Controversial Role of Gamma-Glutamyl Transferase Activity in Cisplatin Nephrotoxicity

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Summary

Nephrotoxicity of chemotherapeutics is a major hindrance in the treatment of various tumors. Therefore, test systems that reflect mechanisms of human kidney toxicity are necessary. Cell culture based systems have to be developed to reduce animal testing. Renal proximal tubular epithelial cells are of specific interest in this regard, as they reabsorb substances from human primary urine filtrates and thus are exposed to urinary excreted xenobiotics and are a major target of cisplatin toxicity. While animal studies using gamma glutamyl transferase (GGT) knock-out mice or GGT inhibitors show that GGT activity increases kidney toxicity of cisplatin, the use of various cell models gives contradictory results. We therefore used a cell panel of immortalized human renal proximal tubular epithelial (RPTECs) cell lines differing in GGT activity. Low GGT activity resulted in high cisplatin sensitivity, as observed in RPTEC-SV40 cells or after siRNA mediated knock-down of GGT in RPTEC/TERT1 cells that have high GGT activity. However, the addition of GGT did not rescue but also increased cisplatin sensitivity, and adding GGT inhibitor as well as substrate (glutathione) or product (cysteinyl-glycine) of GGT resulted in decreased sensitivity. While our data suggest that the use of cell panels is of value in toxicology and toxicogenomics, they also emphasize the complex interplay of toxins with the intracellular and extracellular microenvironment. We hypothesize that especially epithelial barrier formation and polarity of RPTECs need to be considered in toxicity models to validly predict the *in vivo* situation.

Keywords: GGT, nephrotoxicity, RPTEC/SV40, RPTEC/TERT1, HEPTEC/TERT

1 Introduction

Development of novel drugs is very cost intensive and time consuming. About one third fails due to toxicological concerns or lack of clinical safety (Kola and Landis, 2004; Jiang et al., 2007). Therefore, suitable model systems for reliable pre-clinical testing are of major importance to validly predict clinical safety and can reduce the costs of bringing new products to the market. The 3R principles to reduce animal testing were first formulated in 1959 (Russell and Burch, 1959) and efforts to this effect were intensified with the European Directive 86/609/EEC and its revision 2010/63/EU. Still the need for *in vitro* toxicity prediction systems remains urgent (Li, 2004; Dorato and Buckley, 2007). Apart from reducing the need for animal testing, such test systems can help us understand the mechanisms of drug toxicity and will support high throughput screening approaches

(Davila et al., 1998; Hartung, 2001; Dorato and Buckley, 2007; Astashkina et al., 2012).

Nephrotoxicity testing is of special interest as several compound groups, including aminoglycoside antibiotics, calcineurin inhibitors or platinum compounds, are known to be specifically toxic to the kidney. Further, an estimated 18-27% of acute kidney failures are caused by drugs (Loghman-Adham et al., 2012). This indicates that the test systems currently in use only cover certain aspects of nephrotoxic side effects. The major cell type that is hit by nephrotoxic substances is the renal proximal tubular epithelial cell (RPTEC) (Rush et al., 1984; Boogaard et al., 1990). These cells are involved in the blood clearance mechanism and resorption of essential metabolites (Smith et al., 2006) and are exposed to a high blood flow. Therefore, RPTECs seem to be the most appropriate cells for nephrotoxicity model systems (Li et al., 2006 and reviewed by Boogaard et al., 1990).

So far, several RPTEC lines have been established from various organisms.

The well characterized porcine cell line LLC-PK1 (Hull et al., 1976) and rodent primary cells have been used to test cytotoxicity of chemicals and to predict nephrotoxicity. However, these models are limited by the fact that cells from different species may show different responses to the same drug and to other factors such as oxidative stress (Dietrich et al., 2001; Li, 2004; Parinello et al., 2003), therefore the predictivity of animal cells for human cell responses is clearly limited.

