



Drug Discovery–Development Interface

Implementation of a Human Renal Proximal Tubule on a Chip for Nephrotoxicity and Drug Interaction Studies



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ARTICLE INFO

Article history:

Received 23 December 2020

Revised 26 January 2021

Accepted 26 January 2021

Available online: 03 February 2021

Keywords:

Renal-proximal-tubule-on-a-chip

Drug-screening

Drug-transporter interaction

miRNA

Microfluidics

Pharmaceutical

ABSTRACT

Proximal tubule epithelial cells (PTEC) are susceptible to drug-induced kidney injury (DIKI). Cell-based, two-dimensional (2D) *in vitro* PTEC models are often poor predictors of DIKI, probably due to the lack of physiological architecture and flow. Here, we assessed a high throughput, 3D microfluidic platform (Nephroscreen) for the detection of DIKI in pharmaceutical development. This system was established with four model nephrotoxic drugs (cisplatin, tenofovir, tobramycin and cyclosporin A) and tested with eight pharmaceutical compounds. Measured parameters included cell viability, release of lactate dehydrogenase (LDH) and N-acetyl-β-D-glucosaminidase (NAG), barrier integrity, release of specific miRNAs, and gene expression of toxicity markers. Drug-transporter interactions for P-gp and MRP2/4 were also determined. The most predictive read outs for DIKI were a combination of cell viability, LDH and miRNA release. In conclusion, Nephroscreen detected DIKI in a robust manner, is compatible with automated pipetting, proved to be amenable to long-term experiments, and was easily transferred between laboratories. This proof-of-concept-study demonstrated the usability and reproducibility of Nephroscreen for the detection of DIKI and drug-transporter interactions. Nephroscreen it represents a valuable tool towards replacing animal testing and supporting the 3Rs (Reduce, Refine and Replace animal experimentation).

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Introduction

Renal proximal tubules are susceptible to drug-induced kidney injury (DIKI),¹ which can be a dose-limiting factor in pharmacotherapy and lead to kidney failure in patients. Up to 14–26% of acute kidney injury cases are caused by DIKI.^{2–4} This clinical observation is in stark contrast to the low number of drug candidates, 2% of total, that fail in preclinical development due to

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nephrotoxicity.⁵ This disparity demonstrates the need for better clinically predictive models for nephrotoxicity for use in discovery and early development. Screening-friendly, human-relevant *in vitro* test systems will also help replace animal studies in drug discovery and contribute to the 3 Rs (Replacement, Reduction and Refinement of animal studies). One third of drugs and drug candidates tested are (partially) excreted via the urine following dosing to humans.^{6,7} Active secretion of xenobiotics in the kidney takes place mainly via the proximal tubule epithelial cell (PTEC) of the nephron.⁸ A polarized monolayer of PTECs, joined by characteristic epithelial tight junctions, separates the vasculature on the basolateral side from the tubular fluid on the apical side and regulates solutes and water by active transport mechanisms.

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 Currently employed *in vitro* two-dimensional (2D) PTEC models lack important *in vivo* characteristics, such as cell-extracellular matrix (ECM) interaction and fluid shear stress (FSS), which limits their relevance and predictivity with regards to nephrotoxicity.^{1,9,10} Renal proximal tubule-on-a-chip showed improved characteristics, such as increased tight-junction formation (ZO-1 expression), and increased number of cilia and microvilli at the apical membrane.^{11,12} Features like albumin uptake and increased P-glycoprotein (P-gp) activity in renal proximal tubule-on-a-chip, and nephrotoxicity induced by cisplatin showed close resemblance with *in vivo* observations.^{11,12}

Implementation of renal proximal tubules-on-a-chip in large-scale nephrotoxicity screening is limited due to the complexity and low throughput of most models, often consisting of one chip connected to pumps to generate flow.^{11,12} Furthermore, most renal proximal tubules-on-a-chip described lack basolateral and apical compartments,¹¹ or make use of a two-compartmental model separated by cells cultured on a semi-permeable membrane ignoring cell-ECM interaction.¹² Choice of renal cell source is another important factor in the proximal tubule-on-a-chip. Although freshly isolated primary PTECs show more physiological characteristics in a nephrotoxicity screening proximal tubule-on-a-chip model,¹³ availability of primary PTECs limits the throughput of this model. Using immortalized renal PTECs would not only overcome this problem but would also enhance reproducibility across different laboratories.¹³

