# Cisplatin, rather than oxaliplatin, increases paracellular permeability of LLC-PK1 cells via activating protein kinase C

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**Abstract**

The clinical use of cisplatin is limited by its adverse events, particularly serious nephrotoxicity. It was clarified that cisplatin is transported by a kidney-specific organic cation transporter (OCT2). OCT2 also mediates the uptake of oxaliplatin into renal proximal tubular cells; however, this agent does not lead nephrotoxicity. In the present study, we carried out comparative experiments with cisplatin and oxaliplatin using porcine kidney LLC-PK1 cell monolayers. In the fluorescein-labeled isothiocyanate-dextran flux assay, the basolateral application of cisplatin, but not oxaliplatin, resulted in an increase in the paracellular permeability of cell monolayers. Even though the cellular accumulation of platinum at 50 μM oxaliplatin could reach the same level at 30 μM cisplatin, oxaliplatin did not induce hyper-permeability in cell monolayers. Cisplatin, but not oxaliplatin, significantly activated PKC. In addition, the combination of PKC inhibitors recovered the increase in paracellular permeability. In conclusion, pharmacodynamic mechanisms via PKC could explain the difference in nephrotoxicity between cisplatin and oxaliplatin.

**Keywords**

Cisplatin, oxaliplatin, nephrotoxicity, protein kinase C, paracellular permeability

## 1. Introduction

Cisplatin shows a broad spectrum of activity against various cancers and plays a significant function in cancer chemotherapy. Its adverse effects, particularly nephrotoxicity, remain major factors that limit its clinical use in cancer therapy [1,2]. On the other hand, carboplatin shows little nephrotoxicity, even though it produces the same active agent as cisplatin in the cell. Organic cation transporter 2 (OCT2) is predominantly expressed in the basolateral membranes of renal proximal tubular cells and mediates the uptake of cationic drugs [3-7]. Previously, we reported that OCT2 played important roles in kidney accumulation of cisplatin but not carboplatin [8,9]. The substrate specificities of OCT2 among platinum agents can determine cisplatin-specific nephrotoxicity.

On the contrary, another platinum-based antitumor drug, oxaliplatin, is also transported by OCT2, but it does not cause any renal toxicity [9]. The active agent of oxaliplatin is different from those of cisplatin and carboplatin. It has been reported that oxaliplatin exhibited a different cytotoxic mechanism(s) from cisplatin in cancer cells [10,11]. For example, mismatch repair proteins and some damage-recognition proteins bind to cisplatin-GG adducts with higher affinity than oxaliplatin-GG adducts [12]. This is thought to contribute to differences in cytotoxicity and to the range of anticancer activity shown by oxaliplatin and cisplatin. Pharmacodynamic but not pharmacokinetic mechanisms could explain the difference in nephrotoxicity between cisplatin and oxaliplatin.

The protein kinase C (PKC) family consists of 10 related serine/threonine protein kinases, some of which are critical regulators of cell proliferation, survival and death [13]. Its activity and phosphorylation of downstream signals are also associated with regulation of the barrier function of epithelial cells [14-19]. Recently, the PKC family has been shown to attract marked attention regarding cisplatin-induced nephrotoxicity [20-23]. Furthermore, it has been reported that the inhibition of PKCδ reduces cisplatin-induced nephrotoxicity without

blocking chemotherapeutic efficacy in mouse cancer models [24]. PKC could play an important role in cisplatin-specific nephrotoxicity.

Based on this background, we hypothesized that the intracellular mechanisms of nephrotoxicity differ between cisplatin and oxaliplatin. In the present study, we carried out comparative experiments using LLC-PK1 cell monolayers, an epithelial cell line originated from porcine proximal tubular cells which express organic cation transporters.

## 2. Materials and Methods

### *2.1. Materials*

Cisplatin and fluorescein-labeled isothiocyanate (FITC) dextran (average mol. wt.: 4,000) were purchased from Sigma-Aldrich Co. (St. Louis, USA). Oxaliplatin, sotrastaurin, GF109203X and Go6976 were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Rottlerin was obtained from Abcam (Cambridge, UK). Cimetidine was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other compounds used were of the highest purity available.

### *2.2. Cell Culture and Drug Treatment*

LLC-PK1 cells (JCRB Cell Bank, Osaka, Japan) were cultured as described previously [25]. In general, cells were seeded on 6-well polycarbonate membrane filters (3-μm pores, 4.71-$cm^2$ growth area) inside transwell@ cell culture chambers (Corning, Cambridge, MA, USA) at a density of $1.8\times10^6$ cells/well. The cells were used for the experiments on the 6th day after seeding. Cell monolayers were incubated in the culture medium with 10, 30 and 50 μM cisplatin or oxaliplatin added to the basolateral side at 37°C. When inhibitors were used in experiments, they were added to the basolateral side with cisplatin.

### *2.3. FITC-Dextran Flux Assay*

Paracellular permeability of FITC-dextran was measured using monolayer cultures grown in transwell@ chambers, as described previously with some modification [26]. The cell monolayers were incubated in culture medium with cisplatin or oxaliplatin. After removal of the culture medium from both sides of the monolayers, the cells were incubated with incubation buffer (2 mL each side, pH 7.4 buffer on the basolateral side and pH 6.0 buffer on the apical side) at 37°C for 10 min. Then, 2 mL of incubation buffer (pH 7.4) containing FITC-dextran (50 μM) was added to the basolateral side and 2 mL of incubation buffer (pH 6.0) to the apical side. For paracellular flux measurement, the incubation buffer on the apical side was taken at specific time-points (5, 15 and 30 min). The fluorescence intensity of FITC-dextran at 585 nm in the buffer was determined with an excitation wavelength of 485 nm using a Mithras LB 940 multimode microplate reader (Berthold Technologies, Bad Wildbad, Germany).