Thus, human primary cells were assessed as model systems (Boogaard et al., 1990; Cummings et al., 2000; Li et al., 2006; Brown et al., 2008). Even though very valuable, these models are hampered due to limited population doublings, resulting in the need for large amounts of human tissue samples from which the cells must be prepared and therefore high variability in RPTEC cell behavior between isolations from different donors. Also, the human kidney cell line HK-2, immortalized by a recombinant retrovirus containing the human papilloma virus E6/E7 genes, has been used, but controversial results concerning this cell line's RPTEC-like characteristics, including drug transporter expression, have been published (Ryan et al., 1994; Wieser et al., 2008; Jenkinson et al., 2012). Moreover, for all cell lines used for toxicity testing so far alterations of the primary cell-like characteristics due to viral oncogenes used for immortalization were noted and are seen as major shortfalls (Stacey and Hartung, 2006), even though the use of conditional SV40 in combination with telomerase seems promising (Wilmer et al., 2010). Still, telomerase alone is regarded as the most appropriate strategy to immortalize cells without altering the primary cell-like key functionalities, and a large variety of different human cells have been successfully immortalized using hTERT (Bodnar et al., 1998; Chang et al., 2005). We have previously reported that introduction of hTERT alone is sufficient for immortalization and does not change the functional characteristics of epithelial cells of renal proximal tubules (Wieser et al., 2008). However, it may prove useful to employ a panel of cell lines originating from more than one individual to represent different genetic backgrounds and so improve predictivity.

In order to test the value of *in vitro* nephrotoxicity model systems, we have chosen cisplatin, a widely used chemotherapeutic agent against cancers of the testis, cervix, head, neck and lung (Kartalou and Essigmann, 2001; Townsend, 2003; Dos Santos et al., 2012). Moreover, its applicable dose is limited by an extremely narrow ratio of nephrotoxicity to therapeutic effect (Li et al., 2006). An important, but ambiguous, role in cisplatin toxicity has been attributed to GGT, the enzyme that catalyzes the first step of GSH degradation and produces cysteinylglycine (cysgly). GGT is essential for the recycling of cysteine from the extracellular space and provides the cell with cysteine required for *de novo* synthesis of intracellular GSH. However, even if *in vivo* models of GGT knockout mice and the use of GGT inhibitors in mice favor a role of GGT in toxification of cisplatin, results from *in vitro* models do not yet match the *in vivo* results. Specifically, some reports show toxic effects caused by metabolism of cisplatin through

a pathway including GGT (Hanigan et al., 2001), whereas others see GGT as the main detoxification mechanism, where the direct GGT product cysgly or the GGT substrate glutathione (GSH) are covalently attached to cisplatin, rendering it non-toxic (Daubeuf et al., 2002, 2003; Paolicchi et al., 2003).

Here we report the characterization of a cell panel of RPT-ECs derived from different individuals and immortalized by different strategies in regard to their sensitivity to cisplatin. We show a surprising modulation of the cytotoxic effects of cisplatin by GGT, GSH, cysgly and OU749. We also show the effect of siRNA knockdown of the active GGT variant. Taken together, we suggest that GGT plays a complex role in cisplatin detoxification with interactions of the intra- and extracellular environment of cells playing a critical role. In addition, we propose that the novel human model cell lines tested here can be applied for studying GGT related nephrotoxic mechanisms as a cell panel, and propose that such cell panels might be useful for the prediction of nephrotoxicity and mechanisms of toxicity. However, limitations in classical two-dimensional cell culture became apparent from these results and suggest that cultivation conditions that mimic luminal and basolateral sides are necessary especially for polar epithelial cells.

2 Materials and methods

Cell culture conditions

All cell lines used within this study were cultivated at 37°C in a humidified atmosphere containing 7% CO₂. Confluent monolayers were passaged with a split ratio of 1:2-1:4 depending on the cell line. The cells were detached using 0.1% trypsin/0.02% EDTA (Sigma), which was inactivated by addition of soybean trypsin inhibitor (1 mg/ml, Sigma), followed by a centrifugation step (5 min, 170xg), and transfer of the resuspended cells to new culture vessels.

Commercial cell lines

RPTEC/TERT1 cells (Evercyte GmbH) and HEPTEC/TERT1/2 cells (Evercyte GmbH) were cultivated in ProxUp and ProxUp-2 medium (Evercyte GmbH), respectively. Prior to assays, cells were adapted to Optipro SFM™ (Invitrogen). HK-2 cells (ATCC) and RPTEC/SV40 cell lines were maintained in Optipro SFM™ (Invitrogen).

Cell line establishment

The study was approved by the local ethics commission (General Hospital of Vienna, Austria) and donors gave informed consent before providing urine samples. Thus, the study was performed in accordance with the Declaration of Helsinki.

