Erschienen in: Archives of Toxicology ; 93 (2019), 7. - S. 1965-1978
https://dx.doi.org/10.1007/s00204-019-02469-8

Functional transepithelial transport measurements to detect nephrotoxicity in vitro using the RPTEC/TERT1 cell line

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Abstract
The kidney is a frequent target for organ-specific toxicity as a result of its primary function in controlling body fluids, for example, via resorption of amino acids, peptides, nutrients, ions, xenobiotics and water from the primary urine as well as excretion of metabolic waste products and hydrophilic and amphiphilic xenobiotics. Compounds exhibiting dose-limiting nephrotoxicity include drugs from highly diverse classes and chemical structures, e.g., antibiotics (gentamicin), chemotherapeutics (cisplatin), immunosuppressants (cyclosporine A and tacrolimus) or bisphosphonates (zoledronate). All of these compounds elicit nephrotoxicity primarily by injuring renal proximal tubule epithelial cells (RPTECs). However, prediction of a compound's nephrotoxic potential in humans to support early unmasking of risk-bearing drug candidates remains an unmet challenge, mainly due to the complex kidney anatomy as well as pronounced inter- and intraspecies differences and lack of relevant and validated human in vitro models. Accordingly, we used the recently established human RPTEC/TERT1 cell line to carry out toxicity studies with a focus on impairment of functional characteristics, i.e., transepithelial electrical resistance (TEER), vectorial transport of water, cations, and anions. Results were compared to real-time cytotoxicity assessments using cellular impedance (xCELLigence assay) and the routine cell viability readout (MTT). As expected, most toxins caused exposure time- and concentration-dependent cytotoxicity. However, for some compounds (cyclosporine A and tacrolimus), transport processes were strongly impaired in absence of a concomitant decrease in cell viability. In conclusion, these data demonstrate that functional parameters are important, highly sensitive and meaningful additional readouts for nephrotoxicity assessment in human renal proximal tubule epithelial cells.

Keywords Kidney · Nephrotoxicity · Proximal tubule · In vitro · Epithelial transport

Introduction

The special vulnerability of the kidney is a result of its primary function in controlling body fluids, for example, via resorption of amino acids, peptides, nutrients, ions, xenobiotics, and water from the primary urine as well as excretion of metabolic waste products and hydrophilic and amphiphilic xenobiotics. Compounds exhibiting dose-limiting nephrotoxicity include drugs from highly diverse classes and chemical structures, e.g., antibiotics (gentamicin), chemotherapeutics (cisplatin), immunosuppressants (cyclosporine A and tacrolimus) or bisphosphonates (zoledronate). Common to these compounds is that they elicit nephrotoxicity primarily by injuring renal proximal tubule epithelial cells (RPTECs) (Miller et al. 2010; Naesens et al. 2009; Perazella and Markowitz 2008; Quiros et al. 2011). RPTECs are responsible for the lion's share of renal secretion and solute reabsorption (Lepist and Ray 2016) and thus present with high transport capabilities and metabolic rates (Hall and Unwin 2007). As a result of the latter, RPTECs experience a high exposure to drugs thereby rendering these cells especially vulnerable toward drug-mediated adverse effects. Despite their known risk

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of causing kidney injury, many drugs—including those introduced above—are routinely used in clinics, mostly because of a lack of nontoxic or similarly efficient but less-toxic alternatives. Thus, development of new drugs with decreased risk for nephrotoxicity would provide for a huge patient benefit and would widen therapeutic windows. Moreover, elderly patients, usually with a lowered renal function (Pazhayattil and Shirali 2014), not only represent a specially high-risk group, but are often exposed to a multitude of drugs simultaneously, thereby facing an increased risk of renal injury due to drug overdosing resulting from the preexisting reduced renal function or from polypharmacy and thus potential combined drug renal toxicity (Loboz and Shenfield 2005). In contrast to the latter needs, prediction of a compound's nephrotoxic potential in humans to support early de-risking of drug candidates remains an unmet challenge, mainly due to the complex kidney anatomy as well as pronounced inter- and intraspecies differences and lack of relevant, robust, sensitive, and validated human in vitro models (Tiong et al. 2014). With regard to human-derived cells, the latter is mainly due the unavailability of functionally differentiated continuous cell lines (Tiong et al. 2014) and the rapid dedifferentiation of primary RPTECs in culture (Pfaller and Gstraunthaler 1998). Thus human cell lines that maintained key functionalities including xenobiotic uptake and secretion are of key interest especially for pharmaceutical research and development. The RPTEC/TERT1 cell line was recently established by immortalizing primary human RPTECs via telomerase (TERT1) overexpression (Wieser et al. 2008). Subsequent gene expression analysis revealed remarkable concordance of RPTEC/TERT1 cells with primary human RPTECs. Furthermore, RPTEC/TERT1 cells retain important functional characteristics such as dome formation (Wieser et al. 2008), water (Wilmes et al. 2014) and cation transport (Aschauer et al. 2015a). Consequently, this cell line has been used for in-depth repeated dose nephrotoxicity studies (Aschauer et al. 2015b) as well as to study the underlying mechanisms of cyclosporine A (Wilmes et al. 2013) and cisplatin (Wilmes et al. 2015) toxicity. However, the latter was carried out primarily with a focus on transcriptomic, metabolomic, and proteomic approaches and not with a focus on functional characteristics. While functional readouts are commonly applied for the in vitro detection of neuro- (Hausherr et al. 2014) or cardiotoxicity (Sirenko et al. 2017), such approaches have so far been rarely applied for the detection of nephrotoxicity and, when applied, were basically limited to measurements of transepithelial electrical resistance (Duff et al. 2002). Thus, in the present study, we investigated whether the combination of functional measurements, e.g., water reabsorption and cation secretion with classical cytotoxicity endpoints, while using a limited set of nephrotoxic and non-nephrotoxic reference substances, could provide for improved sensitivity in the detection of drug-induced nephrotoxicity.

Results

RPTEC/TERT1 cell viability upon single or multiple exposures with known nephrotoxicants

Cultivation of RPTEC/TERT1 cells on standard tissue culture plates for 16 days resulted in a monolayer of non-dividing, differentiated cells reminiscent of fully functional primary human proximal tubule cells (Aschauer et al. 2015a). We exposed these differentiated RPTEC/TERT1 cells to a set of test compounds comprised of nephrotoxicants with reported primary proximal tubule toxicity (cisplatin, zoledronate, gentamicin, tacrolimus, and cyclosporine A) and compounds being specifically handled by the kidney yet lacking nephrotoxicity (dexamethasone and probenecid). Cells were exposed either for 24 h or 14 day with treatment renewal every 48 h followed by determination of cell viability via MTT reduction assay. Remarkably, cisplatin, gentamicin, and zoledronate were more cytotoxic when applied repeatedly for 14 days than for 24 h (Fig. 1), whereas the immunosuppressants tacrolimus and cyclosporine A showed limited or no cytotoxicity irrespective of single or repeated exposure. Indeed, repeated treatment with cisplatin (Fig. 1a) and gentamicin (Fig. 1b) resulted in reducing the lowest observed effect concentration (LOEC) by almost 200-fold (from 300 to 1.6 µM) and 60-fold (from 22.5 mM to 350 µM), respectively, when compared to the LOEC of the 24 h treatment. Zoledronate (Fig. 1c) did not impair cell viability in the 24 h exposure, even when tested up to the limit of compound solubility, but significantly reduced cell viability, with a LOEC of 12.5 µM, when tested for 14 day. Tacrolimus treatment (Fig. 1d) resulted in a very steep concentration–response curve irrespective of the exposure duration and single or multiple treatment, i.e., showing no effect at 63 µM but complete loss of viability at 100 µM. In contrast, despite being tested up to the limit of solubility, the known nephrotoxicant cyclosporine A did not affect cell viability at all (Fig. 1e). Similarly, the non-nephrotoxic probenecid (Fig. 1f) and dexamethasone (Fig. 1g) had no effect on cell viability when tested up to 1 mM or 500 µM in the 24 h or 14 day repeated exposures. On the contrary, repeated dexamethasone exposure appeared to result in increased MTT conversion rates.

