTX/Fol- $c_1$ - $\beta$ -CyD uptake in these cells. By contrast, in A2780 cells, only PCFT knockdown significantly decreased the cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD, indicating that PCFT but not FR $\alpha$  is involved in PTX/Fol- $c_1$ - $\beta$ -CyD uptake in these cells. In addition, we tested the effect of RFC depletion on the cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD (Figure S3). Transfection of RFC-targeting siRNA efficiently reduced RFC expression (Figure S3A), but the cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD was not suppressed in A2780 or SK-OV-3 cells (Figure S3B), indicating that RFC is not involved in PTX/Fol- $c_1$ - $\beta$ -CyD uptake in A2780 or SK-OV-3 cells. These data suggest that PTX/Fol- $c_1$ - $\beta$ -CyD could be taken up via a PCFT-dependent pathway in

A2780 cells or via an FR $\alpha$ /PCFT-dependent pathway in SK-OV-3 cells.

We wondered why treatment with the FR $\alpha$ -targeting siRNA decreased the growth of A2780 cells, in which no FR $\alpha$  expression was detected. Hence, we tested the effect of another FR $\alpha$ -targeting siRNA (Figure S4). The commercially available siRNA suppressed proliferation of A2780 cells similarly to the siRNA that we designed (Figure S4A), arguing against a secondary effect. Next, we speculated that extremely low levels of FR $\alpha$  expressed in A2780 cells were still functional, and that this expression could be knocked down by FR $\alpha$ -targeting siRNA. If so, growth promotion by folate supplementation would be suppressed following siRNA treatment (Figure S4A). However, folate supplementation promoted the growth of A2780 cells, and the degree of growth promotion rate did not significantly differ among the conditions. In addition, treatment with the FR $\alpha$ -targeting siRNA did not affect the expression levels of RFC or PCFT (Figure S4B).

### 3.4 | PTX/Fol- $c_1$ - $\beta$ -CyD is taken up into A2780 cells via PCFT

Because the transport activity of PCFT is higher under acidic conditions,<sup>27</sup> we investigated whether the cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD was elevated at lower pH. We first asked whether A2780 and SK-OV-3 cells could grow under acidic pH, and found that proliferation of A2780 cells was almost completely suppressed at pH 6.6 (Figure S5A), whereas SK-OV-3 cells were not affected at those



**FIGURE 3** Folate receptor alpha (FR $\alpha$ ) is dispensable for the cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD in A2780 cells but indispensable in SK-OV-3 cells. FR $\alpha$ -targeted (siFR $\alpha$ ), proton-coupled folate transporter (PCFT)-targeted (siPCFT) or control (siASN) siRNA was transfected into A2780 and SK-OV-3 cells, which were incubated in folate-free DMEM. A, B, Forty-eight hours after transfection, immunoblot assays were conducted to detect the FR $\alpha$ , PCFT and GAPDH proteins. C, A2780 and SK-OV-3 cells transfected with the indicated siRNA were incubated in folate-free DMEM containing 20  $\mu$ M Fol- $c_1$ - $\beta$ -CyD (white bars) or 20  $\mu$ M PTX/Fol- $c_1$ - $\beta$ -CyD complex (equivalent to PTX concentration, black bars). Forty-eight hours after transfection, the in vitro antitumor activity assays were conducted. Data are shown as fold change relative to siASN/Fol- $c_1$ - $\beta$ -CyD and shown as means  $\pm$  SEM ( $n = 3$ ). \*\* $P < .01$ ; \* $P < .05$ ; n.s., no significant difference

pH conditions (Figure S5B). Therefore, we tested the cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD at pH 7.6 and pH 7.1. Cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD was significantly higher at pH 7.1 than pH 7.6 in A2780 cells, whereas in SK-OV-3 cells no difference was observed between pH conditions (Figure 4A). In addition, the PCFT inhibitor DIDS partially suppressed the cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD in A2780 cells (Figure 4B). Taken together, these data suggest that PTX/Fol- $c_1$ - $\beta$ -CyD is taken up into A2780 cells via PCFT.