### *2.4. Cellular Platinum Uptake Experiment*

For measurement of platinum accumulated in cells, uptake studies were performed as described previously [27]. The cell monolayers were incubated in culture medium with cisplatin or oxaliplatin for 1 hour. After removal of the culture medium from both sides of the monolayers, cells were rapidly washed twice with ice-cold incubation buffer containing 3% bovine serum albumin and then washed three times with ice-cold incubation buffer. The filters with monolayers were detached from chambers, the cells on the filters were solubilized in 0.5 mL of 0.5 N NaOH, and the amount of platinum was determined using inductively coupled plasma-mass spectrometry (ICP-MS) (Agilent7700/MassHunter, Agilent Technologies, California, USA). The protein contents of solubilized cells were determined by

the method of Bradford with a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA).

### *2.5. PKC Kinase Activity Assay*

The amount of PKC kinase activity in each sample was measured using a specific PKC assay kit (ab139437, Abcam, Cambridge, UK) according to the manufacturer's instructions. Cell monolayers were incubated in a culture medium with cisplatin or oxaliplatin for 2 hours. After removal of the culture medium from both sides of the monolayers, cells were homogenized with lysis buffer and centrifuged at 13,000 g for 15 min at 4°C. Supernatants were used for the assay with the kit; the absorbance was measured at 450 nm using a VERSAmax tunable microplate reader (Molecular Devices, San Jose, CA, USA).

### *2.6. Lactate Dehydrogenase Release (LDH) Assay*

The cytotoxicity of cisplatin was measured with monolayer cultures grown in transwell@ chambers using a LDH Cytotoxicity Detection Kit (Takara, Shiga, Japan), as described previously with some modification [8]. Cells were incubated in the medium containing cisplatin with or without rottlerin for 24 hours. After the incubation, the medium was collected, and the lactate dehydrogenase activity in the medium was measured, according to the manufacturer's instructions.

### *2.7. Statistical Analysis*

Data are expressed as the mean ± S.D. Data were analyzed using the unpaired Student's t-test or multiple comparisons with Dunnett's two-tailed test after a one-way ANOVA. Probability values of less than 0.05 were considered to be statistically significant.

## 3. Results

### *3.1. Paracellular Permeability of FITC-Dextran in LLC-PK1 Cell Monolayers after Cisplatin Exposure*

Paracellular permeability of FITC-dextran was evaluated in LLC-PK1 cell monolayers treated with cisplatin for 24 hours. The basal-to-apical flux of FITC-dextran in monolayers with cisplatin was much larger than that in control monolayers, and its rate was nearly constant up to 30 min (Fig. 1a). The effect of the cisplatin exposure duration was also determined. Compared with the control group, significant increases of FITC-dextran flux at 30 min were observed in the cell monolayers treated with cisplatin for 10 and 24 hours (Fig. 1b).

### *3.2. Comparative Studies between Cisplatin and Oxaliplatin Using LLC-PK1 Cell Monolayers*

The cytotoxicities of cisplatin and oxaliplatin were compared by FITC-dextran flux assay. Cell monolayers treated with 30 or 50 μM cisplatin became more permeable to FITC-dextran, while oxaliplatin-treated cell monolayers showed little permeability to FITC-dextran (Fig. 2).

The cellular accumulation of platinum was examined in LLC-PK1 cells treated with cisplatin or oxaliplatin (Fig. 3). Then, accumulation of cisplatin increased in a dose-dependent manner, whereas slight increases in oxaliplatin accumulation were also observed. The uptakes of cisplatin at 30 and 50 μM were larger than those of oxaliplatin at corresponding concentrations, although that of oxaliplatin at 50 μM reached the same level as the cellular accumulation of cisplatin at 30 μM.

PKC activities were measured in LLC-PK1 cell monolayers using a PKC kinase activity assay kit. As a result, cisplatin, but not oxaliplatin, significantly activated PKC (Fig. 4a), and

the activation of PKC was increased in a dose-dependent manner as well as the FITC-dextran permeability increased by cisplatin (Fig. 4b).

### *3.3. Effect of Inhibitors in LLC-PK1 Cell Monolayers*

The inhibitory effects of PKC inhibitors on cisplatin-induced hyper-permeability were examined in LLC-PK1 cell monolayers. Sotrastaurin (AEB071, 5 μM), GF109203X (GFX, 2 μM), and rottlerin significantly inhibited the cisplatin-induced hyper-permeability. Go6976 (3 μM) showed a weaker inhibitory effect compared with the other PKC inhibitors (Fig. 5a). These inhibitors also reduced the PKC activation increased by cisplatin (Fig. 5b). However, rottlerin did not affect the LDH release in LLC-PK1 cell monolayers treated with 30 μM cisplatin (31.9 ± 5.5% vs 41.8 ± 2.9%).

The inhibitory effects of an OCT2 inhibitor, cimetidine, on paracellular permeability and cellular accumulation in cell monolayers were studied. Both the fluxes of FITC-dextran (Fig. 6a) and intracellular accumulation of cisplatin (Fig. 6b) in LLC-PK1 cell monolayers were significantly decreased by co-treatment with cimetidine. On the other hand, rottlerin did not reduce the cellular accumulation of platinum (0.12 ± 0.01 nmol/μg protein vs 0.14 ± 0.01 nmol/μg protein).