Cellular impedance as a real-time measure of cytotoxicity in RPTEC/TERT1 cells

The xCELLigence system provides a real-time, label-free and non-invasive readout, expressed as cell index, that

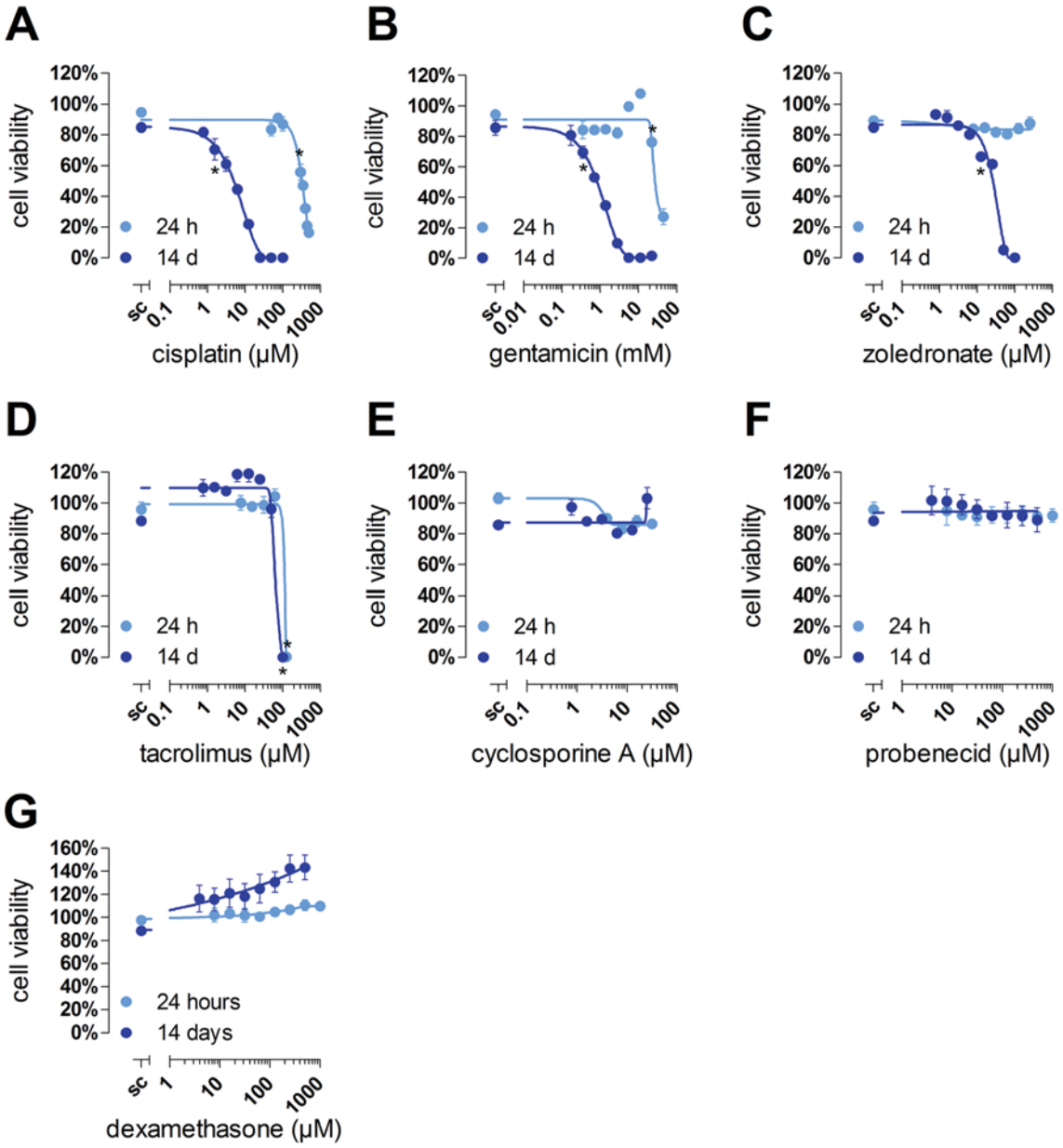

Fig. 1 Viability of RPTEC/TERT1 cells exposed to test compounds. Differentiated RPTEC/TERT1 cells (16 day) were exposed to (**a**) cisplatin, **b** gentamicin, **c** zoledronate, **d** tacrolimus, **e** cyclosporine A, **f** probenecid or **g** dexamethasone for 24 h or 14 d with treatment renewal every 2 days in presence of 1% DMSO (24 h; sc) or 0.5% DMSO (14 day; sc). Cell viability was determined via MTT reduction. Data show mean ± SEM ($n = 3$) and was normalized to non-treated cells. *$P < 0.05$, ANOVA with Dunnett's post-test. The lowest concentration with significant reduction in viability is marked with an asterisk

primarily depends on cellular adherence to the plate and on the intensity of cell–cell connections, thus reflecting a direct relationship to cell density and cell layer integrity (Asphahani and Zhang 2007). We thus hypothesized that cellular impedance measurements in repeated exposure experiments, using the xCELLigence system, would allow for sensitive detection of subtle changes in cell–cell interactions and cellular adhesion that would not necessarily lead to overt cell death in RPTEC/TERT1 cells. A strengthening of cell–cell interactions, i.e., tight junctions, would be represented by an increase in cell index, while a weakening of cell–cell interactions or even loss of cells would result in a decreased cell index. The absolute value of the cell index varies between cell lines and thus has no absolute but rather a relative value. Following 8–10 days of culture, RPTEC/TERT1 cells seeded at different densities, i.e., initially covering 10–50% of the surface area, developed a cell index that remained stable for the subsequent 20 days of culture (Fig. 2a). The periodically