### 3.5 | HEY cells overexpressing FR $\alpha$ and PCFT are targeted by PTX/Fol- $c_1$ - $\beta$ -CyD

We next tested the effect of forced expression of FR $\alpha$  and/or PCFT on the cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD. For this purpose, we transfected HEY cells with FR $\alpha$  or PCFT expression vector, and then monitored the protein levels of each gene (Figure 5A,B) and the cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD (Figure 5C). PTX/Fol- $c_1$ - $\beta$ -CyD exerted cytotoxicity only when expression of FR $\alpha$  and PCFT was

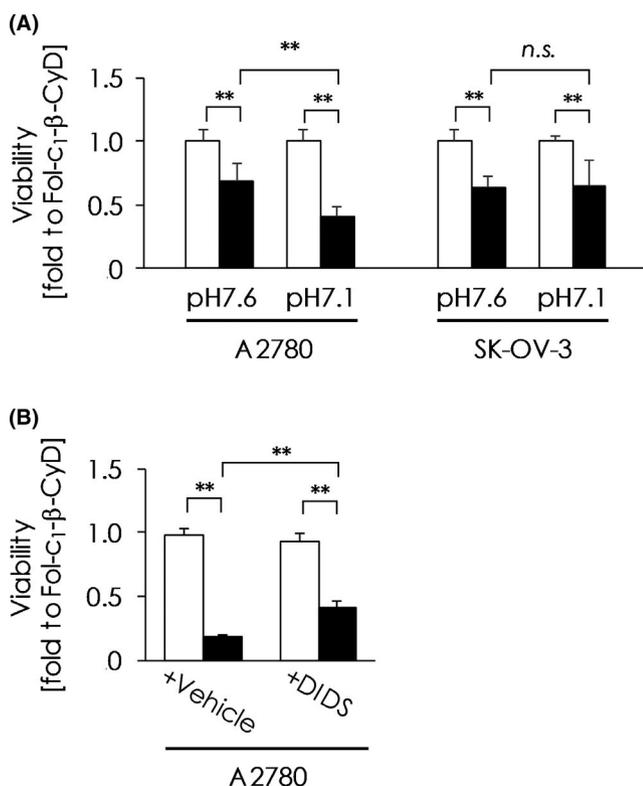
induced simultaneously, suggesting that both proteins are necessary for uptake of PTX/Fol- $c_1$ - $\beta$ -CyD in HEY cells.