This study aimed to assess a high throughput, 3D-microfluidic platform (Nephroscreen) for the detection of drug-induced nephrotoxicity: This platform was specifically designed to fulfill requirements of pharmaceutical companies and promote alternative methods to animal testing in support of the 3Rs. Nephroscreen was first challenged with four selected model human nephrotoxic drugs (cisplatin, tenofovir, tobramycin and cyclosporin A) that affect the proximal tubule. This was complemented with eight additional compounds provided by three pharmaceutical companies for evaluation in a blinded manner. The data were generated at several laboratories to ensure that the platform is robust and transferable.

In Nephroscreen we combined a microfluidics platform with suitable cell lines and appropriate assays. An automatable, microfluidic plate consisting of multiple chips, the OrganoPlate, was combined with renal PTEC cell lines, exposed to FSS induced through passive levelling by gravity.^{14–16} The chosen cells, conditionally immortalized PTEC overexpressing OAT1 (ciPTEC-OAT1) or pseudo-immortalized renal PTEC (RPTEC), are able to establish a polarized epithelium expressing functional transporters.^{15,16} The ciPTEC-OAT1 has been engineered to overexpress OAT1, in addition to other transporters, such as P-gp and OCT2, expressed in the parental ciPTEC line.^{17,18} Therefore, they are an ideal cell line to

study the toxicity of substances requiring transport into the cell via these transporters. RPTEC, on the other hand, were chosen due to their performance in establishing leak-tight epithelial barriers. Thus, both implemented cell lines show different, complementary strengths that were exploited by measuring suitable endpoints: ciPTECs were utilized for cytotoxicity, biomarker, and transporter activity measurements while RPTECs performed very well in functional assays such as barrier function.

Cellular damage was studied using various read-outs, such as enzymatic production of formazan (WST-8 assay), indicator of cell viability and release of lactate dehydrogenase (LDH) and N-acetyl- β -D-glucosaminidase (NAG), measures for membrane integrity. Molecular markers of cellular stress included extracellular levels of specific miRNAs: mir34a, mir21, mir192, and mir29a,^{14,15} as well as gene expression of heme oxygenase 1: HMOX1 and neutrophil gelatinase-associated lipocalin: NGAL.¹⁹ Functional parameters for the epithelial monolayer consisted of barrier integrity testing as well as interactions with P-gp and multidrug resistance-associated proteins 2 and 4 (MRP2/4).^{15,16}

This study was part of the NC3Rs (<https://www.nc3rs.org.uk/>) crackIT challenge *Nephrotube*, launched to generate predictive, animal free-systems for the detection of nephrotoxicity. The results showed the potential of the Nephroscreen for predictive, animal free-detection of nephrotoxicity and drug-transporter interactions.

Materials and Methods

Experimental Workflow: Multi-laboratory Collaboration

The combination of assays described previously,^{14–16} used towards a functional Nephroscreen, were performed in laboratories at the School of Life Sciences, University of Applied Sciences Northwestern Switzerland in Muttenz, Switzerland (lab B), the department of Pharmacology and Toxicology, Radboud University Medical Center, Nijmegen, The Netherlands (lab N) and Mimetax, Leiden, The Netherlands (lab L). Transferability, robustness and reproducibility of Nephroscreen was established by performing experimental procedures in the three laboratories using two renal cell lines (ciPTEC and RPTEC). The experimental workflow is depicted in Fig. 1. For cell viability assessment, tubules of both cell lines were cultured and then exposed to nephrotoxicants for 24 or 48 h (lab L). Measurement of cell viability included enzymatic production of formazan using WST-8 (Lab L), LDH release (lab N) and gene expression of toxicity markers (lab L and N). In addition, RPTEC were tested for their barrier function (Lab L). In lab N, the effect of the nephrotoxicants on the transporter functionality of P-gp and MRP2/4 was assessed in ciPTEC-OAT1. The release of selected miRNAs (mir-21, -29a, -34a and -192) and secretion of the enzyme N-acetyl- β -D-glucosaminidase (NAG) in ciPTEC-OAT1 were measured in lab B.