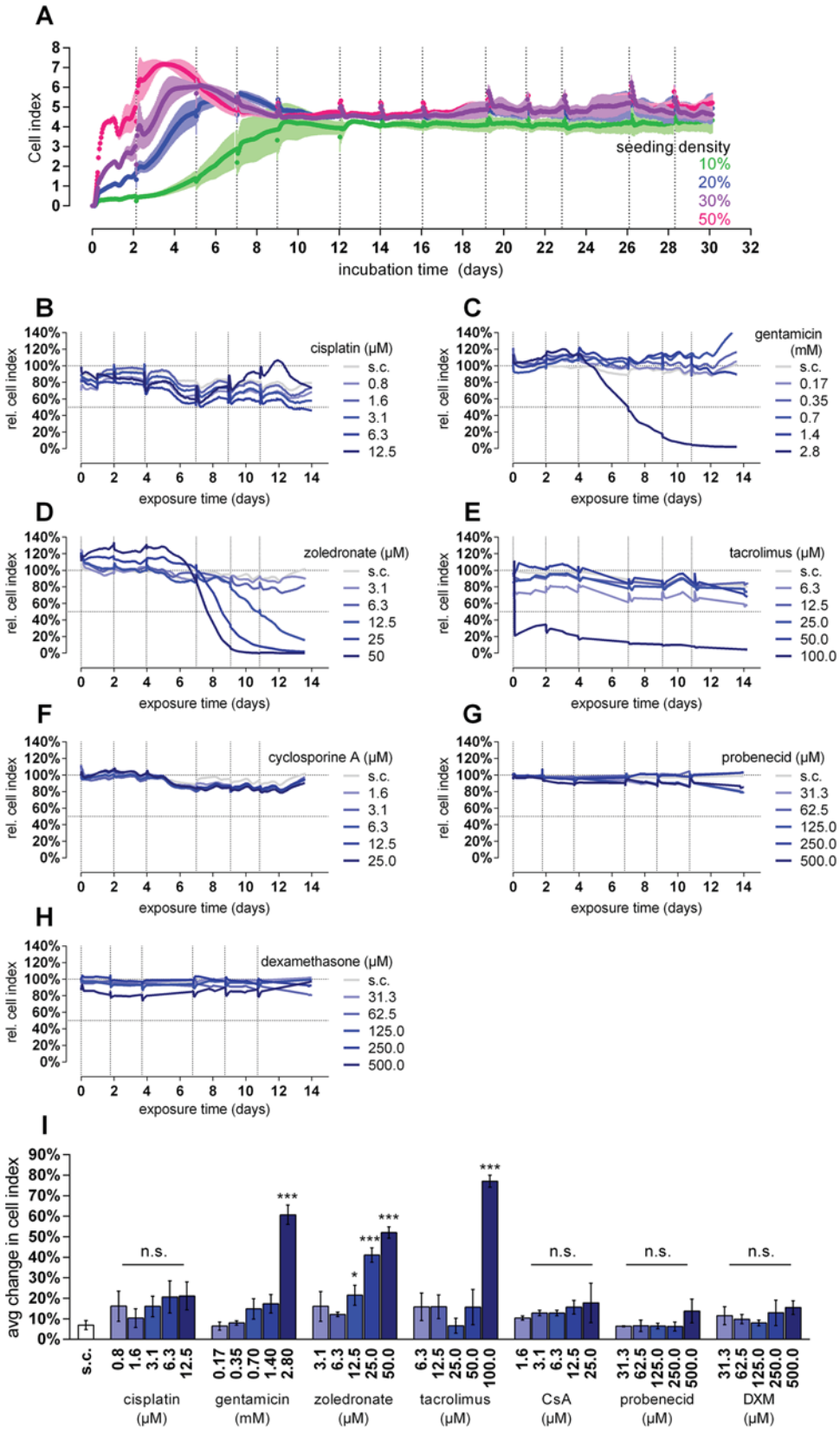

Fig. 2 Impedance measurement of RPTEC/TERT1 cells using xCELLigence. **a** Cell index of RPTEC/TERT1 seeded at different densities monitored over 30 days. Data represent mean ± SEM ($n = 3$). **b**–**h** Change in cell index of RPTEC/TERT1 cells exposed to different concentrations of **b** cisplatin, **c** gentamicin, **d** zoledronate, **e** tacrolimus, **f** cyclosporine A, **g** probenecid or **h** dexamethasone in presence of 0.5% DMSO. Based on results depicted in **a**, RPTEC/TERT1 cells were seeded at 30% and cultured for 16 day prior to exposure (day 0). Data show mean values of a representative experiment performed in quadruplicates and is normalized to time-matched values obtained for non-treated cells. Vertical dotted lines indicate medium or treatment renewal. **i** Average change of cell index of exposed cells from non-treated cells over the complete experimental duration (14 day). Data represent mean ± SEM ($n = 3$). $^{*}P < 0.05$; $^{***}P < 0.001$; *n.s.* not significant compared to s.c. (0.5% DMSO), ANOVA + Dunnett's post-test

occurring peaks observed (Fig. 2a) originated from medium renewal (indicated by the vertical dotted lines). Next, we exposed differentiated RPTEC/TERT1 cells (cultivated for 16 days) repeatedly for 14 days to the test compounds at concentrations that were identified as cytotoxic (MTT reduction) in the multiple exposure experiment (Fig. 1) and continuously quantified cell indices. For compounds that showed no effect on MTT reduction, the highest five concentrations (Fig. 1) were selected for the xCELLigence experiment. The nephrotoxins cisplatin (Fig. 2b) and gentamicin (Fig. 2c) showed surprisingly little effect on the cell index. While cisplatin did not impact the readout up to 12.5 µM, gentamicin caused a progressive reduction of the cell index at the highest concentration (2.8 mM) employed and only after the third treatment renewal. In case of zoledronate (Fig. 2d), good concordance between MTT and impedance measurements was observed since both readouts identified 12.5 µM as the lowest effect concentration for the 14-day exposure period (Figs. 1c, 2d, i). Additionally, the xCELLigence experiment nicely revealed that zoledronate toxicity strongly depends on exposure time and/or dosing frequency. Indeed, a reduction of the cell index was initially observed at day 6 (after three treatments) for 50 µM and approximately 1 day later for 25 µM. At 12.5 µM, progressive decrease in cell index was not observed until day 9 (five treatments). Interestingly, the treatment with 100 µM tacrolimus (Fig. 2e) caused an immediate loss of cellular impedance while up to 50 µM did not show any effect even not after multiple exposures. The latter corresponded well with the MTT data (Fig. 1d) that showed the complete loss of viability at 100 µM after 24 h but unimpaired viability at 50 µM after 14 days. The other nephrotoxic calcineurin inhibitor, cyclosporine A, did not affect cellular impedance (Fig. 2f) similar to the non-nephrotoxic compounds dexamethasone (Fig. 2g) and probenecid (Fig. 2h) thereby mirroring the data obtained from the MTT assays.

Statistical mean values were employed to better enable identification of whether or not a treatment caused an alteration in the cell index. Thus, an internal normalization of the individual experiments was unavoidable. For the latter we decided to calculate the change (increase and decrease) in cell index caused by a defined treatment by dividing the cell index of the treated cells at a given timepoint by the value obtained for non-treated cells on the same plate at the same timepoint. The average change in cell index was then calculated by building the mean across the changes at all timepoints of each experiment (see formula in Fig. S1). This normalization showed that the average change in cell index caused by the solvent control (s.c.; 0.5% DMSO) compared to non-treated cells was below 10% (Fig. 2i). Statistically significant changes in cell index were detected for gentamicin (2.8 mM), zoledronate (starting at 12.5 µM), and tacrolimus (100 µM) (Fig. 2i).

Transepithelial electrical resistance as a functional toxicity readout using RPTEC/TERT1 cells

The proximal tubule is a "leaky" epithelium, characterized by intense cell–cell contacts including tight junctions which, however, still allow for paracellular transport required for the functionality of the proximal tubule epithelium, i.e., reabsorption and secretion of solutes. Thus, cultivation on trans-well membrane filter inserts is considered the gold standard for renal proximal tubule cells since it allows the basolateral-apical polarization of the cells commensurate with their anatomical situation and functional organization. The integrity of the renal proximal epithelium-like monolayer on membrane filters can be quantified by its insulating property as transepithelial electrical resistance (TEER). When seeded on trans-well membrane filters at 100% confluence, RPTEC/TERT1 cells build up electrical resistance, stabilizing between 120–140 $\Omega \times cm^2$ at approximately 14 days and thereafter (Fig. 3a). To determine the effect of potential nephrotoxic compounds on the "leakiness" of the renal proximal tubule cell epithelium using TEER, we repeatedly exposed confluent, 23 days-cultured RPTEC/TERT1 to the test compounds at concentrations that were determined to have no effect (or little effect in case of zoledronate) by the xCELLigence assay (Fig. 2i) and quantified TEER every 2–3 days. To ensure for a stable baseline in TEER prior to the exposure period, measurements were initiated 9 days prior to exposure initiation (day −9). An increase in TEER would represent an increased tightening of the cell–cell contacts and thus reduced leakiness of the monolayer, while a decreased TEER would suggest increased leakiness thus increased weakening of the tight junctions or even cell death.

