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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 μ M oxaliplatin could reach the same level at 30 μ M 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 PKC δ 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- μ m pores, 4.71-cm² growth area) inside transwell[®] cell culture chambers (Corning, Cambridge, MA, USA) at a density of 1.8×10^6 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 μ M 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.

89

90 **2.3. FITC-Dextran Flux Assay**

91 Paracellular permeability of FITC-dextran was measured using monolayer cultures grown
92 in transwell® chambers, as described previously with some modification [26]. The cell
93 monolayers were incubated in culture medium with cisplatin or oxaliplatin. After removal of
94 the culture medium from both sides of the monolayers, the cells were incubated with
95 incubation buffer (2 mL each side, pH 7.4 buffer on the basolateral side and pH 6.0 buffer on
96 the apical side) at 37°C for 10 min. Then, 2 mL of incubation buffer (pH 7.4) containing
97 FITC-dextran (50 µM) was added to the basolateral side and 2 mL of incubation buffer (pH
98 6.0) to the apical side. For paracellular flux measurement, the incubation buffer on the apical
99 side was taken at specific time-points (5, 15 and 30 min). The fluorescence intensity of
100 FITC-dextran at 585 nm in the buffer was determined with an excitation wavelength of 485
101 nm using a Mithras LB 940 multimode microplate reader (Berthold Technologies, Bad
102 Wildbad, Germany).

103

104 **2.4. Cellular Platinum Uptake Experiment**

105 For measurement of platinum accumulated in cells, uptake studies were performed as
106 described previously [27]. The cell monolayers were incubated in culture medium with
107 cisplatin or oxaliplatin for 1 hour. After removal of the culture medium from both sides of
108 the monolayers, cells were rapidly washed twice with ice-cold incubation buffer containing
109 3% bovine serum albumin and then washed three times with ice-cold incubation buffer. The
110 filters with monolayers were detached from chambers, the cells on the filters were solubilized
111 in 0.5 mL of 0.5 N NaOH, and the amount of platinum was determined using inductively
112 coupled plasma-mass spectrometry (ICP-MS) (Agilent7700/MassHunter, Agilent
113 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 $\pm$ 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.

139

140 3. Results

141 3.1. *Paracellular Permeability of FITC-Dextran in LLC-PK1 Cell Monolayers after* 142 *Cisplatin Exposure*

143 Paracellular permeability of FITC-dextran was evaluated in LLC-PK1 cell monolayers
144 treated with cisplatin for 24 hours. The basal-to-apical flux of FITC-dextran in monolayers
145 with cisplatin was much larger than that in control monolayers, and its rate was nearly
146 constant up to 30 min (Fig. 1a). The effect of the cisplatin exposure duration was also
147 determined. Compared with the control group, significant increases of FITC-dextran flux at
148 30 min were observed in the cell monolayers treated with cisplatin for 10 and 24 hours (Fig.
149 1b).

150

151 3.2. *Comparative Studies between Cisplatin and Oxaliplatin Using LLC-PK1 Cell* 152 *Monolayers*

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

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

162 PKC activities were measured in LLC-PK1 cell monolayers using a PKC kinase activity
163 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 μ M), GF109203X (GFX, 2 μ M), and rottlerin significantly inhibited the cisplatin-induced hyper-permeability. Go6976 (3 μ M) 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 μ M cisplatin ($31.9 \pm 5.5\%$ vs $41.8 \pm 2.9\%$).

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/ μ g protein vs 0.14 ± 0.01 nmol/ μ g 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 μ M, 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 , β I, β II, 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 PKC δ 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 PKC δ , 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

239 **Conflicts of interest**

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

241

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245

References

- [1] Decatris MP, Sundar S, O'Byrne KJ. Platinum-based chemotherapy in metastatic breast cancer: current status. *Cancer Treatment Reviews* 2004;30:53–81. doi:10.1016/S0305-7372(03)00139-7.
- [2] Pabla N, Dong Z. Cisplatin nephrotoxicity: Mechanisms and renoprotective strategies. *Kidney International* 2008;73:994–1007. doi:10.1038/sj.ki.5002786.
- [3] Okuda M, Saito H, Urakami Y, Takano M, Inui K. cDNA cloning and functional expression of a novel rat kidney organic cation transporter, OCT2. *Biochem Biophys Res Commun* 1996;224:500–7. doi:http://doi.org/10.1006/bbrc.1996.1056
- [4] Urakami Y, Okuda M, Masuda S, Saito H, Inui KI. Functional characteristics and membrane localization of rat multispecific organic cation transporters, OCT1 and OCT2, mediating tubular secretion of cationic drugs. *J Pharmacol Exp Ther* 1998;287:800–5.
- [5] Inui KI, Masuda S, Saito H. Cellular and molecular aspects of drug transport in the kidney. *Kidney International* 2000;58:944–58. doi:10.1046/j.1523-1755.2000.00251.x.
- [6] Sugawara-Yokoo M, Urakami Y, Koyama H, Fujikura K, Masuda S, Saito H, et al. Differential localization of organic cation transporters rOCT1 and rOCT2 in the basolateral membrane of rat kidney proximal tubules. *Histochem Cell Biol* 2000;114:175–80. doi:10.1007/s004180000186.
- [7] Urakami Y, Okuda M, Masuda S, Akazawa M, Saito H, Inui K. Distinct characteristics of organic cation transporters, OCT1 and OCT2, in the basolateral membrane of renal tubules. *Pharm Res* 2001;18:1528–34. doi:10/1023/A:1013070128668.