### 3.6 | PTX/Fol- $c_1$ - $\beta$ -CyD specifically targets EOC cells expressing FR $\alpha$ or PCFT in vivo

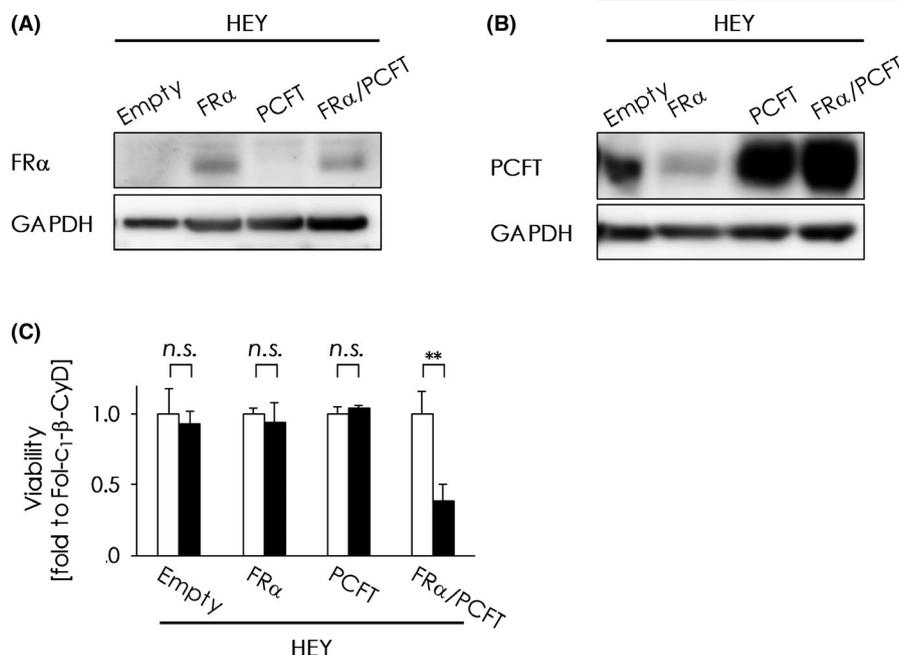
We then tested the antitumor activity of PTX/Fol- $c_1$ - $\beta$ -CyD in vivo (Figure 6, Figure S6). Because metastasis of ovarian cancer is generally confined to the peritoneal cavity,<sup>28,29</sup> we inoculated SK-OV-3-Luc.ip1 or A2780-Luc.ip1 cells intraperitoneally; 1 week after inoculation, saline, PTX or PTX/Fol- $c_1$ - $\beta$ -CyD was administered intraperitoneally. Except on day 30 in SK-OV-3 inoculated mice, PTX alone exhibited no significant antitumor effect relative to the control (Figure 6A,C and Figure S6). In contrast, PTX/Fol- $c_1$ - $\beta$ -CyD significantly inhibited tumor growth relative to PTX alone, with no significant alteration in body weight (Figure 6B,D). We then tested the effect of Fol- $c_1$ - $\beta$ -CyD on the level of neutrophils using healthy immunocompetent ICR mice (Figure 6E). No significant change in neutrophil ratio to total white blood cells was observed in mice receiving PTX/Fol- $c_1$ - $\beta$ -CyD relative to vehicle-treated mice. These results suggest that inclusion of PTX in Fol- $c_1$ - $\beta$ -CyD increases antitumor activity while decreasing the adverse effect of PTX in vivo.

## 4 | DISCUSSION

In this study, we assessed the specificity and efficacy of Fol- $c_1$ - $\beta$ -CyD, a folate-conjugated-CyD carrier, toward EOC cell lines in vitro and in vivo. We found that PCFT is also targeted by the carrier. This is the first report to demonstrate an FR $\alpha$ -independent and PCFT-dependent transport pathway for a folate-conjugated drug carrier. In SK-OV-3 cells, cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD was decreased by knockdown of FR $\alpha$  (Figure 3) but was not affected by the pH of the media (Figure 4A); therefore, PTX/Fol- $c_1$ - $\beta$ -CyD uptake in SK-OV-3 cells may be mediated mainly through FR $\alpha$ . Interestingly, knockdown of PCFT also decreased the cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD in SK-OV-3 cells, suggesting that PCFT is also necessary for the efficacy of PTX/Fol- $c_1$ - $\beta$ -CyD. Consistent with our results, FR $\alpha$ -mediated growth inhibition by ZD9331 also requires co-expression of PCFT.<sup>30</sup> PCFT also promotes transport of 5-formyltetrahydrofolate (leucovorin) from the membrane vesicle into the cytosol in a HeLa subline, and FR $\alpha$  and PCFT are co-localized to the endosomal compartment within another HeLa subline that is stably transfected with PCFT, indicating a role for PCFT as a pathway for escape from FR $\alpha$ -dependent endosomes.<sup>31</sup> Zhao *et al*<sup>32</sup> reported that M160-8, a pemetrexed-resistant HeLa subline, is collaterally sensitive to EC0905, which consists of DAVLBH (a lipophilic microtubule inhibitor) connected to folic acid with a linker containing a sulfhydryl bond. Both pemetrexed and EC0905 are antifolates and are internalized within FR $\alpha$ -mediated endosomes in M160-8 cells. In the case of EC0905, the sulfhydryl bond is cleaved upon acidification, and the lipophilic DAVLBH moiety passively diffuses out of the endosome



