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Current Opinion in Toxicology

Nephroscreen: A robust and versatile renal tubule-on-a-chip platform for nephrotoxicity assessment

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Abstract

Proximal tubule epithelial cells are the main driver of renal transport and secretion of xenobiotics, making them susceptible to drug-induced kidney injury. Cell-based assays are a meaningful alternative to animal testing to detect nephrotoxicity and contribute to the 3Rs (refine, reduce, replace animal experimentation). Here we report on a high-throughput, three-dimensional microfluidic platform (Nephroscreen) to detect drug-induced nephrotoxicity. Toxicologically relevant parameters were used to assess cell viability, functional epithelial barrier integrity, and interactions with specific transporters (P-glycoprotein: P-gp and multidrug resistance–associated protein 2/4: MRP2/4). Nephroscreen allowed the combination of a variety of read-outs, including imaging, extracellularly released markers, intracellular markers, and functional assays. Nephroscreen is compatible with automated pipetting, proved to be amenable to long-term experiments (at least 11 days), and was easily transferred between laboratories. The compelling data originate from several published reports on the development and implementation of this platform to detect nephrotoxicity and drug–transporter interactions. The reports demonstrate that Nephroscreen could be used to detect the nephrotoxic liabilities of the tested compounds. Future directions should include additional test compounds and thorough validation of its performance.

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Current Opinion in Toxicology 2021, **25**:42–48

This review comes from a themed issue on **Translational Toxicology**

Edited by **Roos Masereeuw** and **Damiën van Berlo**

For complete overview of the section, please refer the article collection - Translational Toxicology

Available online 22 March 2021

https://doi.org/10.1016/j.cotox.2021.03.001

Keywords

Renal proximal tubule, Nephrotoxicity, Drug screening, Drug–transporter interaction, miRNA, Kidney-on-a-Chip, Microfluidics.

Background

Renal proximal tubules are susceptible to drug-induced kidney injury (DIKI) [1], which can be a dose-limiting factor in pharmacotherapy and potentially lead to organ failure [2–4]. The clinical incidence of DIKI is in the range of 14%–26% and much higher than the number of drug candidates (around 2%) that fail in preclinical development because of the risk of nephrotoxicity [5]. This mismatch demonstrates the need for better, predictive, human-relevant models to screen for nephrotoxic liabilities of substances during the drug discovery and development process. The kidney is not only a target organ for potential toxicities but is also actively involved in the excretion of substances. One-third of drugs and drug candidates tested are (partially) excreted via the urine after dosing to humans [6,7]. Active excretion of xenobiotics in the kidney occurs mainly via secretion by the proximal tubule epithelial cell (PTEC) of the nephron [8]. This epithelium consists of a polarized monolayer of cells joined by tight junctions that form a barrier, separating

the vasculature on the basolateral side from the tubular fluid on the apical side. A human-relevant *in vitro* test system can help avoid or reduce the number of animal studies in drug discovery and therefore contribute to the 3Rs (replacement, reduction, and refinement of animal studies). Such an optimized *in vitro* system should not only help detect the nephrotoxic potential of substances but also uncover specific features related to their renal excretion.

In recent years, three-dimensional (3D) microfluidic *in vitro* models of PTECs, also referred to as "proximal tubule-on-a-chip," gained significant interest as predictive platforms for nephrotoxicity in drug development [9]. These 3D microfluidic models are expected to outperform *in vitro* two-dimensional (2D) PTEC models. The latter lack important *in vivo* characteristics, such as cell-extracellular matrix (ECM) interaction, tubular architecture, and constant fluid shear stress. These shortcomings limit their relevance and predictivity with regard to nephrotoxicity [1,9,10]. In contrast, renal proximal tubule-on-a-chip displays more physiological characteristics, such as increased tight-junction formation (zonula occludens-1 expression) and increased number of cilia and microvilli at the apical membrane [11,12]. Moreover, functional features such as albumin uptake and increased P-glycoprotein (P-gp) activity as well as nephrotoxicity induced by cisplatin show close resemblance with clinical observations [11,12].