- 270 [8] Yonezawa A, Masuda S, Nishihara K, Yano I, Katsura T, Inui K-I. Association
271 between tubular toxicity of cisplatin and expression of organic cation transporter
272 rOCT2 (Slc22a2) in the rat. *Biochem Pharmacol* 2005;70:1823–31.
273 doi:10.1016/j.bcp.2005.09.020.
- 274 [9] Yonezawa A, Masuda S, Yokoo S, Katsura T, Inui K-I. Cisplatin and oxaliplatin, but
275 not carboplatin and nedaplatin, are substrates for human organic cation transporters
276 (SLC22A1-3 and multidrug and toxin extrusion family). *J Pharmacol Exp Ther*
277 2006;319:879–86. doi:10.1124/jpet.106.110346.
- 278 [10] Wang D, Lippard SJ. Cellular processing of platinum anticancer drugs. *Nature*
279 *Reviews Drug Discovery* 2005;4:307–20. doi:10.1038/nrd1691.
- 280 [11] Kelland L. The resurgence of platinum-based cancer chemotherapy. *Nat Rev Cancer*
281 2007;7:573–84. doi:10.1038/nrc2167.
- 282 [12] Chaney SG, Campbell SL, Bassett E, Wu Y. Recognition and processing of cisplatin-
283 and oxaliplatin-DNA adducts. *Crit Rev Oncol Hematol* 2005;53:3–11.
284 doi:10.1016/j.critrevonc.2004.08.008.
- 285 [13] Reyland ME. Protein kinase C isoforms: Multi-functional regulators of cell life and
286 death. *Front Biosci (Landmark Ed)* 2009;14:2386–99.
- 287 [14] Banan A, Zhang LJ, Shaikh M, Fields JZ, Choudhary S, Forsyth CB, et al. theta
288 Isoform of protein kinase C alters barrier function in intestinal epithelium through
289 modulation of distinct claudin isotypes: a novel mechanism for regulation of
290 permeability. *J Pharmacol Exp Ther* 2005;313:962–82. doi:10.1124/jpet.104.083428.
- 291 [15] Koizumi JI, Kojima T, Ogasawara N, Kamekura R, Kurose M, Go M, et al. Protein
292 Kinase C Enhances Tight Junction Barrier Function of Human Nasal Epithelial Cells
293 in Primary Culture by Transcriptional Regulation 2008;74:432–42.
294 doi:10.1124/mol.107.043711.

- 295 [16] Groschwitz KR, Hogan SP. Intestinal barrier function: Molecular regulation and
296 disease pathogenesis 2009;124:3–20. doi:10.1016/j.jaci.2009.05.038.
- 297 [17] Boratkó A, Csontos C. PKC mediated phosphorylation of TIMAP regulates PP1c
298 activity and endothelial barrier function. *Biochim Biophys Acta Mol Cell Res*
299 2017;1864:431–9. doi:10.1016/j.bbamcr.2016.12.001.
- 300 [18] Ogasawara N, Kojima T, Go M, Ohkuni T, Koizumi J-I, Kamekura R, et al.
301 PPARgamma agonists upregulate the barrier function of tight junctions via a PKC
302 pathway in human nasal epithelial cells. *Pharmacol Res* 2010;61:489–98.
303 doi:10.1016/j.phrs.2010.03.002.
- 304 [19] Clarke H, Soler AP, Mullin JM. Protein kinase C activation leads to
305 dephosphorylation of occludin and tight junction permeability increase in LLC-PK1
306 epithelial cell sheets. *Journal of Cell Science* 2000;113 (Pt 18):3187–96.
- 307 [20] Zhang J-G, Lindup WE. Cisplatin-induced nephrotoxicity in vitro: increases in
308 cytosolic calcium concentration and the inhibition of cytosolic and mitochondrial
309 protein kinase C. *Toxicology Letters* 1996;89:11–7. doi:10.1016/S0378-
310 4274(96)03776-9.
- 311 [21] Ikeda S, Fukuzaki A, Kaneto H, Ishidoya S, Orikasa S. Role of protein kinase C in
312 cisplatin nephrotoxicity. *Int J Urol* 1999;6:245–50. doi/10.1046/j.1442-
313 2042.1999.00058.x
- 314 [22] Jaiman S, Sharma AK, Singh K, Khanna D. Signalling mechanisms involved in renal
315 pathological changes during cisplatin-induced nephropathy. *European Journal of*
316 *Pharmacology* 2013;69:1863–74. doi:10.1007/s00228-013-1568-7.
- 317 [23] van de Water B, Tijdens IB, Verbrugge A, Huigsloot M, Dihal AA, Stevens JL, et al.
318 Cleavage of the actin-capping protein alpha -adducin at Asp-Asp-Ser-Asp633-Ala by
319 caspase-3 is preceded by its phosphorylation on serine 726 in cisplatin-induced