Normal human renal proximal tubular epithelial cells (nRPT-ECs) and human exfoliated proximal tubular epithelial cells (HEPTECs) were isolated from urine samples as described in detail previously (Wieser et al., 2008, 2014) and cultivated in ProxUp-Pri medium (Evercyte GmbH).

nRPT-ECs were transfected with a plasmid encoding SV40 early region (Voglauer et al., 2005) using Lipofectamin 2000 (Invitrogen) according to the manufacturer's instructions. Non-

transfected cells were overgrown by SV40 early region expressing cells and these cells were further propagated as mass culture designated RPTEC-SV40 as described in detail previously (Fliedl et al., 2014).

For immortalization of HEPTECs, cells from 2 different donors were transduced with retroviruses encoding the catalytic subunit of human telomerase as described (Wieser et al., 2014). Twenty-four hours post transduction positively transduced cells were selected using 100 μ g/ml G418 (Invivogen) and arising clones were cultivated as mass culture designated HEPTEC/TERT1 and HEPTEC/TERT2.

GGT assay

The protocol for determination of GGT activity was adapted from Meister et al. (1981). In brief, cells were cultured in 12-well plates to full confluence and incubated with substrate (1 mM G-glutamyl-para-nitroanilide, 60 mM Tris-HCl pH 8.0 and 20 mM glycyl-glycine) for 20 min at RT. The enzymatic process was stopped by addition of 10% acetic acid (1:2). Para-nitroanilide release was determined with Tecan Infinite M200 at 405 nm. Values were normalized to cell number as determined by Vicell. All tests were performed with biological and technical triplicates. For biological replicates, cells were grown and treated separately and technical replicates were three parallel measurements at the end of the protocol. While technical replicates were only used to evaluate stability of measurement, biological replicates were used to evaluate standard deviation. For supplementation experiments GGT (Sigma Aldrich; stock solution 10 units/ml in 0.2 M 2-amino-2-methyl-1,3-propanediol buffer pH 8.5) or OU749 (Cayman Chemical; stock solution 50 mM in DMSO) were used in the concentrations indicated there.

Cytotoxicity assay

Cells were grown in 96-well plates. Before reaching confluence (approximately 90-95%) they were incubated with the indicated concentrations of cisplatin (stock solution 3.33 mM in DMSO) for 24 h followed by addition of 10 μ l MTT (5 mg/ml) per well for 4 h. After solubilization of formazan by SDS in 0.1 M HCl results were measured with Tecan Infinite M200 at 570 nm and

reference value at 690 nm. For supplementation experiments cisplatin was coincubated with 1.5 mM of cysteinylglycine (Sigma Aldrich; 6 mM in PBS), 833 μ M GSH (Sigma Aldrich; 5 mM in PBS), 1.66 U/ml GGT or 200 μ M OU749 for 2 h before addition to the cells. Vehicle controls were added accordingly in the respective assays. All tests were performed with biological triplicates, which were used to evaluate standard deviation. For calculation, values were standardized using the appropriate controls.

GGT knockdown

Cells were seeded 24 h prior to transfection with siRNA to reach approximately 50% confluence. For specific knock-down a pool of 4 pre-designed IBONI® siRNA (ribocxx Life sciences) targeting GGT1 or GGT7 were used at a final concentration of 20 nM as recommended by the manufacturer. Transfection was done according to the manufacturer's protocol using ribocxxfect100 (ribocxx Life sciences). GGT assay and cytotoxicity assays were performed 48 h after transfection.

RNA isolation, quantification and quality control

Total RNA was isolated using Tri reagent (Sigma) according to the manufacturer's instructions. RNA concentration was determined by absorbance measurement on a Nanodrop ND-1000 spectrophotometer.

QPCR

For quantification of mRNA expression, 500 ng of total RNA were reverse transcribed using the Dynamo cDNA synthesis kit (Fynnzymes). Quantitative real-time PCR was performed on a Rotor-Gene real-time cycler (Corbett Research) using 5x HOT FIREPol EvaGreen® qPCR Mix Plus (NO ROX) as indicated by the manufacturer. For quantification, serial dilutions of a standard were included in each run. Copy numbers were calculated after dynamic tube normalization and slope correction. Expression values were normalized to GAPDH and indicated as fold changes relative to control.