Cell Culture

CiPTEC-OAT1 cells (Cell4Pharma, Oss, NL) were cultured in T75 flasks in a 1:1 mixture of Dulbecco's modified Eagle's medium and nutrient mixture F-12 without phenol red (DMEM-F12, Gibco, ThermoFisher Scientific, Carlsbad, USA, 11039-021) supplemented with insulin-transferrin-sodium selenite media supplement (Sigma-Aldrich, I1884, insulin 5 μ g/mL; transferrin 5 μ g/mL; sodium selenite 5 ng/mL), 36 ng/mL hydrocortisone (Sigma-Aldrich, H0135), 10 ng/mL epidermal growth factor (Sigma-Aldrich, E9644), 40 pg/mL 3-iodothyronine (Sigma-Aldrich, T5516), 10% v/v fetal bovine serum (FBS, Gibco, 16140-071 or Greiner Bio-One, Alphen aan den Rijn, The Netherlands, 758093), and 1% v/v penicillin/streptomycin (P/S, Sigma-Aldrich, P4333), referred to as ciPTEC

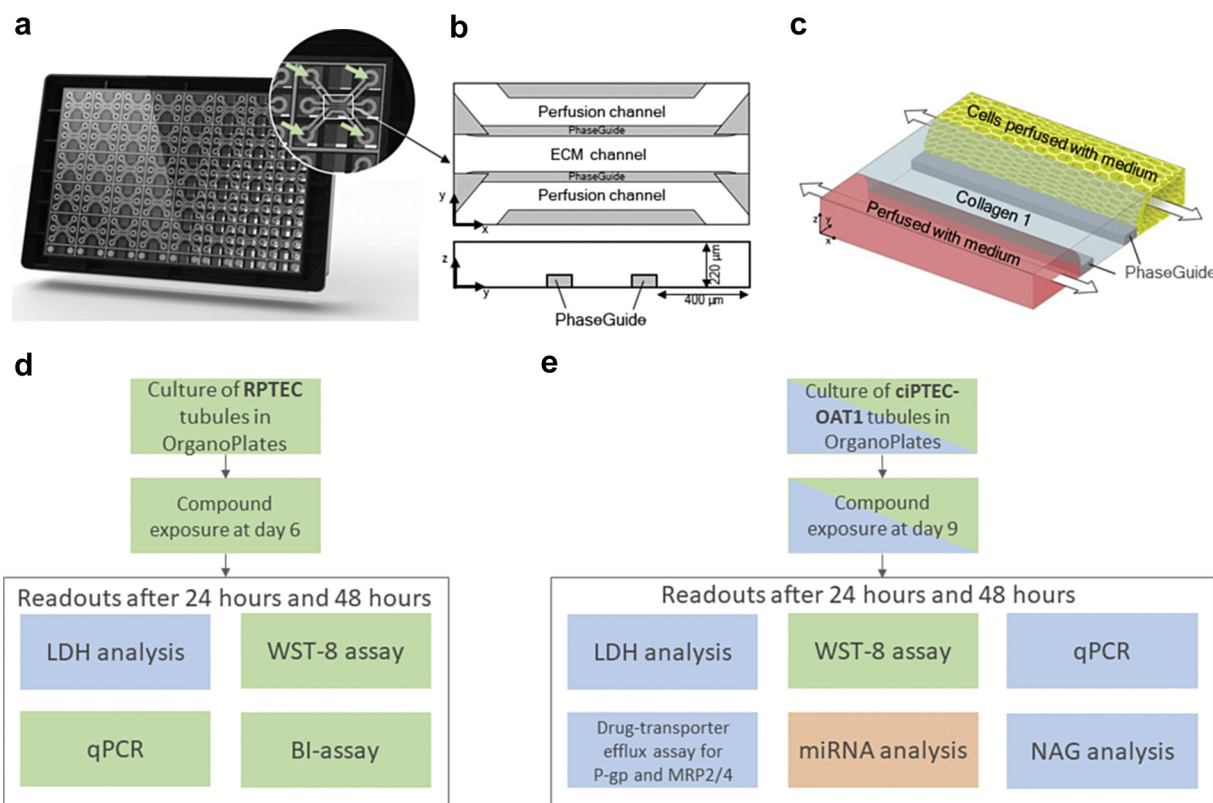


Fig. 1. Overview of the microfluidics platform, the cell culture, exposure, and read-out methods performed on ciPTEC-OAT1 tubules and RPTEC tubules. (a) Image of the back side of the OrganoPlate 3-lane. The microfluidic network is positioned in-between a glass sandwich of two microscope grade glass plates 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 as 8 “wells” compose one chip. Green arrows indicate the inlets used for compound dosing. (b) Schematic of one chip presenting two perfusion channels and the extracellular matrix (ECM) channel in the middle. Channels are divided by 55 μm high ridges called PhaseGuide (grey bars) with act as pressure barriers. (c) Artist impression of one chip. The chip was loaded with collagen 1 (blue) to the ECM channel and proximal tubule cells (yellow) were seeded to the top channel. After cell attachment medium was added to both perfusion channels and perfusion was started (indicated by white arrows). (d) and (e) Flow charts indicating the culture of RPTEC and ciPTEC-OAT1, respectively. Each cell line was cultured following optimized conditions before exposure to compounds for 24 and 48 h. (d) Readouts for RPTEC included lactate dehydrogenase (LDH)-release, cell viability assay using WST-8 assay, determination of barrier integrity (BI) and collection of total RNA for quantitative PCR (qPCR). (e) Readouts for ciPTEC-OAT1 included lactate dehydrogenase (LDH)-release, N-acetyl- β -D-glucosaminidase (NAG) release, cell viability assay using WST-8 assay, determination of drug-transporter efflux assay, collection of release RNA for miRNA determination, and collection of total RNA for quantitative PCR (qPCR). Assays were performed and optimized in three laboratories and the obtained results are colour coded: Lab B, orange, Lab L (green), Lab N (blue).