Exposure to cisplatin (12.5 µM) (Fig. 3b) caused a significant but transient increase in TEER between day 2 and 7 of exposure only to return to baseline for days 9–14. As expected, increasing the concentration of cisplatin to 25 and 50 µM resulted in a decrease in TEER at later timepoints suggesting cytotoxicity (Fig. 3b). In contrast, gentamicin (700 µM) (Fig. 3c) caused an increase in TEER on days 9 and 12 of exposure, while zoledronate (12.5 µM) (Fig. 3d) did not affect TEER at any timepoint. Tacrolimus (50 µM), on the other hand, caused an immediate elevation of TEER (Fig. 3e), already detectable after 9 h of exposure and maintained until day 9, after which normalization to baseline occurred. 10 µM tacrolimus (Fig. 3e), however, had no effect on TEER. Cyclosporine A at 5 µM (Fig. 3f) immediately (after 9 h) elevated TEER over the entire exposure period of 14 days. At 25 µM, TEER peaked at 425 $\Omega \times cm^2$ at day 2 before it returned to normal around day 9. The two non-nephrotoxic compounds probenecid (Fig. 3g) and dexamethasone (Fig. 3h) both caused transient increases of TEER at 500 µM but not at 50 µM. None of the test compounds induced a reduction of TEER at concentrations deemed

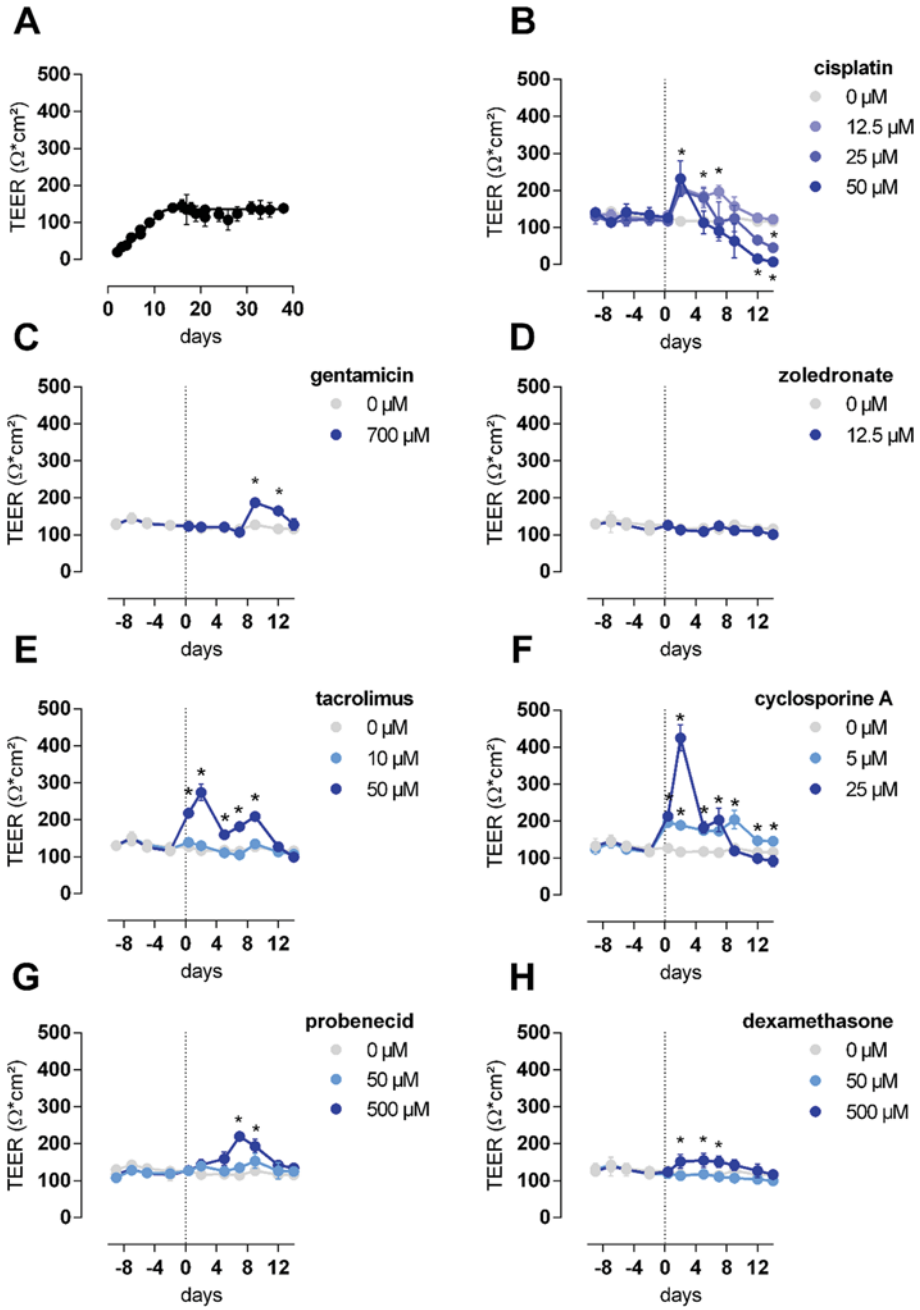

Fig. 3 Transepithelial electrical resistance (TEER) measurements of RPTEC/TERT1 cells. **a** RPTEC/TERT1 cells were seeded on membrane filter inserts at 100% confluence and TEER was measured over a period of 38 days. Data represent mean ± SEM of ($n = 20$–130 filter inserts per timepoint). **b**–**h** Changes in TEER due to exposure to **b** cisplatin, **c** gentamicin, **d** zoledronate, **e** tacrolimus **f** cyclosporine A, **g** probenecid or **h** dexamethasone. Exposure was initiated at day 23 after seeding (indicated as day 0) and TEER quantification was started 9 days prior to exposure (day −9). Exposure was performed for 14 days with medium renewal every 2–3 days. Data represent mean ± SD ($n = 3$). *$P < 0.05$ compared to time-matched controls (0.5% DMSO), two-way ANOVA + Bonferroni's post-test

non-cytotoxic. The latter suggests that RPTEC/TERT1 cells do not exhibit higher sensitivity on trans-well membrane filter inserts when compared to 2D plastics exposures, as used in the xCELLigence assay.

Vectorial transport of RPTEC/TERT1 cells

Beyond the pronounced cell–cell contacts of an established renal proximal tubule epithelial cell layer, active vectorial transport processes represent the key functionality of the proximal tubule epithelium (Morrissey et al. 2013). Indeed, when RPTEC/TERT1 cells were cultured on trans-well membrane inserts, active vectorial water transport was readily apparent, intensified with increasing cultivation time and was quantifiable simply by measuring water removal from the apical compartment (insert) or water addition to the basolateral compartment (well) using an analytical scale (Fig. 4a). Water reabsorption is a passive process that primarily depends on the establishment of a confluent and polarized cell layer, tight junctions, expression of aquaporins and an osmotic gradient foremost established by the Na^+/K^+-ATPase expressed at the basolateral membrane, illustrated by the complete