## 4. Discussion

In the present study, we carried out comparative experiments using LLC-PK1 cell monolayers to demonstrate that cisplatin and oxaliplatin exhibited differential cytotoxic mechanisms in the kidney. It was shown that the basolateral application of cisplatin, but not oxaliplatin, resulted in an increase in paracellular permeability of cell monolayers. Cisplatin but not oxaliplatin induced PKC activation in cells. Although accumulated platinum in cells

reached the same level when cisplatin or oxaliplatin was exposed at 30 and 50 μM, respectively, the hyper-permeability was only observed in cell monolayers treated with cisplatin. In addition, the combination of PKC inhibitors recovered the increase in paracellular permeability induced by cisplatin. These results strongly suggest that pharmacodynamic mechanisms via PKC could explain the difference in nephrotoxicity between cisplatin and oxaliplatin.

Several molecular mechanisms leading to cytotoxicity have been suggested to be involved in platinum-based antitumor agent treatments against tumor cells, such as DNA damage, mitochondrial dysfunction, and signal responses to DNA damage [2,28]. In recent years, it has been reported that PKC is activated in response to cisplatin [29]. We confirmed that cisplatin, but not oxaliplatin, enhanced PKC activity in LLC-PK1 cell monolayers. It was also demonstrated that cimetidine recovered the hyper-permeability, suggesting that cisplatin entered the cells via OCT2 and activated PKC. To distinguish the subtype of PKC, several inhibitors were used in the FITC-dextran flux assay. Sotrastaurin [30,31] and GF109203X [32,33] have been reported to be inhibitors of both classical (α, ßI, ßII, γ) and novel (δ, ε, θ, η) PKC. Both of them made largely recoveries from the hyper-permeability induced by cisplatin exposure. Go6976 [34,35], an inhibitor of classical PKC, showed a weaker inhibitory effect than the others. Although the selectivity of rottlerin in inhibiting the PKCδ isoform has recently been questioned [36-39], rottlerin showed a potent inhibitory effect on cisplatin-induced hyper-permeability in the present study. These results suggest that novel PKC, particularly PKCδ, could be one of major factors in cisplatin-specific nephrotoxicity.

Renal epithelial cells, such as LLC-PK1, show extensive lateral interdigitations between neighboring cells. Evaluation of cell-to-cell connections can be useful as an in vitro cytotoxicity assay. There are several techniques, such as measurements of transepithelial/transendothelial electrical resistance (TEER) and the permeability of labeled

marker molecules, are widely accepted to measure the integrity of tight junction dynamics [40,41]. It has also been reported that the basolateral application of cisplatin significantly reduces the TEER of MDCK-C7 cell monolayers [42]. Similarly, in the present study, cisplatin increased the paracellular permeability of FITC-dextran by PKC kinase activation in LLC-PK1 cell monolayers however, the molecular mechanism underlying hyper-permeability via PKC pathway remains unclear. Oxaliplatin did not increase the paracellular permeability. On the other hand, our previous study has shown that cisplatin and oxaliplatin induces cytotoxicity evaluated by the lactate dehydrogenase release assay in HEK293 cells expressing OCT2 [9]. In addition, oxaliplatin shows more potent cytotoxicity than cisplatin against human tumor cells by conventional cytotoxicity assays [43-45]. These results suggested that oxaliplatin could show a different cytotoxic mechanism(s) from cisplatin in tumor cells and normal tissues. Evaluation of cell-to-cell connections can be useful as in vitro nephrotoxicity assay to measure the cisplatin-induced nephrotoxicity.

In conclusion, cisplatin activated PKC and induced tight junction disruption in renal epithelial cells, while oxaliplatin did not. Pharmacodynamic mechanisms, but not pharmacokinetic mechanisms, may explain the difference in nephrotoxicity between cisplatin and oxaliplatin.

**Author contributions**

Wrote Manuscript: YZ, AY, KM

Designed Research: AY

Performed Research: YZ, AY, SN, SI

Analyzed Data: YZ, AY, DM, TO, TN

## Conflicts of interest

The authors declare no conflicts of interest associated with this manuscript.

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**Figure Legends**

***Fig. 1 Paracellular permeability of FITC-dextran in LLC-PK1 cell monolayers after cisplatin exposure.*** (a) LLC-PK1 cells were incubated in culture medium with 30 μM cisplatin added to the basolateral side for 24 hours at 37°C. The cell monolayers were incubated in the buffer containing 50 μM FITC-dextran added to the basolateral side. The fluorescence intensity of FTIC-dextran on the opposite side was periodically measured. (b) Cell monolayers were incubated in culture medium with 30 μM cisplatin for 1, 2, 6, 10, and 24 hours at 37°C. The cell monolayers were incubated in the buffer containing 50 μM FITC-dextran added to the basolateral side. The fluorescence intensity of FTIC-dextran on the opposite side was measured at 30 min. Each point and each bar represent the mean ± SD of three monolayers. **P<0.01, ***P<0.001, significantly different from control cells.