complete medium. Cells were incubated in a humidified incubator (33 °C, 5% v/v CO₂) and every 2–3 days ciPTEC complete medium was changed. At 90–100% confluency cells were washed with HBSS (Sigma-Aldrich, H6648 or Gibco, 14025-100), detached with accutase (Sigma-Aldrich, A6964), pelleted (200–300 \times g, 5 min), and used for passaging (10,000–20,000 cells/cm²) or for seeding in the OrganoPlate. Cells were used for experiments between passage 52 and 65.

RPTEC cells (Kidney PTEC Control Cells, SA7K Clone, Sigma-Aldrich, Schnellendorf, Germany, MTOX1030) were seeded in T75 cell culture flasks coated with PureCol (Advanced BioMetrix, 5005-B, diluted 1:30 in cold Hank's balanced salt solution (HBSS, Sigma-Aldrich, H6648), 20-min incubation at 37 °C) in MEME alpha Modification (Sigma-Aldrich, M4526) supplemented with RPTEC Complete Supplement (Sigma-Aldrich, MTOXRCSUP), L-glutamine (1.87 mM, Sigma-Aldrich, G7513), Gentamicin (28 $\mu\text{g}/\text{mL}$, Sigma-Aldrich, G1397) and Amphotericin B (14 ng/mL, Sigma-Aldrich, A2942), referred to as RPTEC complete medium. Cells were incubated in a humidified incubator (37 °C, 5% v/v CO₂) and every 2–3 days RPTEC complete medium was changed. At 90–100% confluency cells were washed with HBSS, detached with accutase (Sigma-Aldrich, A6964), pelleted (140 \times g, 5 min), and used for seeding in the OrganoPlate. Cells for experiments were used up to passage 3.

OrganoPlate Culture

For all experiments, plates were seeded as described in our previous work.^{14–16} Briefly, a three-lane OrganoPlate (Mimetas BV, 4003 400B, Fig. 1) with a channel width of 400 μm and a height of 220 μm was loaded with 1.6–2 μL of ECM gel composed of 4 mg/mL collagen I (AMSbio Cultrex 3D Collagen I Rat Tail, 3447-020-01), 100 mM HEPES (Life Technologies, ThermoFisher Scientific, 15630), and 3.7 mg/mL sodium bicarbonate (Sigma-Aldrich, 320 S5761) to the middle inlet of all 40 chips. After polymerization of the ECM, HBSS was added on top of the collagen I and the plate was incubated in a humidified incubator (37 °C, 5% v/v CO₂) overnight. RPTEC or ciPTEC-OAT1 were detached from culture flasks and resuspended at a concentration of 10×10^6 cells per mL in RPTEC complete medium or 20×10^6 cells per mL in ciPTEC complete medium, respectively. Of the cell suspension, 2 μL was injected into each top inlet, followed by an addition of 50 μL medium to the same well. For control chips, no cell suspension was added. Subsequently, the OrganoPlate was placed for 4 h at an angle of about 75° into a humidified incubator (37 °C, 5% v/v CO₂ for RPTEC or 33 °C, 5% v/v CO₂ for ciPTEC-OAT1). After attachment of the cells, which was confirmed by light microscopy, 50 μL medium was added to the top outlet, bottom inlet and bottom outlet. The OrganoPlate was placed flat in an incubator (37 °C, 5% v/v CO₂ for RPTEC or 33 °C, 5% v/v CO₂