Primers

See Table 1

Tab. 1: Primers used in the study

target	Accession number	forward	reverse
ANPEP	NM_001150.2	GTCCAGGGTCCAGGTTCCA	CTCCAACAGGCGAAGGTCAC
CCBL1	NM_004059.4	TTGGGAACGAGGGGTGAAGC	TTCCACGGCAAAGTCTGGTG
CCBL2	NM_001008661.2	CGCACCGGATAGCATGTTTTT	GGTCTGCAGCCAATTTGGTAA
SCLC22A2	NM_003058.3	GACCGCATCGGACGCCGTTA	ACCAAGCCAAGCACGCCGAA
GGT1	NM_001032364.2	CTGCTGGACGAGCCATGAAG	CACACAACAGGGCTGCAATG
GGT7	NM_178026.2	CAATTACAGCGCCCTTGTGG	TGTTCTCGGGATACCAGGCT

Data analysis

All data were analyzed with GraphPad Prism. 4-parameter fit curves were used to calculate the IC_{50} values of cytotoxicity assays. Significance for comparison of IC_{50} values was calculated using t-tests. For evaluation of significance of GGT assays one-way ANOVA was used.

3 Results

3.1 GGT determines cisplatin sensitivity in different immortalized human PTECs

In order to test if different proximal tubular cell models (nRPTECs, HK-2, RPTEC/TERT1, RPTEC/SV40, HEPTEC/TERT1, HEPTEC/TERT2) respond differently to substances of known nephrotoxicity, we challenged them with cisplatin and measured the resulting cytotoxicity using the MTT assay (Mosmann, 1983; Daubeuf et al., 2003). The IC_{50} was between 200 and 450 μM in all cell lines except for one: RPTEC/SV40 had a lower IC_{50} value of 80 μM , demonstrating a higher sensitivity to cisplatin (Fig. 1).

Since GGT has been reported as a key enzyme in the cisplatin metabolizing mechanism, we analyzed GGT activity in all cell lines. Indeed, we observed a difference in GGT activity between RPTEC/SV40 and the other cell lines: While all other cell lines produced para-nitroanilide (pNA) levels of

0.25–0.56 nmol/min/ 10^5 cells, the RPTEC/SV40 cell line only reached 0.03 nmol/min/ 10^5 cells (Fig. 2).

In order to test whether cells are sensitized to cisplatin by reduction of GGT, we performed siRNA knock-down of GGT in RPTEC/TERT1 cells. Expression of GGT variants was analyzed by conventional PCR in the cell panel as well as in kidney tissue (data not shown). siRNAs against the expressed GGT variants (GGT1 and GGT7) were used for specific knock-down. In GGT assays, the effects of untransfected control-siRNA, GGT1-siRNA and GGT7-siRNA were compared 48 h after transfection of RPTEC-TERT1. Negative control and GGT7 showed no effect, whereas GGT activity was decreased by 37% by siRNA-GGT1 (Fig. 3A). When cisplatin was added to siRNA-GGT1 transfected cells, the sensitivity to cisplatin was increased, as was expected from the results with the low GGT active RPTEC/SV40 cell line. The 37% reduction in GGT by siRNAs also led to an around 40% decrease in the IC_{50} (Fig. 3B).

3.2 GGT in the extracellular space increases cisplatin sensitivity

After demonstrating that knock-down of GGT increased cisplatin sensitivity, we expected that more GGT activity would decrease the sensitivity. GGT is a membrane bound enzyme with extracellular activity. We therefore supplemented cultures of the low GGT/highly sensitive RPTEC/SV40 cell line with purified GGT. GGT activity was observed after its addition to the supernatants

Fig. 1: Viability curves of human renal cell lines treated with different concentrations of cisplatin

Viability curves of human renal cell lines treated with different concentrations of cisplatin were determined by MTT assay and the IC_{50} values were calculated as 4 parameter fit curves. (A) nRPTECs (B) RPTEC/TERT1 (C) RPTEC/SV40 (D) HK-2 (E) HEPTEC/TERT1 (F) HEPTEC/TERT2. Biological triplicates were used to evaluate standard deviation.

Fig. 2: GGT activity of the human renal cell line panel

GGT activity of all human renal cell lines in our panel. Biological triplicates were used to evaluate standard deviation.