***Fig. 2 Effect of cisplatin and oxaliplatin on paracellular permeability in LLC-PK1 cell monolayers.*** Cell monolayers were incubated in culture medium with 10, 30, and 50 μM cisplatin or oxaliplatin added to the basolateral side for 24 hours at 37°C. The cell monolayers were incubated in the buffer containing 50 μM FITC-dextran added to the basolateral side. The fluorescence intensity of FTIC-dextran on the opposite side was measured at 30 min. Each bar represents the mean ± SD of three monolayers. *P<0.05, ***P<0.001.

***Fig. 3 Concentration-dependence of platinum accumulation in LLC-PK1 cell monolayers.*** Cell monolayers were incubated in culture medium with 10, 30, and 50 μM cisplatin or oxaliplatin added to the basolateral side for 1 hour at 37°C. The amount of platinum in the cells was determined by ICP-MS. Each bar represents the mean ± SD of three monolayers. **P<0.01, ***P<0.001.

***Fig. 4 PKC kinase activity induced by cisplatin and oxaliplatin.*** LLC-PK1 cell monolayers were incubated in culture medium (a) with 30 cisplatin or oxaliplatin or (b) with 10, 30, and 50 μM cisplatin or oxaliplatin added to the basolateral side for 2 hours at 37°C. PKC kinase activity in cell monolayers was measured. Each bar represents the mean ± SD of four monolayers. **P<0.01, ***P<0.001, significantly different from control cells.

***Fig. 5 Effect of PKC inhibitors on LLC-PK1 cell monolayers treated with cisplatin.*** (a) LLC-PK1 cell monolayers were incubated in culture medium with 30 μM cisplatin added to the basolateral side in the presence or absence of sotrastaurin (AEB071, 5 μM), GF109203X (GFX, 2 μM), Go6976 (3 μM), or rottlerin (ROT, 10 μM) for 24 hours at 37°C. The cell monolayers were preincubated in the incubation buffer for 10 min and then were incubated in the buffer containing 50 μM FITC-dextran added to the basolateral side. The fluorescence intensity of FTIC-dextran on the opposite side was measured at 30 min. (b) Cell monolayers were incubated in culture medium with 30 μM cisplatin added to the basolateral side in the presence or absence of inhibitors for 2 hours at 37°C. PKC kinase activity in cell monolayers was measured. ***P<0.001, significantly different from cisplatin-treated cells.

***Fig. 6 Effect of cimetidine on paracellular permeability and platinum accumulation in LLC-PK1 cell monolayers treated with cisplatin.*** (a) Cell monolayers were incubated in culture medium with 30 μM cisplatin added to the basolateral side in the presence or absence of 1 mM cimetidine for 24 hours at 37°C. The cell monolayers were incubated in the buffer containing 50 μM FITC-dextran added to the basolateral side. The fluorescence intensity of FTIC-dextran on the opposite side was measured at 30 min. Each bar represents the mean ± SD of three monolayers. ***P<0.001, **P<0.01. (b) Cell monolayers were incubated in

culture medium with 30 μM cisplatin added to the basolateral side in the presence or absence of 1 mM cimetidine for 1 hour at 37°C. The amount of platinum in the cells was determined by ICP-MS. Each bar represents the mean ± SD of three monolayers. ***P<0.001.