for ciPTEC-OAT1) on an interval rocker platform ($\pm 7^\circ$ angle, 8 min interval) enabling a bidirectional flow through the perfusion channels. At day 3, antibiotic free medium was used to refresh the medium in the chips, and the ciPTEC-OAT1 plates were transferred to a humidified incubator (37 °C, 5% v/v CO₂).

Compound Exposure

Cells were exposed for 24 or 48 h to four model nephrotoxicants and eight blinded compounds following the general experimental design depicted in Fig. 1. The selection of the test concentrations was based on doses where toxicological effects were seen *in vivo* and on preliminary cytotoxicity data in 2D obtained with ciPTEC-OAT1. Briefly, cells were seeded in 96-well flat-bottom plates at 35000 cells/cm², maintained until confluency, subsequently exposed for 24 or 48 h to a concentration range of each of the compounds diluted in ciPTEC complete medium and viability was determined (WST-8 assay). For Nephroscreen testing, a low and a high toxicity concentrations were selected. In absence of detectable toxicity, the two highest concentrations were chosen (data not shown). Detailed solvent, dilutions, and culture conditions for each substance are described in Table S1, while data on previous studies used for the determination of potential test concentrations are listed in Table S2, together with relevant toxicity information disclosed after data acquisition and analysis of data had been completed. For compounds R1 and R2, additional experiments including more concentrations and additional time points were performed in lab B. For R1, cells were exposed for 48 h and for R2, cells were exposed for 11 days, with medium changes every 2–3 days.

Using a pipette, 50 µL of the model compounds (cisplatin, tenofovir, tobramycin and cyclosporin A) or the eight blinded compounds (G1, G2 and G3; R1, R2 and R3; and P1 and P2) diluted in medium were dosed via each of the 4 inlets guiding to the microfluidic channels (details in Fig. 1). Medium from both the apical and basolateral perfusion channels was refreshed before exposure to the nephrotoxicants. For RPTEC, the exposure was started at day 6 and dilutions of nephrotoxicants were prepared in TOX medium (MEME alpha Modification (Sigma-Aldrich, M4526) supplemented with RPTEC Tox Supplement (Sigma-Aldrich, MTOXRTS), L-glutamine (1.87 mM, Sigma-Aldrich, G7513). For ciPTEC-OAT1, cells were exposed on day 9 and dilutions of nephrotoxicants were prepared in ciPTEC complete medium.

WST-8 Assay

Cell viability was determined using the Cell Counting Kit-8 (WST-8, Sigma-Aldrich, 96992) as described previously.^{14,15} Briefly, the WST-8 solution was diluted with ciPTEC complete medium or RPTEC TOX medium (ciPTEC-OAT1 or RPTEC respectively) and added to the channels of the OrganoPlate. After 20–60 min, absorbance was measured at 450 nm with a Multiskan™ FC Microplate Photometer (ThermoFisher Scientific) or with a Benchmark Plus microplate spectrophotometer (Bio-Rad, Veenendaal, The Netherlands). Viability was expressed as % of the control.

LDH Activity in Medium

LDH activity in the culture medium is a measure for membrane integrity. Medium was collected on ice after compound exposure and stored at –80 °C until further processing. After thawing, LDH activity was measured using an activity assay kit (Sigma-Aldrich, MAK066), following the manufacturer's protocol. In brief, 5 µL per sample was added to a flat bottom 96-well plate. In addition, a

calibration curve using a NADH (1.25 mM) standard was prepared. Assay buffer was added to a final volume of 50 µL per well and then a master reaction mix was added per well (1:1, v/v). After 3 min, absorbance was measured at 450 nm on a Benchmark Plus microplate spectrophotometer (Bio-Rad) every 5 min until absorbance measured in a sample was higher than the highest level of NADH in the calibration curve (12 nmol/well). Extracellular LDH activity was expressed as mU/mL.