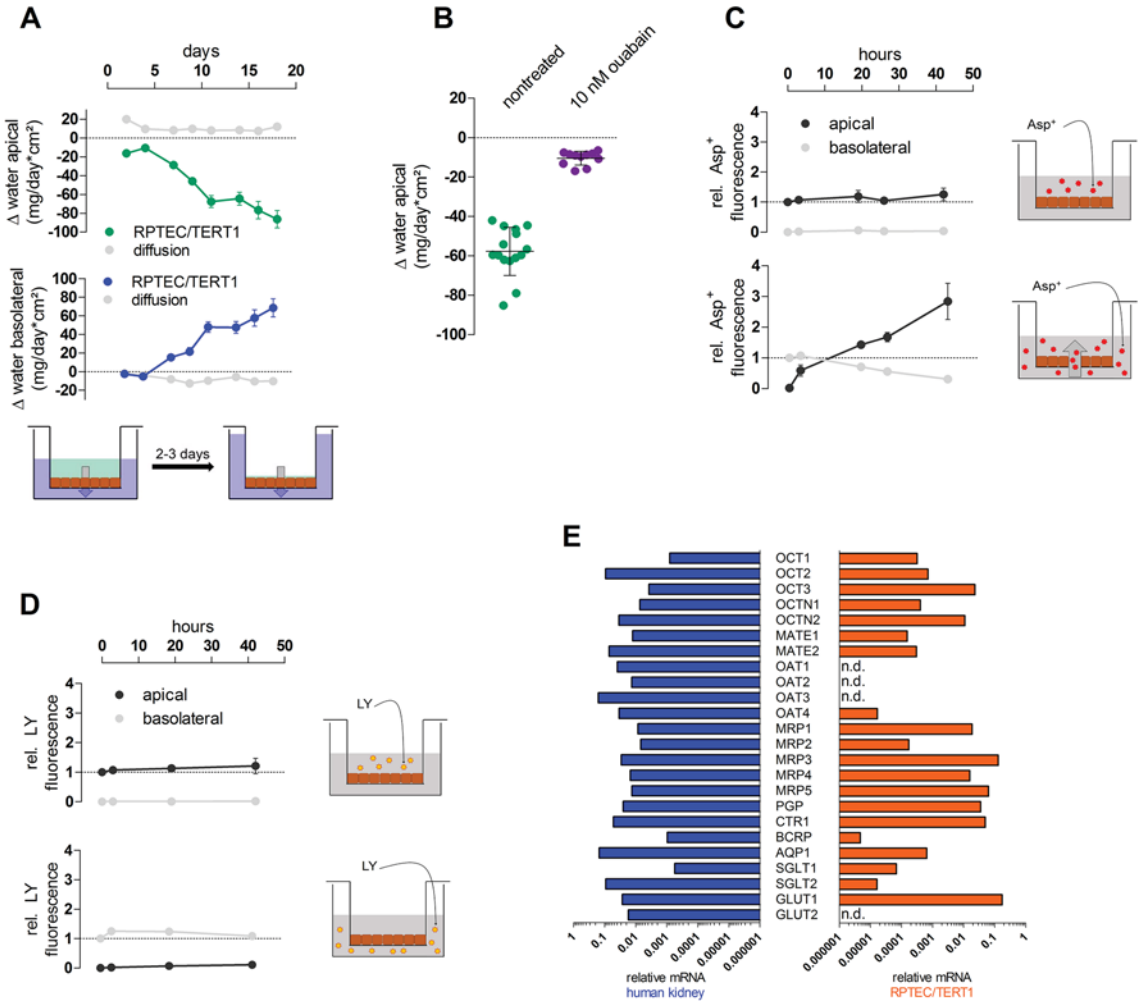

Fig. 4 Vectorial transport processes of RPTEC/TERT1 cell on membrane filters. **a** Water transport of RPTEC/TERT1 cells over time quantified as removal from apical side or addition to basolateral side in mg per day and membrane surface area in cm^2. Data represent mean ± SEM ($n = 4$). Diffusion shows water exchange in wells without cells. **b** Water transport in the presence and absence of the Na^+/K^+-ATPase inhibitor ouabain. **c** Vectorial transport of 4-Di-1-ASP (Asp^+) by RPTEC/TERT1 cells in (upper panel) apical-to-basolateral direction and (lower panel) basolateral-to-apical direction. Data represent mean ± SD of 3 membrane filters. **d** Vectorial transport of Lucifer Yellow (LY) by RPTEC/TERT1 cells in (upper panel) apical to basolateral direction and (lower panel) basolateral to apical direction. Data represent mean ± SD of 3 membrane filters. **e** Gene expression of various transporters in RPTEC/TERT1 cells cultured on membrane inserts and human kidney. Data show mean expression of four independent RPTEC/TERT1 samples and one human kidney sample analyzed in duplicates. *n.d.* not detected

abrogation of water transport by inhibition of the Na^+/K^+-ATPase with ouabain (Fig. 4b) (Wilmes et al. 2014).

Functional water reabsorption suggests in vivo-like polarization of RPTEC/TERT1 cells on trans-well membrane inserts and hence it was suggested that the cells might also be capable of secreting xenobiotics. To determine the capacity of RPTEC/TERT1 to actively secrete xenobiotics, we used the cationic fluorescent probe 4-Di-1-ASP (Asp^+), a substrate for the OCT/MATE transport axis (Aschauer et al. 2015a; Sauzay et al. 2016) and the fluorescent anion Lucifer yellow (LY), secreted via the OAT/MRP axis (Masereeuw et al. 1999). As expected for a substance that undergoes secretion, Asp^+ remained in the apical compartment with no fluorescence detectable in the basolateral medium (Fig. 4c, upper panel). However, when added to the basolateral compartment, Asp^+ was efficiently and actively transported into the apical compartment (Fig. 4c, lower panel). LY on the other hand was not transported in either direction (Fig. 4d), suggesting absence of functional anion transport. Subsequently, gene expression analysis of transporters in the confluent RPTEC/TERT1 cell monolayer, in contrast to human cortical kidney (Fig. 4e), demonstrated that the confluent RPTEC/TERT1 cell epithelium lacked expression of OATs 1–3. The absence of OAT1 and OAT3, as the primary anion importers (Morrissey et al. 2013), would explain the lack of LY transport. However, apart from the latter deficit, RPTEC/

TERT1 cells appear to express most of the important renal transporters including OCT1-3, MATE1-2, MRP1-5, P-gp, AQP1, etc. (Fig. 4e).

Vectorial transport as a functional readout for the detection of nephrotoxicity

Since cellular dedifferentiation and concomitant impairment in functionality are common features of early toxicity (Puri et al. 2015), it was hypothesized that an impairment of water transport or Asp^+ secretion would be a sensitive indicator of toxicity. To test this, confluent RPTEC/TERT1 cells cultured on trans-well membrane inserts were exposed to the test compounds for 9 consecutive days followed by a 14-day recovery period. Water reabsorption was quantified throughout the whole study period and Asp^+ secretion was measured at three distinct time points throughout exposure and recovery (Fig. 5a). Exposure to 12.5 µM cisplatin (Fig. 5b) and 700 µM gentamicin (Fig. 5c) resulted in gradually reduced water reabsorption during the exposure phase, whereby no overt recovery was observed up until day 23 (14 day post exposure recovery period). Conversely, 12.5 µM zoledronate (Fig. 5c) had no effect on water reabsorption at any time point investigated. The most dramatic effects were observed with 50 µM tacrolimus (Fig. 5e) and 25 µM cyclosporine A (Fig. 5f). Both compounds caused an immediate reduction of water transport that peaked at day 9 of exposure when only 30–40% of the water transport capacity remained. Interestingly, upon removal of the compounds water transport gradually recovered and returned back to normal at 10 days of recovery. Exposure to 500 µM probenecid (Fig. 5g) decreased water reabsorption gradually during the exposure phase; however, the cells did not recover until day 23. Conversely, exposure to 50 µM probenecid led to a very slight reduction of water transport, which appeared fully reversible. The other typically non-nephrotoxic compound, dexamethasone (500 µM), strongly decreased water transport in a linear fashion until day 9, when only 40% of the water transport capacity remained. However, the water transport capacity gradually recovered during the recovery period reaching 80–90% of the original capacity at day 14 of recovery. At a lower concentration of 50 µM, the effect of dexamethasone was less pronounced, decreasing water transport to 70% after 9 days of exposure with cells recovering completely after 14 days. Secretion of Asp^+ was determined from day 7 to 9 of exposure and, in case of the known nephrotoxicants, during recovery from day 12 to 14 and day 21 to 23 (Fig. 5a). The comparison of Asp^+ transport of dye diffusion through trans-well membrane filters without cells (Fig. 6a, diffusion) with transport in DMSO-treated cells (Fig. 6a, 0.5% DMSO), demonstrated the active transport of the dye when cells are present. RPTEC/TERT1 cells treated for 7 days with gentamicin, cisplatin, probenecid, and zoledronate showed a slightly elevated Asp^+ transport (Fig. 6a) although this effect was only significant for gentamicin (Fig. 6b). Interestingly, exposure to 500 µM dexamethasone resulted in a transport kinetic that was strikingly similar to the diffusion control (Fig. 6a), suggesting that dexamethasone rendered the monolayer permeable to Asp^+ and consequently impaired active transport, as was also indicated by quantification of water transport (Fig. 5h). The strongest effects on Asp^+ secretion were again observed for cyclosporine A and tacrolimus, both almost completely abolishing Asp^+ transport after 7 days of exposure (Fig. 6a).

To accurately quantify Asp^+ secretion over the 48 h period, the simultaneously occurring water transport had to be taken into account. Asp^+ fluorescence determined at the last time-point (47 h) was therefore, corrected for the medium volume present in the apical compartment at that specific timepoint (Fig. 6b, c). Even after this correction, cyclosporine A and tacrolimus almost completely eliminated relative Asp^+ transport on day 7 of treatment (Fig. 6c). Gentamicin, on the other hand, significantly increased Asp^+ secretion, while none of the other compounds changed the total amount of Asp^+ transported. Both increased and decreased Asp^+ secretion returned back to normal within the recovery phase (from day 9 to 23, see Fig. 5a). In case of cyclosporine A, reduced transport was still visible after 3 days of recovery (day 12) but returned back to normal after 12 days recovery (day 21), while the effects of tacrolimus and gentamicin were already abolished at day 12.