**Figure 1**

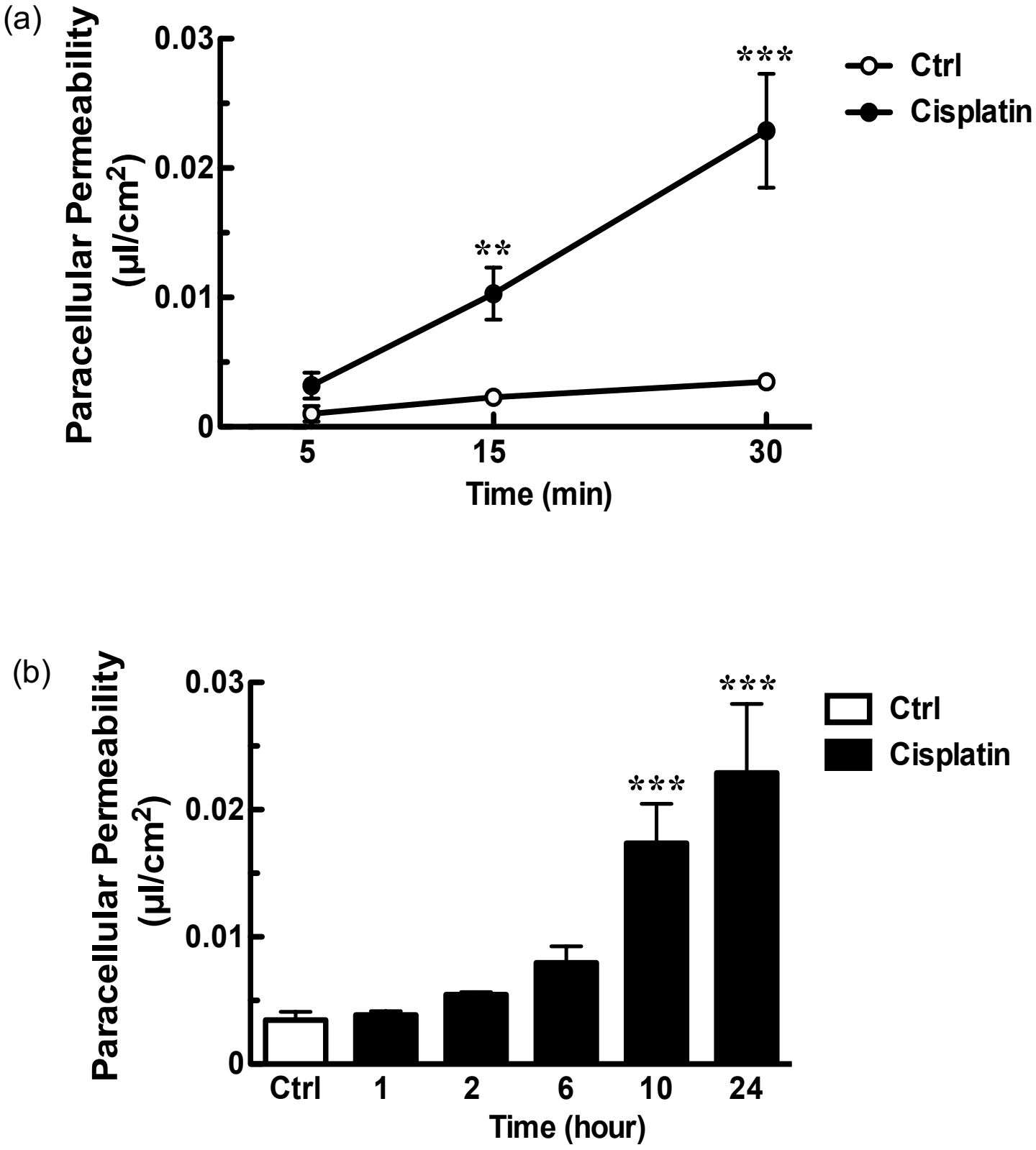

(a)
Paracellular Permeability
(μl/cm2)
0.03
0.02
0.01
0
5
15
30
Time (min)
**
***
Ctrl
Cisplatin
(b)
Paracellular Permeability
(μl/cm2)
0.03
0.02
0.01
0
Ctrl
1
2
6
10
24
Time (hour)
***
***
Ctrl
Cisplatin

**Figure 2**

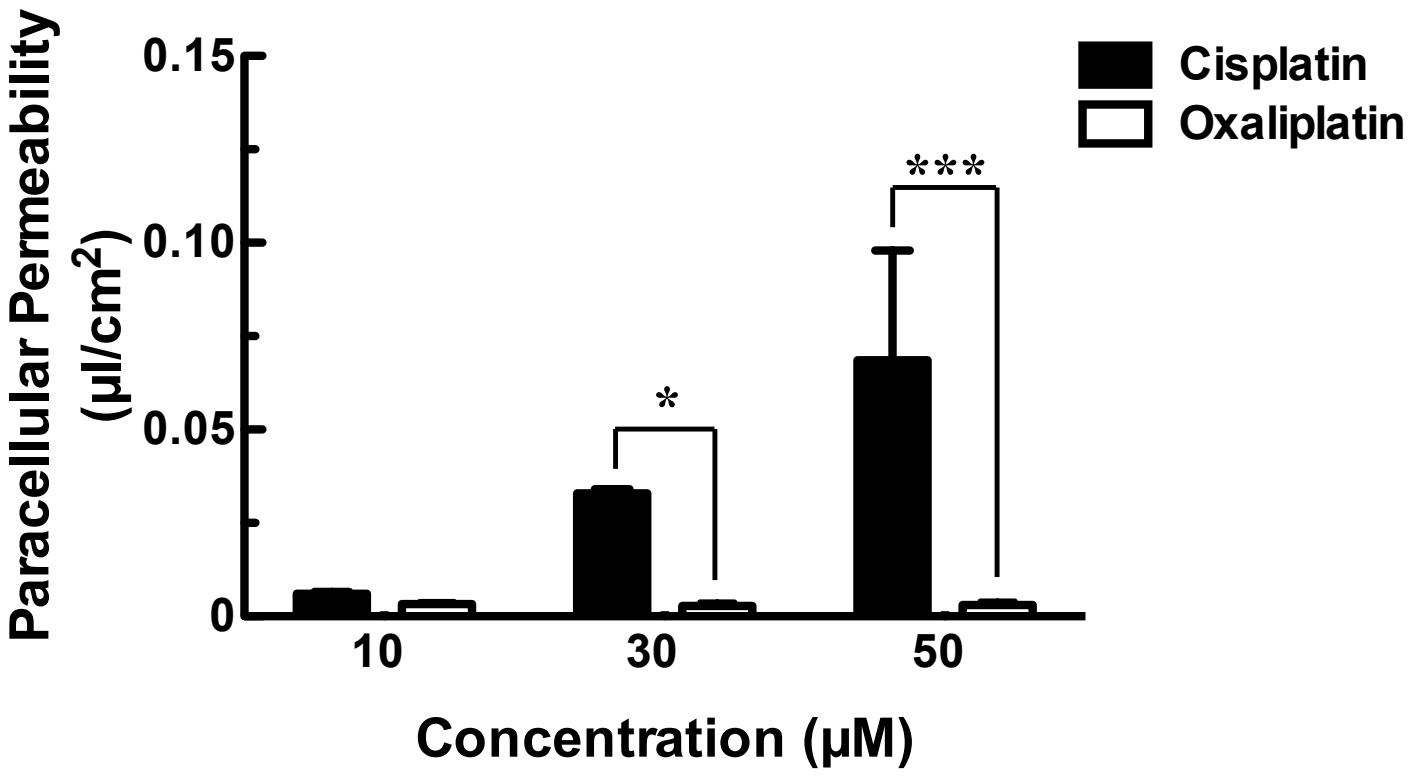

Paracellular Permeability
(μl/cm²)
0.15
0.10
0.05
0
*
***
10
30
50
Concentration (μM)
Cisplatin
Oxaliplatin