Despite encouraging developments, implementation of a renal proximal tubule-on-a-chip in pharmaceutical industry nephrotoxicity screening is limited by the complexity and low throughput of most models, as they often consist of one chip connected to pumps and tubing to generate and maintain flow [11,12]. This reduces throughput and leads to technical hurdles such as increased drug substance requirements and larger media volumes to perfuse the system and to problems such as clogging and bubble formation in the perfusion tubes. Furthermore, most renal proximal tubule-on-a-chip models described so far lack basolateral and apical compartments [11] or make use of a two-compartmental model separated by cells cultured on an artificial semipermeable membrane ignoring cell−ECM interactions [12]. Here, we discuss the recent application of the OrganoPlate, a three-lane microfluidic platform able to accommodate 40 independent chips in the footprint of a 384-well microtiter plate (OrganoPlate, Mimetas BV, 4003 400B; Figure 1). The culture and treatment of PTECs within this microfluidic system and the subsequent detection of cellular responses are the basis for the Nephroscreen platform. The choice of an appropriate renal cell source is another important factor in this proximal tubule-on-a-chip for toxicity testing. Although freshly isolated primary PTECs show physiological characteristics in a nephrotoxicity screening proximal tubule-on-a-chip model [13], their scarce availability limits the throughput of this model and donor-to-donor variabilities impair the interpretation of the data. Using suitable, immortalized renal PTECs helps overcome these problems and enhances reproducibility across different laboratories [13]. The 3D microfluidic platform (Nephroscreen) for the detection of drug-induced nephrotoxicity was specifically designed to fulfill the requirements of drug discovery, putting emphasis on defining standardized experimental procedures (standard operating procedures) and ensuring the transferability between laboratories. Nephroscreen can also be considered a tool to replace animal experiments, as it represents an alternative toward animal testing for the detection of nephrotoxicity and thus supports the 3Rs.

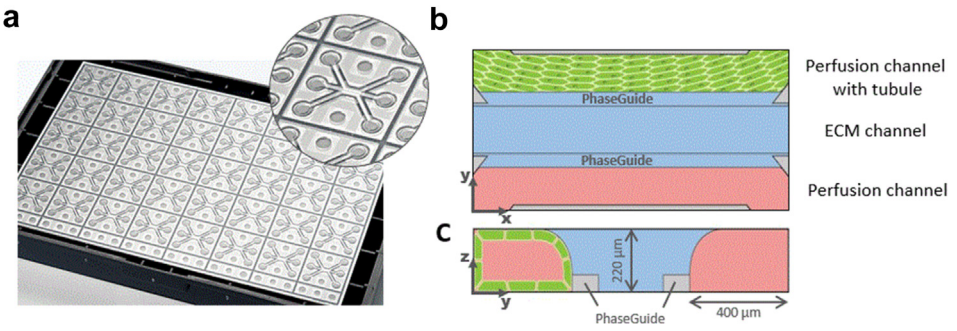
Here, we summarize the results of several publications on this platform. Nephroscreen was first implemented in different laboratories to perform short-term experiments with four selected model human nephrotoxic drugs (cisplatin, tenofovir, tobramycin, and cyclosporin A), known to damage the renal proximal tubule. Subsequently, a similar methodological approach simulating "real-life" compound testing was applied to assess the nephrotoxic potential of eight substances (R1, R2, R3, G1, G2, G3, P1, and P2) provided by pharmaceutical companies in a blinded manner [14]. These compounds were selected based on prior knowledge of their nephrotoxic potential from preclinical and clinical data. Finally, the platform was also amenable to the performance of subchronic, long-term (11 days) *in vitro* exposures to cefepime [14].

Experimental approach

Most data presented in this review were generated in a multilaboratory collaboration. A combination of assays described previously [15−17] and used in the context of the screening platform named Nephroscreen was performed at three different experimental facilities: the laboratories of the School of Life Sciences (FHNW, Muttenz, Switzerland), the Department of Pharmacology and Toxicology (Radboud University Medical Center, Nijmegen, the Netherlands), and the facilities of Mimetas (Leiden, The Netherlands).