***, $p < 0.05$ vs. other cell lines.

Fig. 3: Effects of siRNA treatment on GGT activity and cisplatin toxicity to RPTEC-TERT1

(A) GGT assay after siRNA transfection of RPTEC-TERT1. ***, $p < 0.05$ vs. ctr-siRNA;

(B) effect of GGT1-siRNA on cisplatin toxicity on RPTEC-TERT1. ***, $p < 0.05$.

Fig. 4: Influence of GGT enzyme on GGT activity and cisplatin toxicity to RPTEC-SV40

(A) GGT assay showing the increased GGT activity caused by addition of the indicated units of the enzyme to RPTEC-SV40.

***, $p < 0.05$ vs. ctr. (B) MTT assay of cisplatin treated cells after pre-incubation of RPTEC-SV40 with 1.66 units/ml GGT.

Biological triplicates were used to evaluate standard deviation. ***, $p < 0.05$.

in a dose dependent way (Fig. 4A). When RPTEC-SV40 were incubated with 1.66 units/ml of GGT it resulted in higher activity, similar to RPTEC/TERT1. Surprisingly, however, GGT addition did not rescue from cisplatin sensitivity, but rather slightly increased it by decreasing the IC₅₀ by about 35% (Fig. 4B).

In order to test if GGT inhibition would decrease sensitivity of the cells, we used the recently described specific GGT inhibitor OU749 (King et al., 2009). The GGT assay showed a dose dependent inhibition of GGT activity by OU749 using either RPTEC-SV40 (Fig. 5A) or RPTEC-TERT1 (Fig. 5C). Preincubation of cells before cisplatin treatment indeed rescued from cisplatin sensitivity by more than 200-fold in RPTEC/SV40

(Fig. 5B), i.e., the IC₅₀ of RPTEC/SV40 cells was increased from 51.2 to around 10,000; even more striking RPTEC/TERT1 cells were completely rescued from cisplatin toxicity over the time period tested (Fig. 5D).

In order to test if substrate or product of GGT would have corresponding effects to overexpression or inhibition of GGT, we tested addition of GSH or cystyly before addition of cisplatin. When GSH was used for pre-incubation before cisplatin addition, a markedly decreased sensitivity of the cell line by more than 3.5 fold was observed (Fig. 6A). This protective effect is in line with published data. A consequence of an unbalanced high GGT activity in the extracellular space might be deple-

Fig. 5: Effect of GGT inhibitor OU749 on GGT activity and cisplatin toxicity of RPTEC-SV40 and RPTEC-TERT1

Fold change of GGT activity of RPTEC-SV40(A) and RPTEC-TERT1(C) treated with the indicated concentrations of OU749.

***, $p < 0.05$ vs ctr. Effect of 200 μM OU749 on cisplatin toxicity in RPTEC-SV40(B) and RPTEC-TERT1(D). Biological triplicates were used to evaluate standard deviation. ***, $p < 0.05$.

Fig. 6: Influence of substrate and product of GGT on cisplatin toxicity

MTT assay of cisplatin treated RPTEC-SV40 cells after pre-incubation with (A) 833 μM glutathione (GSH) and (B) 1.5 mM cysteinylglycine (cysgly). Biological triplicates were used to evaluate standard deviation. ***, $p < 0.05$.

tion of GSH resulting in increased sensitivity. Pre-incubation of the cells with cystgly resulted again in a decreased cisplatin sensitivity and a more than 6-fold increase in IC_{50} from 87.42 to 596.1 (Fig. 6B). Thus, blocking of GGT would also block cystgly formation and thus contribute to cisplatin sensitivity. These data would suggest that both GSH and the GGT product cystgly are capable of detoxifying cisplatin, while “unbalanced” addition of GGT to the supernatants might deplete GSH by its activity, but without supplying sufficient cystgly that might be further processed.

Furthermore, we tested mRNA levels of the following proteins possibly involved in the toxicity of cisplatin by qPCR: aminopeptidase N (ANPEP) is expressed in various cell types including the renal tubuli, cleaving peptides to enable cellular uptake; cystein-conjugate beta-lyase (CCBL) is an intracellular enzyme which metabolizes cysteine conjugates of certain halogenated alkenes and alkanes which may lead to formation of reactive metabolites causing nephron- and neurotoxicity and occurs in two variants; organic cation transporters OCT-2 (SLC22A2) in the kidney and other organs are responsible for excretion of many organic cations, drugs and environmental toxins. No correlation was observed between the mRNA expression of these proteins and the different sensitivity to cisplatin noted for the cell panel (Fig. S1 in supplementary file on <http://www.altex-edition.org>).