**Figure 3**

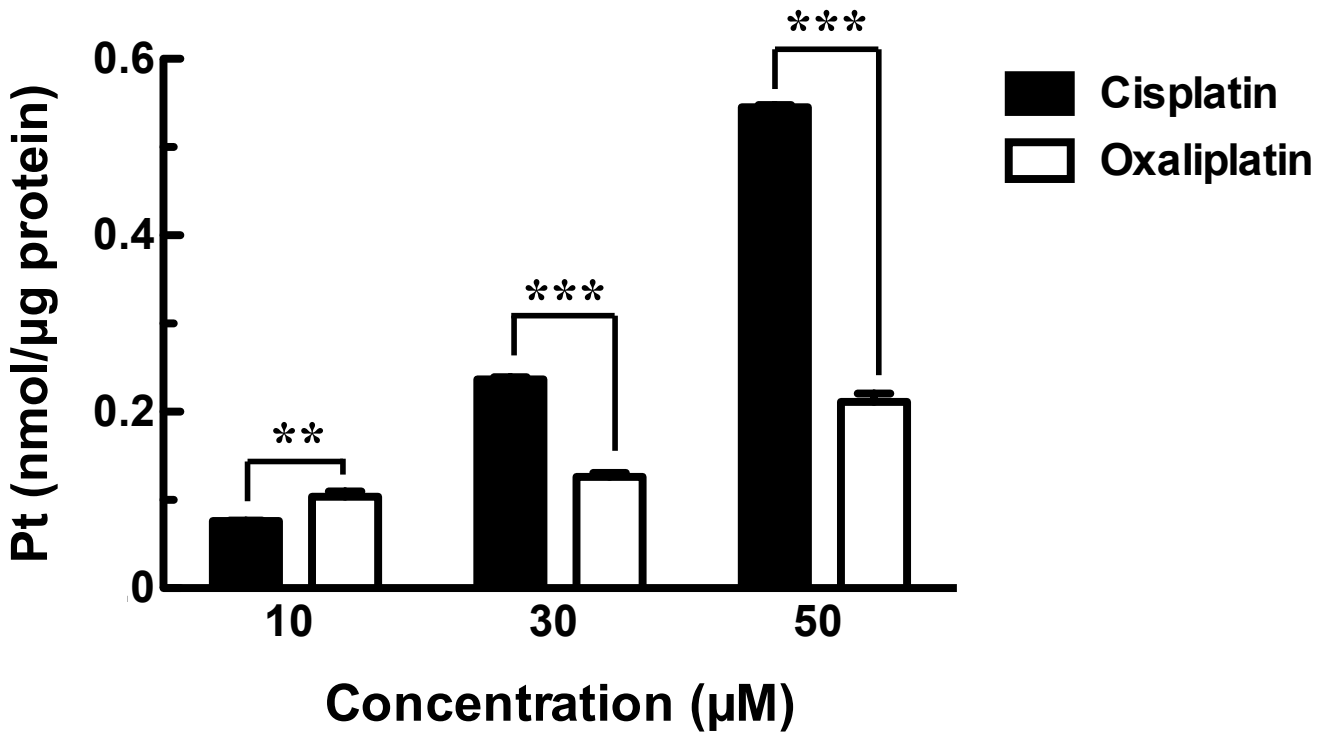

0.6
0.4
0.2
0
Pt (nmol/μg protein)
**
***
***
10
30
50
Concentration (μM)
Cisplatin
Oxaliplatin