The experiments included implementing two human renal tubular cell lines: (1) ciPTEC-OAT1, conditionally immortalized PTEC overexpressing the organic anion transporter 1 (OAT-1), Cell4Pharma.com, and (2) RPTEC, Kidney PTEC Control Cells, SA7K Clone, Sigma−Aldrich, Schnelldorf, Germany, MTOX1030. The selection of the cell lines, an important methodological consideration, followed the "fit-for-purpose" principle. Both cell lines were of human origin and amenable to medium to high-throughput applications. On the one hand, ciPTEC-OAT1 was optimized by overexpressing drug transporter OAT1, increasing its

Figure 1



Schematic depiction of the used microfluidic device, the OrganoPlate **(a)** Image of the back side of the 3-lane OrganoPlate. The microfluid network is positioned in between a glass sandwich of two microscope grade glass plates, which are attached to the bottom of a standard 384-titer well plate. Access to the microfluidic system is facilitated via the top wells. One OrganoPlate comprises in total 40 chips **(b)** Schematic of one chip presenting two perfusion channels and an extracellular matrix (ECM) channel in the middle. The top perfusion channel represents the apical side of the epithelial barrier **(c)**.

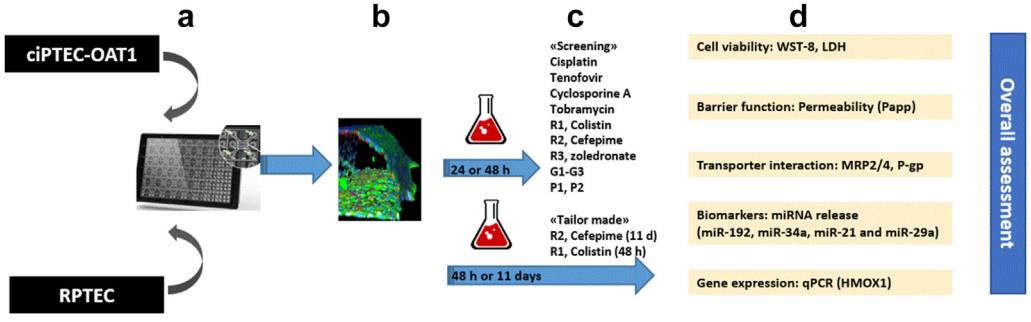
susceptibility to anionic substances such as tenofovir. On the other hand, RPTEC cells showed a very good performance for the assessment of drug–drug interactions. Cell culture conditions and plating on the OrganoPlate have been described in our previous work [15–17]. The kidney tubules developed in the OrganoPlate were kept under flow and exposed to the test compounds apically and basally for 24 or 48 h, although longer incubation times simulating subchronic exposure were also performed. The selection of the test concentrations for each reported substance was based on clinical data (whenever available), *in vivo* toxicity data (in one or two animal species), and preliminary cytotoxicity data in 2D obtained with ciPTEC-OAT1.

As represented in Figure 2, the effects of the treatments on the cells were assessed by a variety of endpoints, including cell viability (WST-8 assay), biochemical parameters (lactate dehydrogenase and β-N-acetylglucosaminidase release), molecular biology measurements (release of microRNA [miRNA] and gene expression), and functional assays (barrier permeability and drug transporter activity). The overall outcome is summarized in Table 1.

Assessment of cytotoxicity and tubular damage

Among the tested substances used to set up the Nephroscreen platform, cisplatin, tenofovir, cyclosporin A, and tobramycin are known to cause mild to severe cytotoxicity in 2D confluent monolayers of ciPTEC-OAT1 [15]. In the 3D microfluidic system discussed here, cell viability was measured upon a 48-h exposure to cisplatin, tenofovir, cyclosporin A, and tobramycin in ciPTEC-OAT1 in the OrganoPlate. The significant reduction in cell viability after 48 h was consistent across three laboratories and agreed with previously reported findings [14,15]. The viability of RPTEC was also affected, but these cells were generally less responsive to the four substances than ciPTEC-OAT1. As expected, the presence of OAT1 is a requirement to detect tenofovir-induced toxicity, as this transporter mediates the drug's uptake, whereas the transporter is absent in RPTEC. These findings clearly underline the