**FIGURE 4** The cytotoxicity of PTX/Fol- $c_1$ - $\beta$ -CyD promoted under the acidic pH (A), but the promotive effect was partially reduced upon treatment with the proton-coupled folate transporter (PCFT) inhibitor DIDS (B) in A2780 cells. The in vitro antitumor activity assay was conducted using the folate-free DMEM containing 20  $\mu$ M Fol- $c_1$ - $\beta$ -CyD (white bars) or 20  $\mu$ M PTX/Fol- $c_1$ - $\beta$ -CyD (equivalent to PTX concentration, black bars) with pH adjusted to 7.1 or 7.6. The effect of the PCFT inhibitor DIDS (0.2 mM) was assessed at pH 7.1. Data are shown as fold change relative to Fol- $c_1$ - $\beta$ -CyD-treated cells, expressed as means  $\pm$  SEM ( $n = 3$ ). \*\* $P < .01$ ; n.s., no significant difference



**FIGURE 5** Simultaneous expression of folate receptor alpha (FR $\alpha$ ) and proton-coupled folate transporter (PCFT) is necessary for the cytotoxicity of PTX/Fol-c<sub>1</sub>- $\beta$ -CyD in HEY cells. HEY cells were transfected with FR $\alpha$ -expressing, PCFT-expressing or empty plasmids (10  $\mu$ g DNA/well in total, each) and incubated for 48 h. Protein levels of FR $\alpha$  (A) and PCFT (B) were checked by immunoblotting. The experiment was repeated at least three times, and representative blots are shown. Meanwhile, 48 h after the transfection, the *in vitro* antitumor activity assays were conducted using folate-free DMEM containing 20  $\mu$ M Fol-c<sub>1</sub>- $\beta$ -CyD (white bars) or 20  $\mu$ M PTX/Fol-c<sub>1</sub>- $\beta$ -CyD complex (equivalent to PTX concentration, black bars). Data are shown as fold change relative to Fol-c<sub>1</sub>- $\beta$ -CyD-treated cells, expressed as means  $\pm$  SEM (n = 3). \*\*P < .01; n.s., no significant difference

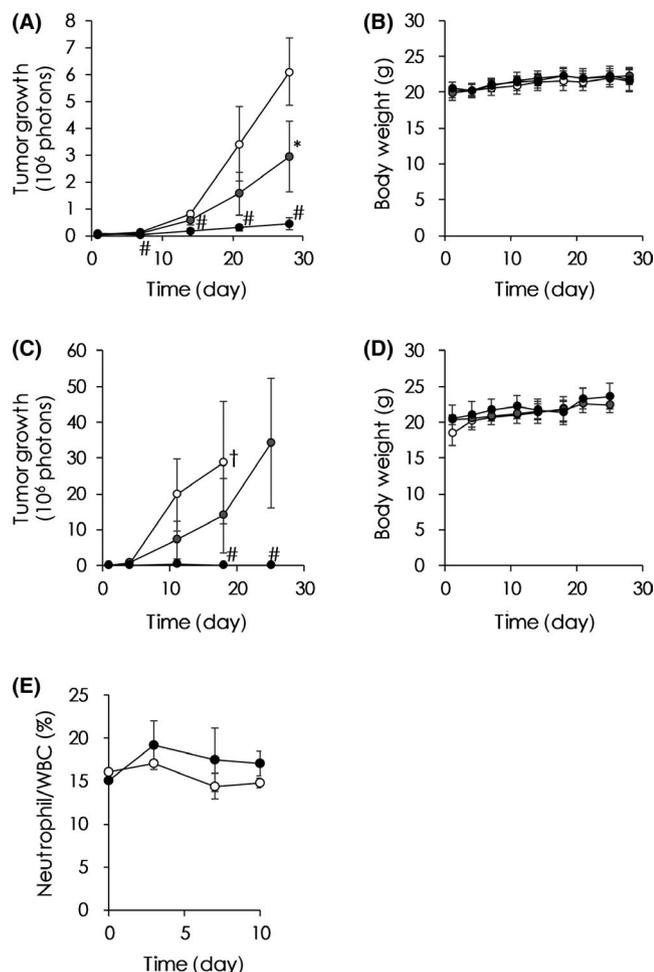
into the cytosol. However, hydrophilic pemetrexed is trapped within the endosome in the absence of PCFT. This may be the case also for Fol-c<sub>1</sub>- $\beta$ -CyD; while the inner cavity of  $\beta$ -CyD backbone is hydrophobic, its outer periphery is hydrophilic.