**Figure 4**

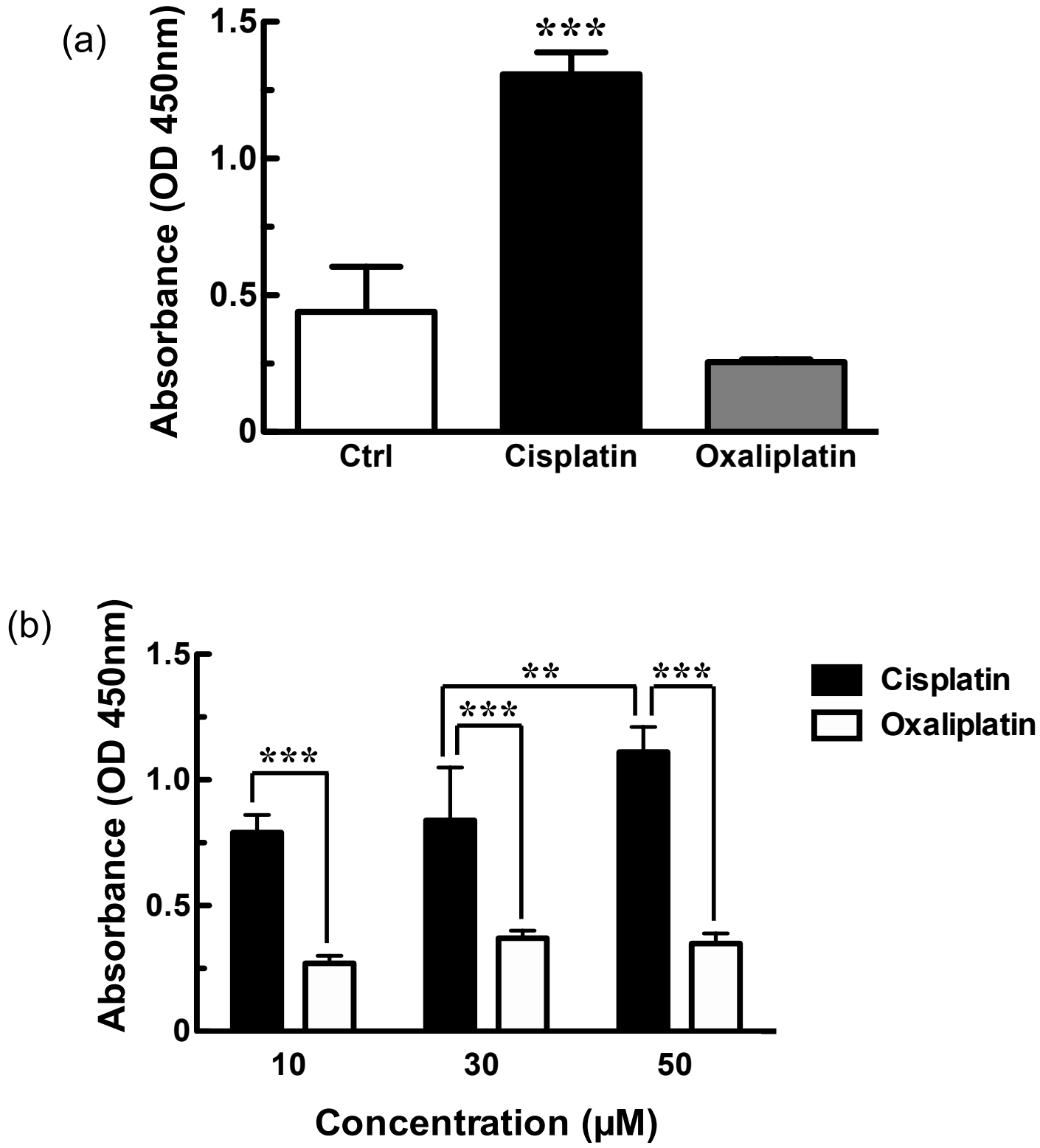
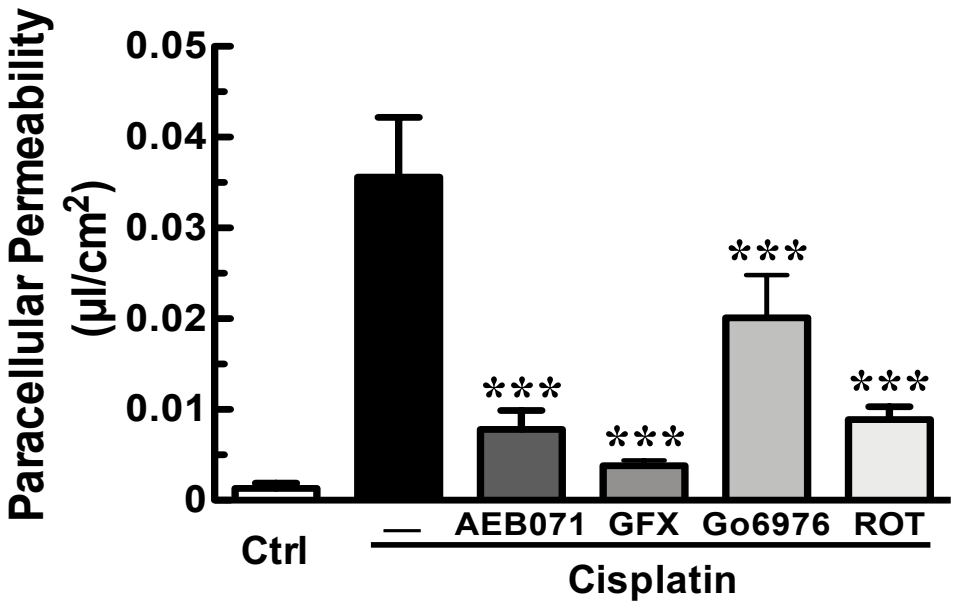

(a)
Absorbance (OD 450nm)
1.5
1.0
0.5
0
***
Ctrl
Cisplatin
Oxaliplatin
(b)
Absorbance (OD 450nm)
1.5
1.0
0.5
0
***
**
***
***
10
30
50
Concentration (μM)
Cisplatin
Oxaliplatin

**Figure 5**

(a)


Paracellular Permeability
(μl/cm²)
0.05
0.04
0.03
0.02
0.01
0
***
***
***
***
Ctrl
—
AEB071
GFX
Go6976
ROT
Cisplatin

(b)