Figure 2



Schematic representation of the experimental procedure **(a)** Either ciPTEC-OAT1 or RPTEC cells were cultured in a three-lane OrganoPlate **(b)** Cells form a perfused tubular structure in the channel and **(c)** are treated for 48 h ("screening") or following a "tailor-made" protocol with several compounds **(d)** Several parameters are analyzed to detect compound-induced toxicity or impaired functionality.

Table 1 Overall assessment of significant effects published by Vormann et al., 2021 [14]. Exposures were generally performed for 48 h. Tailor-made studies: R1 (colistin) dose–response curve (9–1000 μM) over 48 h; R2 (cefepime) dose–response curve (90–1000) over 11 days. miRNA release refers to the combined assessment of a panel of four miRNAs (miR-192, miR-34a, miR-21, and miR-29a).

	Cytotoxicity (LDH, WST) (ciPTEC-OAT1 and RPTEC)	Barrier function (RPTEC)	Interaction with transporters (ciPTEC-OAT1)	miRNA release (ciPTEC-OAT1)	Cytotoxicity (ciPTEC-OAT1) Tailor made	Induction of HMOX1 (ciPTEC-OAT1) Tailor made
Cisplatin	↓ viability			↑ release		
Tenofovir	↓ viability (*ciPTEC-OAT1*)			↑ release		
Tobramycin	↓ viability	↑ Papp		↑ release		
Cyclosporine A	↓ viability	↑ Papp		↑ release		
G1	↓ viability	↑ Papp		↑ release		
G2			MRP2/4			
G3	↓ viability	↑ Papp	MRP2/4 P-gp	↑ release		
R1 (colistin)	↓ viability	↑ Papp		↑ release	↓ viability IC50 ~250 μM	↑ expression ≥ 60 μM
R2 (cefepime)					↓ viability IC50 ~300 μM	↑ expression ≥30 μM
R3 (zoledronate)	↓ viability	↑ Papp		↑ release		
P1			P-gp	↑ release		
P2	↓ viability	↑ Papp	MRP2/4 P-gp	↑ release		

importance of the cell line selection regarding the expression of transporters and their overall physiology. Arguably, cell lines genetically modified to express additional transporters (e.g. OAT-3) could be advantageous for toxicity screening. Future studies could, for example, also assess the performance of a recently developed cell line overexpressing OAT-3 [18].

The reported Nephroscreen also performed well with substances that were assayed in a blinded manner. Most of these eight pharmacologically active substances, provided by pharmaceutical companies, led to specific findings concordant with the toxicity data unveiled *a posteriori* (Table 1). Three of the substances, G2, P1, and R2 (cefepime), did not cause measurable loss of cell viability. However, G2 and P1 led to functional effects on specific transporters (as described below).

The most notable exception was cefepime (R2), which failed to cause any significant changes in PTECs exposed acutely to the compound. Cefepime is a broad-spectrum antibiotic that has been associated with clinical adverse drug reactions such as acute kidney injury [19]. In rats, cefepime caused proximal tubular injury 4 and 8 days after a 5-day treatment [20]. The additional, subchronic (11 days), tailor-made tests performed with cefepime showed that this substance has a liability for nephrotoxicity that was not uncovered with short-term (24 and 48 h) treatments. In the subchronic setting, cytotoxicity was observed with an IC50 of approximately 300 μM accompanied by a dose-dependent increase of expression of heme oxygenase 1 (HMOX1) at concentrations of 30 μM or higher. Similarly, a tailor-made, dose–response evaluation of colistin (R1) showed an IC50 of approximately 250 μM and concomitant induction of HMOX1 expression at concentrations of 60 μM and above.