Discussion

About one third of drug-development failures occurring during clinical studies are due to safety reasons (Waring et al. 2015) and the kidney is one major organ affected by adverse drug reactions (Lin and Will 2012). As nephrotoxicity affects the functionality and viability of RPTECs (Paueksakon and Fogo 2017), depicting the functionality of these cells in vitro is of key importance for the development of alternative test methods to detect potential nephrotoxins. Unfortunately, there is no human RPTEC cell line available with fully preserved capacity for xenobiotic transport (Tiong et al. 2014) thus limiting their suitability for detection of nephrotoxicity. Indeed, one of the most frequently used human tubular epithelial cell lines, HK-2, was reported to lack expression of the key transporters OAT1, OAT3, OCT2, and BCRP (Jenkinson et al. 2012), whereas, more recently established cell lines, including the RPTEC/TERT1 cell line, were reported to have retained important functional parameters (Aschauer et al. 2015a; Wilmes et al. 2014).

Simple cell viability assays are often disparaged due to their unspecific nature and indeed MTT reduction was not a sensitive readout when analyzed after an acute 24 h exposure

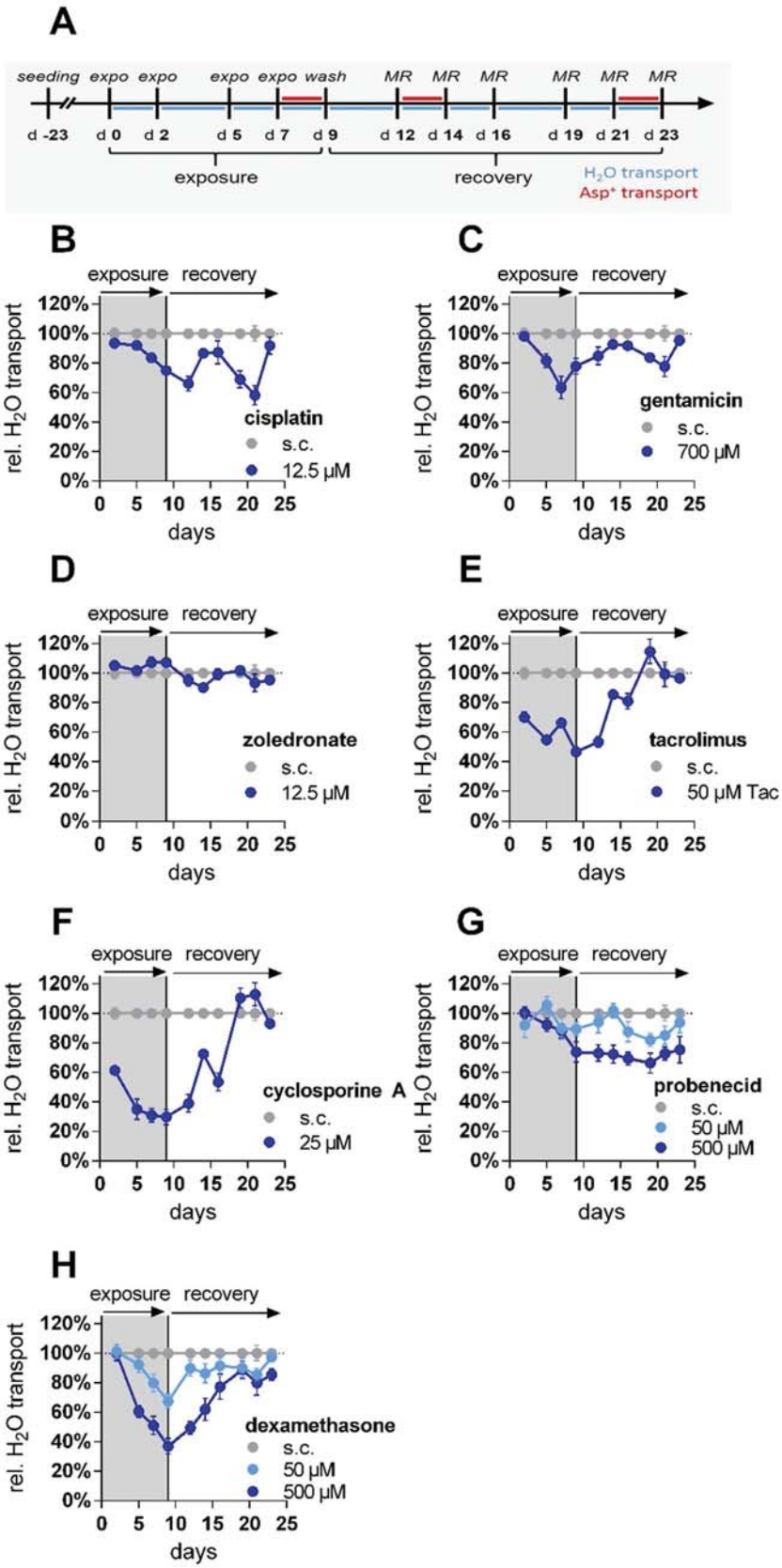

Fig. 5 Vectorial water transport as a functional parameter to detect nephrotoxicity. **a** Schematic of the experimental setup designed to investigate the effects of the test compounds on water and Asp^+ transport. Cells were seeded 23 days prior to exposure initiation. Medium with (exposure phase) or without (recovery phase) compounds was renewed at the days indicated. Water and Asp^+ transport were quantified in the highlighted intervals. **b**–**h** Compound-induced change in water transport as quantified in the apical compartment. Relative water transport of cells exposed to **b** 12.5 µM cisplatin, **c** 700 µM gentamicin, **d** 12.5 µM zoledronate, **e** 50 µM tacrolimus, **f** 25 µM cyclosporine A, **g** 50 µM and 500 µM probenecid and **h** 50 µM and 500 µM dexamethasone. Data represent mean ± SEM ($n = 4$) normalized to time-matched controls (0.5% DMSO)