Finally we evaluated the possible influence of growth rate on the cytotoxicity assays used. Growth rates were calculated from at least 6 serial passages. No significant differences in growth rates were noticed (Fig. S2 in supplementary file on <http://www.altex-edition.org>).

4 Discussion

Nephrotoxicity of cisplatin is a clinically important problem during chemotherapy. Toxicity to renal proximal tubular epithelial cells is a major factor in cisplatin nephrotoxicity and an enzyme specific for this cell type, GGT, has been discussed as a key enzyme in nephrotoxicity. Similarly, GGT seems involved in increasing resistance of tumors to cisplatin treatment.

However, reported results are controversial, claiming GGT activity either to enhance or to decrease cisplatin sensitivity of the kidney and/or RPTECs. We used a renal cell panel to test the potential of the cell lines as predictors of nephrotoxicity in order to elucidate the role of GGT. However, our experiments also result in controversial data, emphasizing that the simple question whether GGT enhances or decreases cisplatin toxicity in human RPTECs has a complex answer.

We here observed that one of the cell lines used, RPTEC-SV40, showed an increased sensitivity to cisplatin. This increased sensitivity was paired with a reduced GGT activity, which was also unique for RPTEC-SV40 compared to other cell lines. In keeping with this, the high GGT active RPTEC/TERT1 cell line became more sensitive after siRNA mediated knock-down of GGT. The cell culture media was then complemented with the product of GGT, cystgly, which also rescued

from increased sensitivity. These data would suggest that GGT is a “good” activity involved in detoxifying cisplatin, and are consistent with previously published proposed mechanisms of GGT-dependent resistance to cisplatin (Daubeuf et al., 2002, 2003; Paolicchi et al., 2003). These proposed mechanisms were all based on findings using 2D cell cultures with cell lines that had varying degrees of similarity with RPTECs down to overexpression of GGT in HeLa cells. These findings also correlate with studies of different tumor and tumorigenic cell lines that have an increased GGT activity and a reduced sensitivity to cisplatin (Godwin et al., 1992; Fan et al., 1994).

In our attempts to more carefully study the mechanistic influence of GGT on proximal tubular epithelial cells, however, we also performed experiments adding purified GGT to the cell supernatants, and inhibiting GGT activity by addition of a specific inhibitor, OU749. Surprisingly, and in contrast to the siRNA experiment described above, GGT addition increased the sensitivity to cisplatin, and its inhibition decreased it, suggesting that GGT is the “bad” activity that activates and enhances cisplatin nephrotoxicity. Again, other recently published work also supports such an interpretation: A very strong indication that GGT is a damaging agent during cisplatin treatment came from GGT knock-out mice, where kidney failure and mortality after cisplatin treatment were observed at lower cisplatin doses (Hanigan et al., 2001), similar to when acivicin was employed as a GGT inhibitor (Hanigan et al., 1994). These results led Hanigan and co-workers to the conclusion that cisplatin is successively metabolized by GGT, dipeptidase and finally cystein-conjugate beta-lyase, finally forming an active thiol that causes nephrotoxicity (Townsend, 2003; Zhang and Hanigan, 2003). Paolicchi et al. (2003) reinterpreted these findings, as it had already been shown that systemic inhibition of GGT by acivicin lead to increased plasmatic levels of GSH in mice (Griffith and Meister, 1979). This finding was corroborated in GGT knockout mice (Lieberman et al., 1996). Therefore, an alternative explanation for the protective effect in GGT knockout or inhibited mice can be the plasmatic accumulation of GSH, which can offer direct protection (Paolicchi et al., 2003), and is also visible in our model system where addition of GSH is protective. A question that remains to be answered in this scenario would be whether the cisplatin is detoxified in the plasma before it can kill the tumor cells. In addition, an increase in cytotoxicity by addition of exogenous GGT might also come from depletion of GSH by GGT, as addition of GSH indeed also decreases sensitivity to cisplatin in our model systems, similar to the previous proposed models (Daubeuf et al., 2003; Paolicchi et al., 2003 and commentary of Pompella et al., 2006).