NAG Measurement in Medium

The levels of β-N-acetylglucosaminidase (NAG) in supernatant are a measure for membrane integrity and are also used as a biomarker of kidney damage *in vivo*. They were determined using the NAG-Assay Kit (BioVision, K733-100), following provider's instructions. Briefly, 30 µL of the supernatant (cell culture medium) were adjusted to a volume of 70 µL with NAG assay buffer in a 96-well plate. To this, 55 µL substrate were added and incubated for 30 min at 37 °C, followed by the addition of 25 µL stop solution. After another 10 min at 37 °C, absorbance was measured at 400 nm in a Flexstation 3 (Molecular Devices). Absorbance of a standard dilution series of pNP (0–20 nmol of pNP/well) was measured in parallel and used for the calculations. Extracellular NAG activity was calculated (mU/mL) and expressed as % of the control.

Barrier Integrity Assay

The barrier integrity assay was performed as previously published on RPTEC.¹⁵ Briefly, the medium of the perfusion channel (apical side of the cells) was replaced with RPTEC TOX medium containing 0.5 mg/mL tetramethylrhodamine (TRITC)-dextran (4.4 kDa, Sigma-Aldrich, FD20S) and 0.5 mg/mL fluorescein isothiocyanate (FITC)-dextran (150 kDa, Sigma-Aldrich, T1287). Subsequently, plates were imaged with the ImageXpress Micro XLS-C High Content Imaging System (Molecular Devices). Leakage of the dyes from the lumen (apical compartment) to the basal compartment into the ECM was measured over time, and the ratio between the basal and the apical compartment was analyzed with Image J version 1.51n.²⁰ From these measurements, the apparent permeability index (P_{app}) was calculated as previously described,¹⁵ using the following formula:

$$P_{app} = \frac{\Delta C_{receiver} \times V_{receiver}}{\Delta t \times A_{barrier} \times C_{donor}} \left(\frac{cm}{s} \right)$$

$\Delta C_{receiver}$ is the measured normalized intensity difference of the ECM to the donor channel (apical compartment) at t_{0min} and t_{10min} , $V_{receiver}$ is the volume of the measured region in the ECM channel (0.0001 cm²), Δt is the time difference (10 min), $A_{barrier}$ is the surface of the ECM interface with the medium channel (0.0057 cm²), and C_{donor} is the donor concentration of the dextran dyes (0.5 mg/mL).

Gene Expression of Toxicity and Nephrotoxicity Markers

Total intracellular RNA was isolated from cells harvested from perfusion channels in the OrganoPlate using the RNeasy Micro Kit (Qiagen, Venlo, The Netherlands) as previously described.¹⁶ Complementary DNA (cDNA) was synthesized with Moloney Murine Leukemia Virus (M-MLV, Promega, M1705) reverse transcriptase, according to the manufacturer's protocol. Gene expression levels of heme oxygenase 1 (*HMOX1*) and neutrophil gelatinase-associated lipocalin (NGAL), encoded by the lipocalin-2 (*LCN2*), were measured using quantitative PCR (qPCR) with *GADPH* as reference. TaqMan Universal PCR Master Mix (Life Technologies, 4304437)

and gene specific primer-probe sets (*HMOX1*: Hs01110250_m1, *LCN2*: Hs01008571_m1 and *GAPDH*: Hs9999905_m1) were purchased from Applied Biosystems (ThermoFisher Scientific). The real-time PCR was carried out using a fluorometric thermal cycler (Qiagen, Rotor-Gene Q 3000 or Celtic Diagnostics, Corbett research PCR Rotorgene 6000). The PCR program consisted of 10 min of initial denaturation at 95 °C followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Fluorescence was detected at the end of each cycle at 510 nm (excitation 470 nm). Ct values were determined using the second derivative method. For each sample, dCt was calculated using Ct values of the gene of interest and the house-keeping gene (*GAPDH*). Treatment induced differential gene expression was calculated using the $-ddCt$ ($-dCt_{\text{treatment}} - dCt_{\text{control}}$) and fold changes as 2^{-ddCt} .