Because Fol-c<sub>1</sub>- $\beta$ -CyD alone did not exert cytotoxicity in the EOC cell lines in our preliminary experiments (data not shown), the cytotoxicity of PTX/Fol-c<sub>1</sub>- $\beta$ -CyD must be attributed to PTX, which is thought to be pharmacologically inactive when it is incorporated in Fol-c<sub>1</sub>- $\beta$ -CyD. Therefore, PTX/Fol-c<sub>1</sub>- $\beta$ -CyD in cells must dissociate into PTX and Fol-c<sub>1</sub>- $\beta$ -CyD. Currently, we are unsure when PTX/Fol-c<sub>1</sub>- $\beta$ -CyD dissociates in the EOC cell lines. According to a report by Sharma *et al*<sup>33</sup> the apparent stability constant for PTX and microtubules (GMP-cPP) is very high, approximately  $53 \times 10^6$  (M<sup>-1</sup>). In contrast, the stability constant for the doxorubicin complexed with Fol-c<sub>1</sub>- $\beta$ -CyD is  $1.7 \pm 0.3 \times 10^6$  (M<sup>-1</sup>).<sup>17</sup> We tried to determine the stability constant of PTX conjugated with Fol-c<sub>1</sub>- $\beta$ -CyD using the method of continuous variations (Job plot) but were unsuccessful (data not shown). Although the stability constant of the PTX/Fol-c<sub>1</sub>- $\beta$ -CyD complex has not yet been determined, the fact that the persistence of the antitumor effect *in vivo* was shorter than that of doxorubicin/Fol-c<sub>1</sub>- $\beta$ -CyD (Figure 6A,C) implies that the complex is less stable than the doxorubicin/Fol-c<sub>1</sub>- $\beta$ -CyD complex. Therefore, it is possible that PTX in Fol-c<sub>1</sub>- $\beta$ -CyD could be removed from the PTX/Fol-c<sub>1</sub>- $\beta$ -CyD complex by the microtubules of the cancer cells, rather than by spontaneous dissociation.

Functional deficiency of RFC is involved in the resistance of cancer cells to certain antifolates, such as methotrexate and PT523.<sup>27,34</sup>

By contrast, suppression of RFC expression had no significant effect on the cytotoxicity of PTX/Fol-c<sub>1</sub>- $\beta$ -CyD in A2780 or SK-OV-3 cells (Figure S3C,D). In addition, although the expression levels of RFC in each EOC cell line were almost the same (Figure 2), the cytotoxicity of PTX/Fol-c<sub>1</sub>- $\beta$ -CyD was particularly high in A2780, SK-OV-3 and TOV-21G cells (Figure 1B). Okamoto *et al* reported that uptake of Fol-c<sub>1</sub>- $\beta$ -CyD is lower in FR $\alpha$ -negative A549 cells than in FR $\alpha$ -expressing KB cells,<sup>17</sup> whereas the expression levels of RFC in those cell lines are comparable.<sup>35</sup> Based on these reports and our results together, we conclude that RFC is not required for the uptake or cytotoxicity of anticancer drugs conjugated to Fol-c<sub>1</sub>- $\beta$ -CyD.