These results highlight that the Nephroscreen system is not only a suitable screening tool but is also amenable to tailor-made compound-specific evaluations. This is often necessary for compounds, such as cefepime, which require longer times to elicit the response, or tenofovir that requires cells expressing OAT1 to be able to display toxicity [18,21].

Epithelial barrier function

Cell damage is not the only parameter relevant to the function of a complex organ such as the kidney. Renal epithelial cells form a tight barrier that separates the urine on the apical compartment from the blood circulation at the basolateral side [22]. Nephroscreen enables the assessment of the barrier function of renal epithelial cells under flow conditions. Substances leading to cell damage detected by cytotoxicity to ciPTEC-OAT1 also caused a time-dependent, quantifiable increase in the leakiness of the RPTEC barrier, providing an independent measure of their effect on the kidney tubules.

Drug–transporter interactions

Drug interaction with cellular transporters is another important feature that can result in high intracellular

concentrations of xenobiotics and lead to nephrotoxicity. Specific interactions with P-gp and multidrug resistance–associated protein (MRP) 2/4 were determined in ciPTEC-OAT1, as this cell line expresses both efflux pumps [17]. As expected, cyclosporine A, a substrate and inhibitor of P-gp interacted with this transporter in the Nephroscreen model. Among the eight tested substances G2, G3, P1, and P2 interacted either with P-gp and/or MRP2/4. In particular, for G2, this is an interesting finding, as this compound does not seem to cause any toxicity or functional impairment at the tested concentrations, but affected MRP2/4 function. Interactions with the tested transporters were not observed with cefepime (R2). This is in line with the fact that although most of the injectable cephalosporins have an inhibitory effect on MRP4 transport activity, cefepime does not [23].

Novel biomarkers of nephrotoxicity

Small regulatory RNAs known as miRNAs are single-stranded, noncoding RNAs consisting of 21–25 nucleotides. They are not only present in cells but are also released into biofluids, including plasma, serum, urine, and cell culture media [24]. Thus, released miRNAs can act as sensitive biomarkers of cell damage. In Nephroscreen, four miRNAs were selected as potential nephrotoxicity biomarkers (miR-192, miR-34a, miR-21, and miR-29a) [15]. All four miRNAs showed similar secretion patterns and were increased in the medium of cells treated with the compounds G1, G3, R1, R3, P1, and P2. In concordance with the other results, cefepime (R2) did not lead to an increased release of miRNAs. On the other hand, exposure to colistin (R1) led to the release of miRNAs at 125 μM, a concentration considered subtoxic based on all other measured parameters. This further supports the concept that miRNA release may be a more sensitive nephrotoxicity marker [14].

Microphysiological systems for detection of nephrotoxicity

Besides the Nephroscreen platform discussed in this article, other reports have promoted the use of microfluidic and microphysiological systems (MPS) for the reconstruction of a 3D microenvironment that can mimic the structural, mechanical, and physiological properties of human tissue and be applied for toxicity evaluation. A review of several systems has been recently published by Cong et al. [25]. Specifically for the proximal tubule of the kidney, complex *in vitro* systems have also been described [26]. Recently, Lin et al. described a multitissue chip that combined cultured kidney with liver cell lines and was able to detect the toxicity of cyclosporine A [27]. Similarly, a complex coculture system consisting of PTECs and peritubular capillary endothelial cells was recently discussed by Yin et al. [28]. Also, the detection of biomarkers of kidney injury, such as kidney injury marker-1 (KIM-1) from proximal tubular cells in an MPS, has recently been reported [29]. These independent results also show that MPS and 3D cultures are key for appropriate function of PTECs in cell culture and can be used to detect toxicity to the proximal tubule *in vitro*. They also point out the potential of the systems to address specific research questions based on tailor-made studies. However, many systems suffer from a lack of versatility and low throughput, inherent to the underlying, complex technologies. Nephroscreen, in contrast, has been optimized to be used in a medium-throughput screening mode, including diverse endpoints that greatly expand the potential applications.