Detection of miRNAs in Medium

Total RNA was extracted from 80 μ L supernatant using the miRNeasy® Serum/Plasma Kit (Qiagen, 217184) following the manufacturer's protocol. miRNAs were reverse-transcribed using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems®, 4366596) and the miRNA-specific stem-loop primers for the miRNAs-21, -34a, -29a and -192 (Applied Biosystems™, TaqMan microRNA Assays #002438, #000426, #002112, and #000491, respectively). The reaction mix was prepared according to the manufacturer's instructions for a final reaction volume of 10 μ L with 3 μ L RNA extract. The conditions for reverse transcription were set for 30 min at 16 °C followed for 30 min at 42 °C and 5 min at 85 °C.

The real-time PCR was carried out using a fluorometric thermal cycler (Qiagen, Rotor-Gene Q 3000 or Celtic Diagnostics, Corbett research PCR Rotorgene 6000). The reaction mix contained TaqMan® Fast Advanced Master Mix 1x (Applied Biosystems™, 4444557), TaqMan microRNA Assay primer 1x (Applied Biosystems™) and 1.3 μ L cDNA in a final reaction volume of 20 μ L. The PCRs were run at 95 °C for 20 s followed by 40 cycles of 1 s at 95 °C and 20 s at 60 °C. All extracted RNAs were analyzed in technical duplicates; Ct-values of these two measurements were averaged and considered a single value.

Drug Transporter Assays

Drug-transporter interactions with P-gp and MRP2/4 were studied in ciPTEC-OAT1 using calcein-acetoxymethyl (calcein-AM, 2 μ M, Life Technologies, C1430) and 5-chloromethylfluorescein diacetate (CMFDA, 1.25 μ M, Life Technologies, C7025), respectively.^{16,21} Both calcein-AM, a P-gp substrate, and CMFDA are permeable to the cell membrane and are intracellularly metabolized into calcein or glutathione-methylfluorescein (GS-MF), substrates for MRP2/4. PSC833 (10 μ M, Tocris, Bristol, UK, 4042/1) was used as model inhibitor for P-gp, included as positive control for inhibition in each experiment. Transport of calcein and GS-MF is by MRP2/4, P-gp and BCRP and therefore, a mixture of PSC833 (10 μ M), MK571 (10 μ M, Sigma-Aldrich, M7571) and KO143 (10 μ M, Sigma-Aldrich, K2144) was used to selectively inhibit efflux, again as positive control.²¹ Stocks of calcein-AM, CMFDA, PSC833 and KO143 were dissolved in DMSO (Sigma-Aldrich, D5879), MK571 was dissolved in milli-Q water. Test compounds were dissolved as described in Table S1. Work solutions were prepared in freshly-prepared Krebs-Henseleit buffer (Sigma-Aldrich, K3753), supplemented with 10 mM HEPES (Sigma-Aldrich, H3375) at pH 7.4, referred to as KHH. All conditions contained a final concentration of DMSO of 0.6% v/v. Perfusion channels were washed with 50 μ L in the medium-channel inlet and outlet and then perfused twice with KHH. Cells were incubated with calcein-AM and CMFDA with or

without the presence of model inhibitors or nephrotoxicants at 37 °C for 1 h. Next, efflux was arrested using PSC833 (10 μ M), MK571 (10 μ M) and KO143 (10 μ M) in cold (4 °C) KHH. Perfusion during washing and incubation was ensured by adding 80 μ L or 20 μ L to the medium-channel inlet and outlet, respectively.

Intracellular accumulation of calcein and GS-MF in ciPTEC-OAT1 in the OrganoPlate was measured *in situ* on a spinning disk confocal Becton Dickinson (BD) Pathway 855 high-throughput microscope (BD Bioscience, Breda, The Netherlands). A $\times 10$ objective was used with excitation and emission filters set at 488 nm and 520 nm, respectively. Subsequently, bright-field images of each chip were acquired. Fluorescence intensity was determined at the ECM-medium interface in Image J. Intensity per chip was then normalized to fluorescence measured in vehicle in same experiment.