- 320 apoptosis of renal epithelial cells. *J Biol Chem* 2000;275:25805–13.
- 321 doi:10.1074/jbc.M001680200.
- 322 [24] Pabla N, Dong G, Jiang M, Huang S, Kumar MV, Messing RO, et al. Inhibition of
- 323 PKC δ reduces cisplatin-induced nephrotoxicity without blocking chemotherapeutic
- 324 efficacy in mouse models of cancer. *J Clin Invest* 2011;121:2709–22.
- 325 doi:10.1172/JCI45586.
- 326 [25] Urakami Y, Kimura N, Okuda M, Masuda S, Katsura T, Inui K-I. Transcellular
- 327 transport of creatinine in renal tubular epithelial cell line LLC-PK1. *Drug Metab*
- 328 *Pharmacokinet* 2005;20:200–5. doi:10.2133/dmpk.20.200.
- 329 [26] Matsuo Y, Yano I, Ito T, Hashimoto Y, Inui K. Transport of quinolone antibacterial
- 330 drugs in a kidney epithelial cell line, LLC-PK1. *J Pharmacol Exp Ther*
- 331 1998;287:672–8.
- 332 [27] Yokoo S, Yonezawa A, Masuda S, Fukatsu A, Katsura T, Inui K-I. Differential
- 333 contribution of organic cation transporters, OCT2 and MATE1, in platinum agent-
- 334 induced nephrotoxicity. *Biochem Pharmacol* 2007;74:477–87.
- 335 doi:10.1016/j.bcp.2007.03.004.
- 336 [28] Miller RP, Tadagavadi RK, Ramesh G, Reeves WB. Mechanisms of Cisplatin
- 337 Nephrotoxicity. *Toxins* 2010;2:2490–518. doi:10.3390/toxins2112490.
- 338 [29] Muscella A, Vetrugno C, Antonaci G, Cossa LG, Marsigliante S. PKC- δ /PKC- α
- 339 activity balance regulates the lethal effects of cisplatin. *Biochem. Pharmacol*
- 340 2015;98:29–40. doi:10.1016/j.bcp.2015.08.103.
- 341 [30] Wu X, Li J, Zhu M, Fletcher JA, Hodi FS. Protein kinase C inhibitor AEB071 targets
- 342 ocular melanoma harboring GNAQ mutations via effects on the PKC/Erk1/2 and
- 343 PKC/NF- κ B pathways. *Mol Cancer Ther* 2012;11:1905–14. doi:10.1158/1535-
- 344 7163.MCT-12-0121.

- 345 [31] Hung C-H, Chan S-H, Chu P-M, Tsai K-L. Docetaxel Facilitates Endothelial
346 Dysfunction through Oxidative Stress via Modulation of Protein Kinase C Beta: The
347 Protective Effects of Sotrastaurin. *Toxicol Sci* 2015;145:59–67.
348 doi:10.1093/toxsci/kfv017.
- 349 [32] Marano CW, Laughlin KV, Russo LM, Mullin JM. The protein kinase C inhibitor,
350 bisindolylmaleimide, inhibits the TPA-induced but not the TNF-induced increase in
351 LLC-PK1 transepithelial permeability. *Biochemical and Biophysical Research*
352 *Communications* 1995;209:669–76. doi:10.1006/bbrc.1995.1551.
- 353 [33] Yang J, Zhang K, Wu J, Shi J, Xue J, Li J, et al. Wnt5a Increases Properties of Lung
354 Cancer Stem Cells and Resistance to Cisplatin through Activation of Wnt5a/PKC
355 Signaling Pathway. *Stem Cells Int* 2016;2016:1690896–16.
356 doi:10.1155/2016/1690896.
- 357 [34] Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, et al.
358 Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö 6976. *J*
359 *Biol Chem* 1993;268:9194–7.
- 360 [35] Parmentier JH. Calcium and Protein Kinase C (PKC)-Related Kinase Mediate alpha
361 1A-Adrenergic Receptor-Stimulated Activation of Phospholipase D in Rat-1 Cells,
362 Independent of PKC. *J Pharmacol Exp Ther* 2002;303:1206–15.
363 doi:10.1124/jpet.102.041384.
- 364 [36] Soltoff SP. Rottlerin: an inappropriate and ineffective inhibitor of PKCdelta. *Trends*
365 *in Pharmacological Sciences* 2007;28:453–8. doi:10.1016/j.tips.2007.07.003.
- 366 [37] Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of
367 some commonly used protein kinase inhibitors. *Biochem J* 2000;351:95–105.
368 doi:10.1042/bj3510095.