Thus, the paradoxical result of increase in cisplatin toxicity when purified GGT is exogenously added into the supernatant of RPTEC cells, could also be a result of increased oxidative burden caused by GGT (Dominici et al., 2005), even though it was published that overexpression of GGT and consequently higher activity can offer resistance to oxidative stress. Since the increased GGT expression was associated with higher expression levels of catalase (Giommarelli et al., 2008), the higher resistance of intracellular GGT overexpression might be indi-

Fig. 7: Models for mechanism of cisplatin toxicity

(A) Model for 2D cell culture, similar to the previously proposed models, but now including a transporter for intact GSH (GSH-T) that is inhibited by a product of GGT, γ -glutamyl-X. All enzymes are in close proximity to each other and not in separate compartments. (B) *In vivo* 3D model: RPTECs are polar and form tight layers and in contrast to the 2D model GSH-T is located on the basolateral side, while GGT and the dipeptidase are on the luminal side. The different spatial organization as represented in the two models explains why contradictory results can be obtained depending on the model system used. Glutathione (GSH), γ -glutamyltransferase (GGT), cysteinylglycine (cysgly), DiPEP (dipeptidases)

rectly due to catalase activity and oxidative stress resistance. With addition of exogenous GGT the catalase expression would be unchanged and therefore our results might also be interpreted as a consequence of increased oxidative stress, in line with the results of Giommarelli et al. (2008).

How can such controversial data be explained and can GGT now be pinned down as “good” (helping to detoxify) or “bad” (increasing sensitivity of RPTECs) for kidney cells?

The cell culture models used so far all were performed in classical 2D cell culture. If we include data showing that intact GSH is transported into cells by the specific transporter GSH-T (Lash and Jones, 1983; Lash et al., 2007), which is inhibited by γ -glutamyl-X, a product of GGT (Lash and Jones, 1984; Hagen et al., 1988), we might suggest a process as outlined in Figure 7A. Thus, in RPTECs with low GGT activity (by siRNA or after SV40 immortalization), the extracellular GSH present in the medium might be depleted from the extracellular space by transport into the cell by the GSH-T transporter. In contrast, in the RPTECs with higher GGT activity, the GSH is converted to cysgly and γ -glutamyl-X. The γ -glutamyl-X in turn inhibits cellular uptake of intact GSH and thus might keep sufficient GSH in the extracellular space to decrease cisplatin sensitivity.

If however, GGT is added extracellularly, GSH is depleted quickly and efficiently to cysgly, which is further cleaved to the single amino acids, which are taken up. This cysgly cleavage seems to be very fast, so that overall less GSH and cysgly are available for detoxification. Thus the paradoxical situation arises that either reduced expression of GGT or addition of recombinant GGT both decrease sensitivity.

Why now, do we see lower cisplatin sensitivity when we add a specific GGT inhibitor? The answer might lie in the experimental

set-up: GGT activity is blocked after changing the medium, so that already γ -glutamyl-X has been formed, blocking the GSH-T mediated uptake and thus extracellular depletion of GSH.

In vivo, however (Fig. 7B), RPTECs are polar and form tight layers with GSH-T on the basolateral side (Lash and Jones, 1983 and reviewed in Lash, 2010), while GGT and the dipeptidase are on the luminal side. Thus, lacking GGT activity (as in ko mice or using acivicin) reduces sensitivity as GSH levels are not depleted from the extracellular environment, because GSH-T is spatially not available – in contrast to working with 2D cell culture models. It is clear that more detailed studies are necessary to support our model and to reconcile the controversial data. Moreover accumulation of GSH, oxidative stress by GGT activity and reduction of the latter by catalase could also play key roles in the nephrotoxicity of cisplatin.

In any case, we strongly suggest that such studies are undertaken using tight epithelial monolayers as “3-dimensional” models of RPTECs in transwell assays as used, e.g., by Brown et al. (2008). The spatial distribution of transport proteins in polar cells needs to be taken into account in future studies and might explain some differences in cisplatin resistance of cancer cells against cisplatin toxicity of RPTECs.

We here have included immortalized HEPTECs that can be derived non-invasively from any person and due to immortalization with hTERT are standardizable and retain their main RPTEC characteristics over many population doublings, similar to our RPTEC/TERT1 cell line (Wieser et al., 2008, 2014). These cells might turn out to be useful for generating cell panels of donors with different genetic backgrounds, diseases or treatments in the context of toxicogenomics and personalized medicine in general.

Conflict of interest

RG and JG are cofounders of Evercyte GmbH and declare conflict of interest.

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