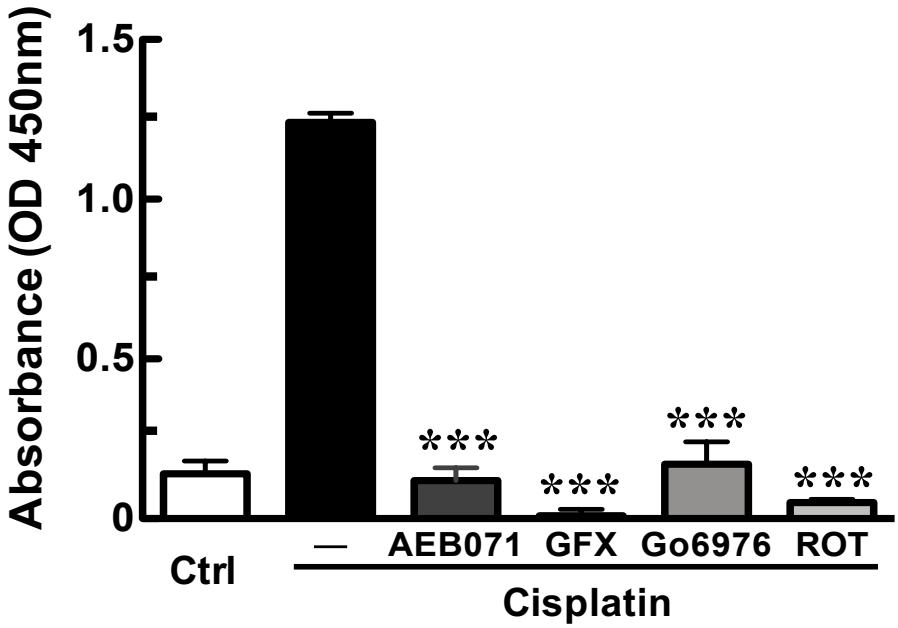
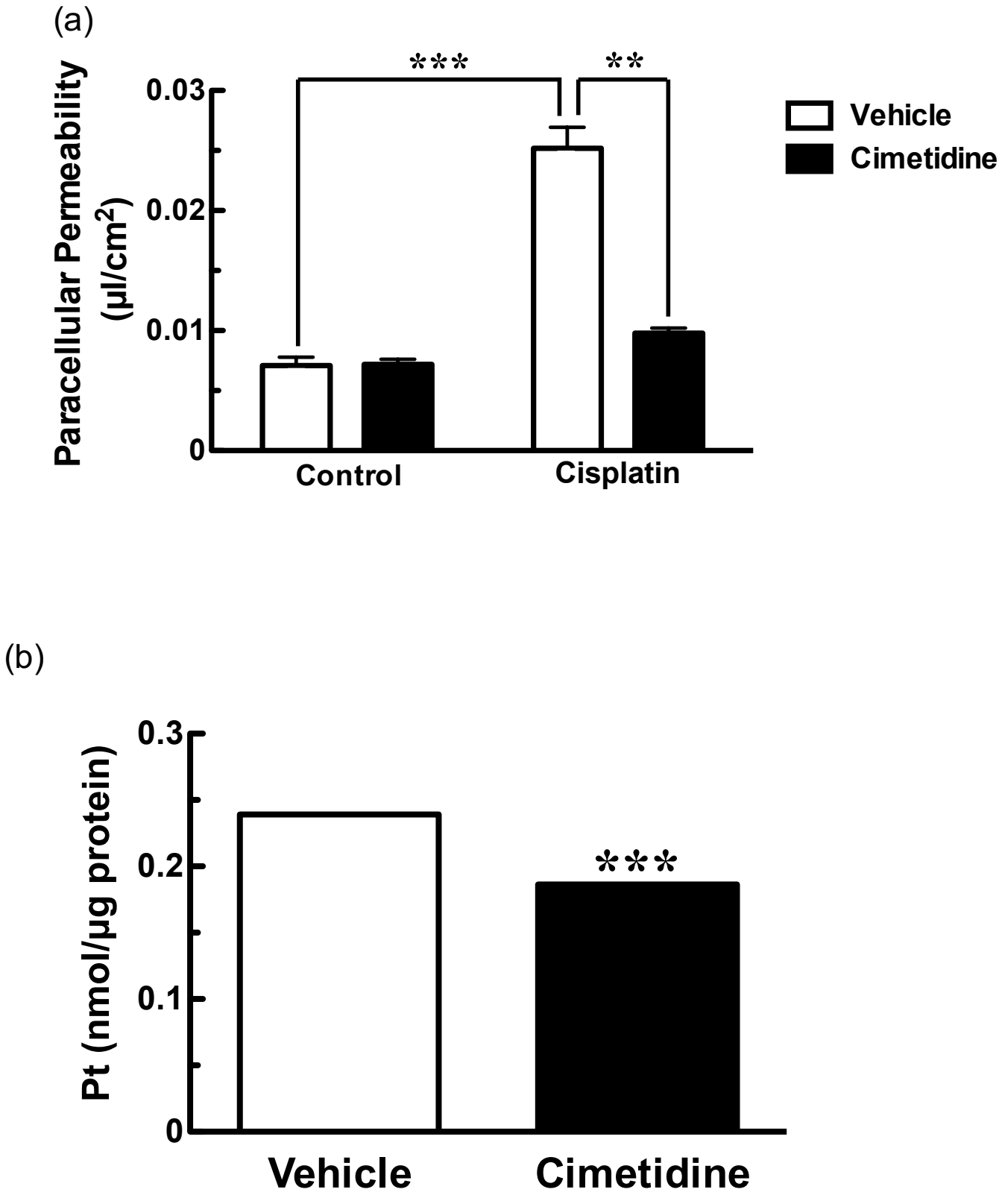

Absorbance (OD 450nm)
1.5
1.0
0.5
0
***
***
***
***
Ctrl
—
AEB071
GFX
Go6976
ROT
Cisplatin

**Figure 6**


(a)
Paracellular Permeability (μl/cm²)
0.03
0.02
0.01
0
***
**
Vehicle
Cimetidine
Control
Cisplatin
(b)
Pt (nmol/μg protein)
0.3
0.2
0.1
0
***
Vehicle
Cimetidine