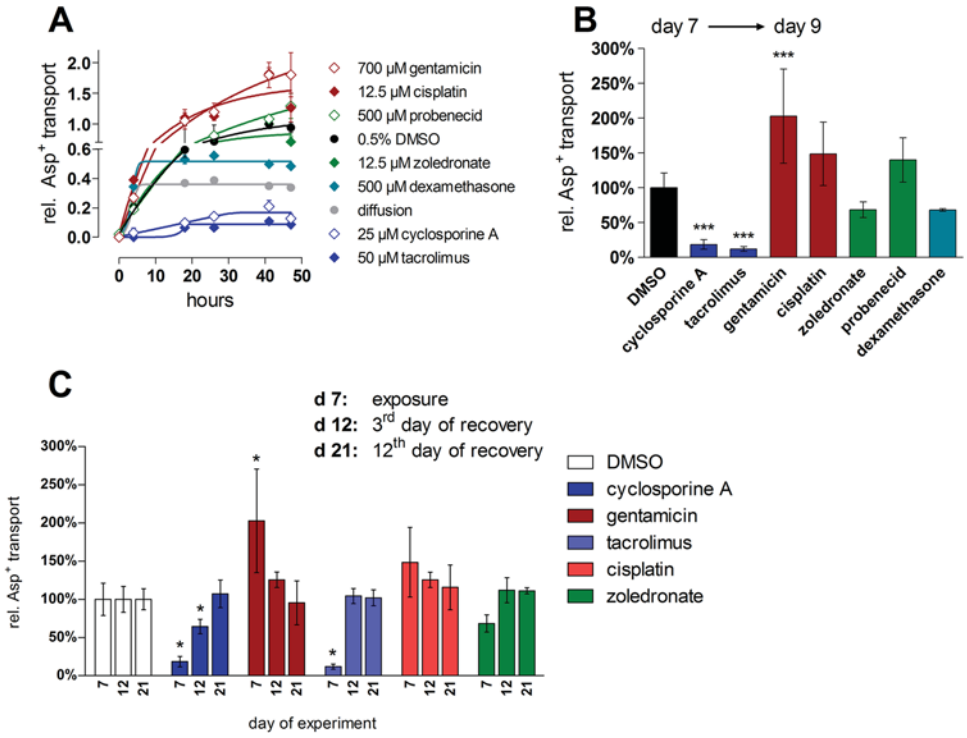

Fig. 6 Asp^+ secretion by RPTEC/TERT1 cells exposed to the test compounds. **a** Time dependency of Asp^+ fluorescence in the apical compartment. Experiment was initiated after 7 days of exposure in presence of the compounds indicated. Fluorescence was normalized towards values obtained for 0 h in the basolateral compartment where the dye was added. **b** Absolute Asp^+ fluorescence in the apical compartment after 47 h (from day 7 to day 9) normalized to water content. **c** Absolute Asp^+ fluorescence in the apical compartment after 47 h normalized to water content at all different timepoints. Data represent mean ± SEM ($n = 4$) normalized to time-matched controls (0.5% DMSO). $^*P < 0.05$; $^{***}P < 0.001$, compared to time-matched controls (0.5% DMSO), two-way ANOVA + Sidak's post-test

to the test compounds in our hands. However, when used at the end of a 14-days repeated exposure experiment with RPTEC/TERT1 cells, the MTT assay was the single most sensitive readout in case of cisplatin and gentamicin (Table 1). For these nephrotoxins, a decrease in MTT reduction occurred in absence of cell death or disintegration of the cell layer as detected by xCELLigence and TEER measurements. Since the MTT assay quantifies mitochondrial reductase activity, this finding likely mirrors the mitochondrial dysfunction reported as proximal events in cisplatin (Zsengeller et al. 2012) and gentamicin (Sepand et al. 2016) nephrotoxicity.

In case of zoledronate, MTT reduction and xCELLigence assay were equally sensitive suggesting that in this case the decrease in MTT reduction is due to cell death. In general, cellular impedance measurements using the xCELLigence system turned out to be not a particularly sensitive readout in our approach but nonetheless delivered unique insight into the kinetics of cytotoxicity by allowing for real-time measurements. For example, we were able to demonstrate that zoledronate-induced cytotoxicity is not only concentration- but also time-dependent, thereby suggesting that it is the cumulative dose over time that eventually elicits cell death. The latter stands in contrast to the results obtained for tacrolimus, which caused immediate cell death after minutes of exposure at 100 µM but had no effect at 50 µM and below, even when the cells were repeatedly challenged with the compound for 14 days. Consequently, real-time impedance measurements can provide some evidence on the type of toxicity, whether it is likely caused by exceeding a tolerable concentration (c_{max} driven) or by cumulative exposure over time (AUC driven).

However, while the xCELLigence measurements failed to reveal alterations in the monolayer integrity apart from cell death, the quantification of TEER showed transient or possibly even persistent increases in monolayer resistance for many of the compounds tested but most prominently for cyclosporine A and tacrolimus which, with the exception of 100 µM tacrolimus, had no effect on cell viability and impedance measurements. Consistent with previous publications (Aschauer et al. 2015b; Wilmes et al. 2014), non-treated RPTEC/TERT1 cells developed a stable TEER value of 120–140 $\Omega \times cm^2$ in the present study. When compared to other cell lines, e.g., intestinal carcinoma Caco-2 cells (Srinivasan et al. 2015), TEER in RPTEC/TERT1 cells is rather low, thus suggestive of the "leakiness" of

Table 1 Overview of the apical and functional results obtained for RPTEC/TERT1 exposed for 24 h or 14 day to different nephrotoxicants

	PT toxicant	MTT				xCELLigence		TEER		H_2O reabsorption		Asp^+ secretion	
		24 h		14 day									
		NoEC	LoEC	NoEC	LoEC	NoEC	LoEC	NoEC	LoEC	NoEC	LoEC	NoEC	LoEC
Cisplatin	Yes	100 µM	300 µM	0.8 µM	**1.6 µM**	> 12.5 µM	n.d	n.d	12.5 µM	n.d	12.5 µM	> 12.5 µM	n.d
Gentamicin	Yes	11.1 mM	22.3 mM	170 µM	**350 µM**	1.4 mM	2.8 mM	n.d	700 µM	n.d	700 µM	n.d	700 µM (incr.)
Zoledronate	Yes	> 250 µM	n.d	6.3 µM	**12.5 µM**	6.3 µM	**12.5 µM**	> 12.5 µM	n.d	> 12.5 µM	n.d	> 12.5 µM	n.d
Tacrolimus	Yes	50 µM	100 µM	50 µM	100 µM	50 µM	100 µM	10 µM	**50 µM**	n.d	**50 µM**	n.d	**50 µM**
Cyclosporine A	Yes	> 25 µM	n.d	> 25 µM	n.d	> 25 µM	n.d	n.d	**5 µM**	n.d	**5 µM**	n.d	25 µM
Probenecid	No	> 1000 µM	n.d	> 500 µM	n.d	> 500 µM	n.d	n.d	500 µM	n.d	**50 µM**	> 500 µM	n.d
Dexamethasone	No	> 1000 µM	n.d	> 500 µM	n.d	> 500 µM	n.d	50 µM	500 µM	n.d	**50 µM**	> 500 µM	n.d

Bold font indicates the lowest observed effect concentration across the different assays tested

the monolayer. A transient increase in TEER in response to a toxic challenge was described previously (Aschauer et al. 2015b) and although the underlying mechanism is not fully understood it most likely involves modifications of tight junctions (Wilmes et al. 2014). A "tightening" of the monolayer, as measured by TEER, would also suggest a concomitantly decreased water permeability, as was demonstrated (Figs. 3b, 4a). Indeed, increased TEER and reduced water transport for cyclosporine A were reported in RPTEC/TERT1 cells earlier and involved a downregulation of the tight junction protein claudin-2, which was identified as a paracellular water channel (Wilmes et al. 2014). The findings reported here suggest that an impairment of water transport is a more general response towards cellular damage and therefore is likely shared by many, however, not all nephrotoxicants. As demonstrated, water transport by RPTEC/TERT1 cells is driven by the osmotic gradient built by the Na^+/K^+-ATPase for which multiple prerequisites must be fulfilled. Water transport requires at least integrity of the cell layer, functional transporters and high metabolic rates to fuel the Na^+/K^+-ATPase. It is probably because of this integration of multiple parameters that water transport proved a sensitive readout to detect toxicity.

Remarkably, the RPTEC/TERT1 monolayer fully recovered from the strong effects of cyclosporine A and tacrolimus on water transport within 10 days after compound removal, a fact that nicely reflects the reversibility of acute calcineurin inhibitor-induced nephrotoxicity observed in vivo (Naesens et al. 2009). Recovery from gentamicin and especially from the genotoxic cisplatin was less pronounced, thereby indicating sustained damage to the RPTEC/TERT1 cells. The fact that high concentrations of probenecid (500 µM exceeding the clinical c_{max} tenfold (Selen et al. 1982)), also reduced water transport and increased TEER, demonstrated that presumably non-nephrotoxic compounds can demonstrate off-target effects when tested at sufficiently high concentrations. A somewhat controversial observation is the fact that a near linear decrease in water transport over time together with the diffusion-like permeation of Asp^+ after 7 days was observed with 500 µM dexamethasone. The latter would suggest that the compound rendered the RPTEC/TERT1 monolayer permeable for small molecules like water and Asp^+, however, in absence of a decrease in TEER, which in fact was slightly elevated. However, as has already been discussed in the case of probenecid, these effects are most likely due to the high concentration of 500 µM dexamethasone that was applied, which exceeded the clinical c_{max} by 50-fold (Wenting-Van Wijk et al. 1999). Most likely clinically relevant concentrations would not show these effects, as indeed 50 µM dexamethasone did not show an effect on TEER and only a limited effect on water permeability.