Summary and conclusions

Nephroscreen was designed as a medium-throughput MPS to assess toxicity to the renal proximal tubule. It was implemented with two well-characterized human kidney cell lines cultured in the OrganoPlate. Several parameters, including nucleic acid analysis (quantitative polymerase chain reaction), biochemical endpoints (enzyme activities and enzyme-linked immunosorbent assay), retention of fluorescent dies (interaction with specific transporters), and imaging (barrier function and morphology), were established to assess cellular toxicity and functional responses to the test compounds. Nephroscreen proved to be a robust and versatile platform, able to support the screening of several substances in parallel. It can also be implemented for more complex mechanistic studies, using a tailor-made design, to provide additional information. As an example, the system could be used for long-term *in vitro* exposures, opening possibilities for kinetic investigations and repeated dosing regimens.

An interesting point is the use and interpretation of the data from Nephroscreen for decision making in the context of pharmaceutical development. In the main cellular models summarized in this short review, the results led to a clear identification of cisplatin, tobramycin, and cyclosporine A as potentially nephrotoxic in both tested cell lines. Tenofovir, on the other hand, was only nephrotoxic to ciPTEC-OAT1. These results met the expectations based on prior knowledge on the expression of functional transporters. For the eight compounds provided by pharmaceutical companies and evaluated in a blinded fashion, five were clearly identified as potentially nephrotoxic: G1, G3, R1 (colistin), R3 (zoledronate), and P2. Compounds P1 and G2 showed interactions with transporters: P-gp and MRP2/4, respectively. P1 also led to a significant increase in miRNAs. In a real-life setting, these findings would warrant additional investigations with alternative study designs. On the basis of our current knowledge from animal studies, P1 and G2 showed the potential of causing damage to the proximal tubule (unpublished data). Compound R2 (cefepime), however, did not

cause any effect in a short-term setting. Longer exposure times were required to uncover its potential toxicity. This antibiotic is in clinical use and has a relatively low incidence of nephrotoxicity based on few clinical studies reporting impaired renal function [30]. Therefore, the classification of Nephrotube seems to be an accurate reflection of the clinical data.

The presented results are very promising; however, the sensitivity and specificity of Nephroscreen need to be further evaluated through the systematic generation of more data from an expanded tool set of substances. Future experiments should include compounds with other target organs of toxicity (non-nephrotoxic) as well as nontoxic compounds. Also, side-by-side comparison with conventional 2D cell cultures could be performed to achieve direct comparison and therefore show the value of this model over simpler systems. However, 2D models are unlikely to compete with the information obtained from Nephroscreen, as this platform is not only useful for drug toxicity assays but also for the determination of drug–transporter interactions and therefore for clinically relevant, potential drug–drug interactions.

Authors' contributions

J.V. contributed to conceptualization, investigation, writing the original draft, methodology, visualization, and formal analysis. M.K.V. contributed to conceptualization, investigation, methodology, visualization, formal analysis, and writing the original draft. H.L.L. contributed to supervision, conceptualization, methodology, review and editing the article, and project administration. J.J. contributed to conceptualization, resources, and reviewing and editing the article. S.J.T. contributed to conceptualization and methodology. F.G.M.R. contributed to conceptualization, supervision, and reviewing and editing the article. B.J. contributed to resources and reviewing and editing the article. A.R. contributed to resources and review and editing the article. S.L. contributed to resources and reviewing and editing the article. J.W.P. contributed to resources and reviewing and editing the article. A.A.N. contributed to resources and review and editing the article. R.M. contributed to supervision, conceptualization, resources, and reviewing and editing the article. M.J.W. contributed to supervision, conceptualization, project administration, and reviewing and editing the article. L.S.-D. contributed to supervision, conceptualization, project administration, and writing the original article.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

M.K.V., H.L.L., J.J., and S.J.T. are employees of Mimetas B.V. The OrganoPlate is a registered trademark of Mimetas B·V. M.J.W. is an employee of Cell4Pharma, which markets the ciPTEC-OAT1 cell line.

Acknowledgements

This project was supported under the Crack-it challenge 15 (Nephrotube) project no. 37497–25920, an initiative of the National Center for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs).

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* of special interest

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