Because the cytotoxicity of PTX/Fol-c<sub>1</sub>- $\beta$ -CyD was decreased by knockdown of PCFT, but not FR $\alpha$ , in A2780 cells, it is likely that PTX/Fol-c<sub>1</sub>- $\beta$ -CyD uptake in A2780 cells is mediated primarily through PCFT (Figure 3C). Aside from in this study, A2780 and SK-OV-3 cells are frequently used as target cells in studies of FR $\alpha$ -targeting drugs for ovarian cancer.<sup>16,36-40</sup> Some studies have compared expression levels of FR $\alpha$  between A2780 and SK-OV-3 cells, and in all of the studies, consistent with our results, A2780 cells expressed lower levels of FR $\alpha$  than SK-OV-3 cells.<sup>16,37,39</sup> Wen *et al*<sup>39</sup> reported that farletuzumab, a humanized monoclonal antibody against FR $\alpha$ , did not decrease either tumor weight or the number of tumor nodules in the A2780 orthotopic mouse model, although it had a significant effect on both parameters in the SK-OV-3 model. These observations, together with the results of this study, suggest that the expression level of FR $\alpha$  in A2780 cells is below the threshold necessary for targeting by FR $\alpha$ -targeting drugs.



**FIGURE 6** Effects of single intraperitoneal administration of PTX and PTX/Fol-c<sub>1</sub>-β-CyD complex on tumor growth (A, C), body weight (B, D) and neutrophil ratio (E) in tumor-bearing mice. SK-OV-3-Luc.ip1 (A, B) or A2780-Luc.ip1 (C, D) cells were intraperitoneally inoculated, and 1 week after inoculation, vehicle (white circles), PTX alone (5 mg/kg, grey circles) or PTX/Fol-c<sub>1</sub>-β-CyD (<5 mg PTX/kg, black circles) was administered to tumor-bearing mice by single intraperitoneal injection. Data for each time point are expressed as means ± SEM (n = 3). \*P < .05 vs vehicle-received mice; #P < .05 vs PTX-received mice. †Euthanized

Proton-coupled folate transporter was necessary but not sufficient for PTX/Fol-c<sub>1</sub>-β-CyD uptake in HEY cells, as indicated by the observation that PCFT expression alone failed to induce cytotoxicity (Figure 5). Histologically, the A2780 cell line is considered to be endometrioid carcinoma, whereas HEY and SK-OV-3 are considered to be serous carcinoma. In light of our results, under the gene expression profile of endometrioid carcinoma (A2780), the PCFT-dependent mechanism would function, whereas under the profile of serous carcinoma (HEY and SK-OV-3), the FRα/PCFT-dependent uptake mechanism would function. Further studies are necessary to clarify the details of the PCFT-dependent transport mechanism.

Desmoulin et al performed a comprehensive analysis of folate transporter expression in 80 cancer cell lines derived from human solid tumors (n = 53) and leukemias (n = 27), and PCFT expression was detected in 52 of 53 tumor cells.<sup>41</sup> PCFT expression was elevated

in EOC, hepatoma, malignant pleural mesothelioma, non-small cell lung cancer and pancreatic cancer cells but low to undetectable in leukemias (including acute lymphoblastic leukemia and acute myeloid leukemia). Furthermore, PCFT, which mediates co-transport of folates and protons, exhibits optimal activity at low pH, and the tumor microenvironment is acidified by the Warburg effect.<sup>42</sup> In comparison with FRα, PCFT has not been as extensively studied as a molecular target for cancer treatment, despite its high expression in several solid tumors and potentially high activity in the cancer microenvironment. Various types of folate-conjugated drugs might be exploited to treat cancers with substantial PCFT expression, as well as those that are FRα-positive.