Similar to water transport, quantification of cation secretion using Asp^+ showed the most dramatic reductions for

cyclosporine A and tacrolimus since both almost abolished cation secretion after a 7-day exposure. Whether this inhibition is due to a direct effect of cyclosporine A on either OCT or MATE transporters is unclear since such interaction is unknown at present. However, cyclosporine A is a potent inhibitor of P-gp and MRP2 (Morrissey et al. 2013) which at least share some substrates with MATEs, e.g., the chemotherapeutic agent topotecan (Morrissey et al. 2013). In contrast to cyclosporine A and tacrolimus, gentamicin significantly increased Asp^+ secretion after 7-day exposure. Since OCTs and MATEs transport both, Asp^+ and gentamicin (Gai et al. 2016), the latter effect might well be explained by an induction of OCT and MATE expression or activity induced by the high concentrations of gentamicin (700 µM) used.

While we could successfully show the existence of a cation secretion axis requiring polarized OCT and MATE expression, we were unable to demonstrate functional anion secretion. Indeed a gene expression analysis revealed absence of OAT1-3, while MRP1-5 and the transporters required for cation secretion were clearly detected. Although another study (Aschauer et al. 2015a) reported OAT1 and OAT3 expression in RPTEC/TERT1 cells based on whole-genome expression data and western blots, functional anion secretion was not observed. Without diminishing the many functional benefits of the RPTEC/TERT1 cell line, the lack of functional OATs is a significant shortcoming. Similarly, the other recently established ciPTEC also lacked OAT1 and OAT3 expression (Wilmer et al. 2010). The present finding on RPTEC/TERT1 cells as well as the reports on ciPTECs might suggest that loss of OAT function could be a general artifact of in vitro culture of RPTECs caused by a yet unknown mechanism. However, since many well-established nephrotoxins are either substrates for OCTs or OATs (Morrissey et al. 2013), expression of both transporter classes is of key importance to obtain a comprehensive proximal tubule model. Nevertheless, the functional cation secretion present in RPTEC/TERT1 cells renders the cell line highly suitable to study drug–drug interactions occurring on this transport axis. Moreover, our recently established 3D cultivation model for RPTEC/TERT1 cells showed induction of many transporters including OATs (Secker et al. 2018) thus highlighting the promising prospects of RPTEC/TERT1 cells for nephrotoxicity testing.

In conclusion, we demonstrated that functional readouts using RPTEC/TERT1 cells add valuable information to standard cytotoxicity measurements. Interestingly, water reabsorption was particularly affected by cyclosporine A and tacrolimus, two nephrotoxins that did not affect cell viability. A promising approach to improve the prediction of nephrotoxicity using in vitro models would therefore combine classical endpoints with functional readouts. Especially the fact that TEER measurements as well as quantification of water and xenobiotic transport can be performed on line without termination of the experiment, renders the combination of these experiments with other readouts such as transcriptomics or proteomics approaches an attractive approach to detect the nephrotoxic potential of xenobiotics and therefore offer advanced opportunities to be used within pharmaceutical toxicity testing.

Material and methods

Chemicals and solutions

Cyclosporine A was purchased from Selleckchem (#S2286) and New England Biolabs (#9973). Tacrolimus was from Selleckchem (#S5003) and Alfa Aesar (#J63571). Zoledronic acid (#Z5744) was from ENZO Life Sciences. Cisplatin (#P4394), gentamicin (#G1264), dexamethasone (#D4902) and probenecid (#P8761) were from Sigma-Aldrich.

Cell culture

RPTEC/TERT1 cells were obtained from Evercyte GmbH (Vienna, Austria). Cells were cultured in a 1-to-1 mixture of DMEM and Ham's F-12 nutrient mix (Thermo Fisher) with 5 mM D-glucose final, supplemented with 2 mM GlutaMAX, 5 µg/ml insulin (Sigma, #I1882), 5 µg/ml transferrin (Sigma, #T2252), 5 ng/ml sodium selenite (Sigma, #S5261), 100 U/ml penicillin and 100 µg/ml streptomycin, 10 ng/ml EGF (Sigma, #E9644) and 36 ng/ml hydrocortisone (Sigma, #H0888). Cells were subcultured after establishing a contact-inhibited monolayer and reseeded at 30% density when used on plastic plates or 100% density for Transwell permeable supports (Corning, polyester membrane, 0.4 µm pore size). RPTEC/TERT1 cells were grown for at least 14 days before initiation of experiments with medium renewal every 2–3 days.

Cell viability

RPTEC/TERT1 cells were cultured on standard tissue culture plates for 16 days followed by exposure to compounds for either 24 h or 14 days with treatment renewal every 48 h. Subsequently, treatment was exchanged for fresh medium containing 0.5 mg/ml MTT followed by incubation for 45 min at 37 °C, 5% CO2. Cells were lysed using 95% isopropanol, 5% formic acid. Formazan absorption was measured at 550 nm using a Tecan M200Pro microplate reader. Absorption obtained for dead cells (treated with 0.1% Triton X-100) was subtracted from all other values and viability was calculated relative to non-treated controls and is given as percent.

xCELLigence assay

If not otherwise indicated, RPTEC/TERT1 cells were seeded on E-plates 96 (ACEA Biosciences Inc., San Diego, CA, USA) at 30% density and placed into the RTCA SP instrument (Roche Diagnostics, Mannheim, Germany). After a growth and maturation period of at least 14 days, cells were treated with compounds or solvent control (0.5% DMSO) or left non-treated. Cellular impedance was measured in intervals of 5 min for a total of 14 days. Treatment was renewed on Mondays, Wednesdays, and Fridays. Cell indices obtained for treated cells were normalized to non-treated cells and are given as percent.

Transepithelial electrical resistance (TEER)

RPTEC/TERT1 cells were seeded on 12-well trans-well permeable supports (Corning, #3460). TEER was quantified manually using a Millicell-ERS (Millipore). TEER measurements were performed approximately 5–6 h after medium or treatment renewal on Mondays, Wednesdays, and Fridays.

Water transport

Medium from apical and basolateral compartments was collected during medium or treatment renewal and quantified using an analytical scale. To analyze compound-induced effects, water transport was normalized to control treated (0.5% DMSO) cells and is given as percent.

Quantitative real-time PCR (qPCR)

Total RNA was isolated from RPTEC/TERT1 cells cultured on 6-well Transwell permeable inserts for 24 days using peqGOLD TriFast (VWR) according to manufacturer's protocol. Human kidney total RNA from a 70 year old Caucasian woman was purchased from life technologies (Product #AM7967; Lot #1,745,517). cDNA was synthesized from 1 µg RNA using the cDNA-Synthesis Kit H Plus (VWR). qPCRs were performed using the KAPA SYBR FAST qPCR Master Mix (VWR) on a CFX Connect real-time system (Bio-Rad) according to manufacturer's protocol. Cq values obtained for genes of interest were normalized to the mean of Cq values obtained for the reference genes HPRT1, βactin and RPL13A and are given as fold of this mean value. Gene-specific primer pairs used for amplification are given in Table S1. Specificity of all primers was confirmed by PCR product sequencing.

Transport of fluorescent probes

Vectorial transport of 4-Di-1-ASP (Asp^+, Molecular Probes, #D288) and Lucifer Yellow (LY, Sigma, #L0259) was analyzed after addition of 12.5 µM to either the apical of basolateral compartment. Fluorescence was quantified in 50 µl aliquots taken from both compartments after 0, 4, 18, 26, 41 and 47 h at 485 nm excitation, 590 nm emission for Asp^+ or excitation 428 nm, emission 540 nm for LY. Fluorescence intensity was normalized to values obtained at 0 h for the compartment where the probe was added. To account for water transport while quantifying compound-induced effect on Asp^+ transport, fluorescence obtained for the apical compartment at the final timepoint (47 h) was multiplied by the amount of medium present at this timepoint.

Statistical analysis

Unless indicated otherwise data are presented as mean ± SEM. Sample size (n) indicates the number of independent experiments performed. Statistical analysis was determined using GraphPad Prism Version 5.04 (La Jolla, CA, USA) using statistical tests as indicated in figure legends.

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