- 369 [38] Bazuine M, van der Zon GCM, van de Ven R, van den Broek PJA, Antonie Maassen
370 J. Rottlerin inhibits multiple steps involved in insulin-induced glucose uptake in 3T3-
371 L1 adipocytes. *Biochem Pharmacol* 2004;68:105–12. doi:10.1016/j.bcp.2004.02.032.
- 372 [39] Inoki K, Zhu T, Guan K-L. TSC2 mediates cellular energy response to control cell
373 growth and survival. *Cell* 2003;115:577–90.
374 doi:[http://doi.org/10.1016/S00928674\(03\)00929-2](http://doi.org/10.1016/S00928674(03)00929-2)
- 375 [40] Srinivasan B, Kolli AR, Esch MB, Abaci HE, Shuler ML, Hickman JJ. TEER
376 measurement techniques for in vitro barrier model systems. *J Lab Autom*
377 2015;20:107–26. doi:10.1177/2211068214561025.
- 378 [41] Horibe Y, Hosoya K, Kim KJ, Ogiso T, Lee VH. Polar solute transport across the
379 pigmented rabbit conjunctiva: size dependence and the influence of 8-bromo cyclic
380 adenosine monophosphate. *Pharm Res* 1997;14:1246–51.
381 doi:10.1023/A:1012123411343.
- 382 [42] Ludwig T, Riethmüller C, Gekle M, Schwerdt G, Oberleithner H. Nephrotoxicity of
383 platinum complexes is related to basolateral organic cation transport. *Kidney*
384 *International* 2004;66:196–202. doi:10.1111/j.1523-1755.2004.00720.x.
- 385 [43] Rixe O, Ortuzar W, Alvarez M, Parker R, Reed E, Paull K, et al. Oxaliplatin,
386 tetraplatin, cisplatin, and carboplatin: Spectrum of activity in drug-resistant cell lines
387 and in the cell lines of the national cancer institute's anticancer drug screen panel.
388 *Biochem Pharmacol* 1996;52:1855–65. doi:10.1016/S0006-2952(97)81490-6.
- 389 [44] Raymond E, Buquet-Fagot C, Djelloul S, Mester J, Cvitkovic E, Allain P, et al.
390 Antitumor activity of oxaliplatin in combination with 5-fluorouracil and the
391 thymidylate synthase inhibitor AG337 in human colon, breast and ovarian cancers.
392 *Anticancer Drugs* 1997;8:876–85.

393 [45] Faivre S, Res JWPAAAC. Oxaliplatin effects on DNA integrity and apoptosis
394 induction in human tumor cells. Biochem Pharmacol 2003;66:225-237.
395 doi:10.1016/S0006-2952(03)00260-0.
396

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 μ M cisplatin added to the basolateral side for 24 hours at 37°C. The cell monolayers were incubated in the buffer containing 50 μ M FITC-dextran added to the basolateral side. The fluorescence intensity of FITC-dextran on the opposite side was periodically measured. (b) Cell monolayers were incubated in culture medium with 30 μ M cisplatin for 1, 2, 6, 10, and 24 hours at 37°C. The cell monolayers were incubated in the buffer containing 50 μ M FITC-dextran added to the basolateral side. The fluorescence intensity of FITC-dextran on the opposite side was measured at 30 min. Each point and each bar represent the mean $\pm$ 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 μ M cisplatin or oxaliplatin added to the basolateral side for 24 hours at 37°C. The cell monolayers were incubated in the buffer containing 50 μ M FITC-dextran added to the basolateral side. The fluorescence intensity of FITC-dextran on the opposite side was measured at 30 min. Each bar represents the mean $\pm$ 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 μ M 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 $\pm$ SD of three monolayers. ** P <0.01, *** P <0.001.

422

423 ***Fig. 4 PKC kinase activity induced by cisplatin and oxaliplatin.*** LLC-PK1 cell monolayers
424 were incubated in culture medium (a) with 30 cisplatin or oxaliplatin or (b) with 10, 30, and
425 50 μ M cisplatin or oxaliplatin added to the basolateral side for 2 hours at 37°C. PKC kinase
426 activity in cell monolayers was measured. Each bar represents the mean $\pm$ SD of four
427 monolayers. **P<0.01, ***P<0.001, significantly different from control cells.

428

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

439

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

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

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6