In contrast, PCFT is expressed in several normal tissues, including proximal jejunum, duodenum, choroid plexus, kidney and liver.<sup>41</sup> Therefore, PCFT may also be expressed in tissues facing the peritoneal cavity. We were concerned that intraperitoneally administered Fol-c<sub>1</sub>-β-CyD could be incorporated into those normal tissues, preventing the cancer cells from being efficiently targeted. However, PTX/Fol-c<sub>1</sub>-β-CyD effectively suppressed the growth of cancer cells without significantly decreasing the neutrophil ratio in the mouse peritoneal cavity. Because we observed no neutropenia upon administration of PTX/Fol-c<sub>1</sub>-β-CyD, we presume that Fol-c<sub>1</sub>-β-CyD suppresses the side effects of PTX. Therefore, PTX/Fol-c<sub>1</sub>-β-CyD could be administered at higher doses than PTX formulated using the conventional Cremophor-EL/ethanol mixture, likely yielding a stronger antitumor effect.

It should be noted that the antitumor activity of PTX/Fol-c<sub>1</sub>-β-CyD was most prominent at 1 week after administration, and the tumor growth rate after that period did not seem to differ significantly from those in the vehicle- or PTX-received groups, indicating that the antitumor activity of PTX/Fol-c<sub>1</sub>-β-CyD lasts for only approximately 1 week. Okamoto *et al* showed that the antitumor activity of doxorubicin conjugated with Fol-c<sub>1</sub>-β-CyD continues for more than 30 days.<sup>17</sup> Moreover, they reported that a stability constant value of more than 10<sup>5</sup> (M<sup>-1</sup>) is required to maintain a stable complex in vivo.<sup>17,43,44</sup> Given that the duration of the antitumor effect is short, it is likely that the stability of the PTX/Fol-c<sub>1</sub>-β-CyD complex is near the minimum value (10<sup>5</sup> M<sup>-1</sup>) that allows the complex to exist in vivo. Alternatively, differences in the administration route may affect the persistence of the antitumor effect; notably in this regard, Okamoto *et al* administered the doxorubicin/Fol-c<sub>1</sub>-β-CyD complex intratumorally or intravenously. In any case, the antitumor activity of PTX/Fol-c<sub>1</sub>-β-CyD in the abdominal cavity could be improved by changing the administration schedule to multiple weekly doses. The dose of PTX/Fol-c<sub>1</sub>-β-CyD used in this study could be sufficient for use in humans; that is, Fol-c<sub>1</sub>-β-CyD would not be inhibited by folate derived from peritoneal fluid. Folate concentrations in the peritoneal fluid of mice and humans have not been reported. However, humans and mice eat comparable amounts of folate (0.4 mg for humans<sup>45</sup> and 0.34 mg for mice; MF chow (Oriental Yeast, Japan) contains 0.17 g/kg of folate, and mice eat 2 g of the chow per day on average); thus, given that the body weight of humans (approximately 60 kg) is much larger than that of mice (approximately 30 g), it is unlikely

that the folate concentration in peritoneal fluid is higher in humans than in mice.

In conclusion, this study revealed that Fol- $c_1$ - $\beta$ -CyD targets PCFT as well as FR $\alpha$ . Moreover, we demonstrated that intraperitoneally administered PTX/Fol- $c_1$ - $\beta$ -CyD suppressed the growth of both FR $\alpha$ -positive and PCFT-positive ovarian cancer cells *in vivo*, suggesting that Fol- $c_1$ - $\beta$ -CyD can survey its target cells throughout the expanse of the abdominal cavity. Because peritoneal dissemination is a major route for ovarian cancer metastasis, FR $\alpha$  expression is maintained on metastatic foci and recurrent tumors,<sup>46</sup> and PCFT has limited distribution in normal tissues but is highly expressed in EOC,<sup>27,41,47</sup> we propose that Fol- $c_1$ - $\beta$ -CyD represents a promising potential treatment for recurrent ovarian cancer. Fol- $c_1$ - $\beta$ -CyD may suppress the significant side effects of hydrophobic anticancer agents, including PTX, while enhancing the antitumor effect when administered intraperitoneally.

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## CONFLICTS OF INTEREST

The authors declare no competing interests.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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