Data Analysis

Data are presented as mean \pm SD for at least three chips ($n = 3-5$) per condition, unless stated otherwise. Statistics were performed using GraphPad Prism version 8 (San Diego, CA, USA). Data was found to be significantly different compared to medium control or corresponding vehicle if $p < 0.05$ using a one-way ANOVA followed by a Tukey's multi comparison *post hoc* test.

Results

Robustness of the System

Cisplatin, tenofovir, cyclosporin A and tobramycin have previously been shown to cause mild to severe cytotoxicity in confluent monolayers of ciPTEC-OAT1 in a 96-well plate.¹⁴ In our work, cell viability was measured upon a 48-h exposure to cisplatin (5 and 30 μ M), tenofovir (15.6 and 1000 μ M), cyclosporin A (5 and 30 μ M), and tobramycin (7.5 and 15 mM) in ciPTEC-OAT1 in the OrganoPlate at three different research sites. At the chosen concentrations, these nephrotoxicants caused a significant reduction in cell viability (Fig. 2) and increased LDH release for tobramycin and cyclosporin A at the highest concentrations tested (Figure S2). As depicted in Fig. 2, we observed similar effects in viability of ciPTEC-OAT1 at all

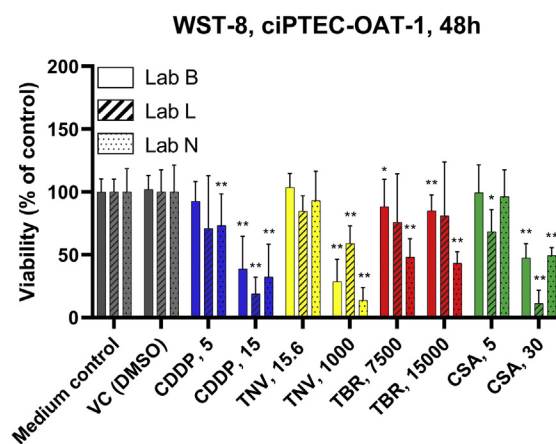


Fig. 2. Side to side comparison of viability data received from the WST-8 assay. Data are from three different labs, performed at FHNW (Lab B), Mimetax (lab L), or at department of Pharmacology and Toxicology, Radboud University Medical Center (lab N). In each site, 1–3 independent experiments with 2–6 replicates (chips) were analyzed. Abbreviations: CDDP, cisplatin; CSA, cyclosporin A; DMSO, dimethyl sulfoxide; TBR, tobramycin; TNV, tenofovir; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfonylphenyl)-2H-tetrazolium, monosodium salt; VC, vehicle control. #: in lab L slightly different concentrations for tenofovir were used, 15 μ M instead 15.6 μ M and 1215 μ M instead of 1000 μ M. Statistically significant compared to corresponding vehicle: * $p < 0.05$, ** $p < 0.01$.

three laboratories, in line with earlier findings reported by lab B.¹⁴ The other PTEC line used in the Nephroscreen, RPTEC, showed decreased viability, assessed with WST-8 and LDH-release, after exposure to tobramycin only, thus appeared to be less sensitive than ciPTEC-OAT1 (Figure S2). ciPTEC-OAT1 exposed to tenofovir in the compound screen showed a higher viability compared to the vehicle control when exposed to the low concentration but a significant loss in viability at 1000 μ M (Fig. 2).

Assessment of Cytotoxicity and Tubular Damage

Based on the data generated with the model nephrotoxics, we performed experiments to ascertain the potential nephrotoxicity of eight test substances. These compounds were provided by

the pharmaceutical companies GlaxoSmithKline (GSK), Roche and Pfizer in a blinded manner, referred to as unknown nephrotoxics, and named as G1, G2 and G3 for compounds provided by GSK, R1, R2 and R3 for compounds provided by Roche, and P1 and P2 for compounds provided by Pfizer.

In a first step, cell viability in confluent monolayers of ciPTEC-OAT1 in 2D was assessed to determine appropriate starting concentrations of the unknown nephrotoxics in the OrganoPlate (data not shown). Cytotoxicity parameters provided a consistent picture and showed reduced cell viability (WST-8) and increased LDH release caused by five of the unknown nephrotoxics (G1, G3, R1, R3, P2) in both cell lines tested (Fig. 3a–h). NAG release was only determined in ciPTEC-OAT1 and yielded concordant results (Fig. 3i and j). In general, toxicity was more marked after 48 h than

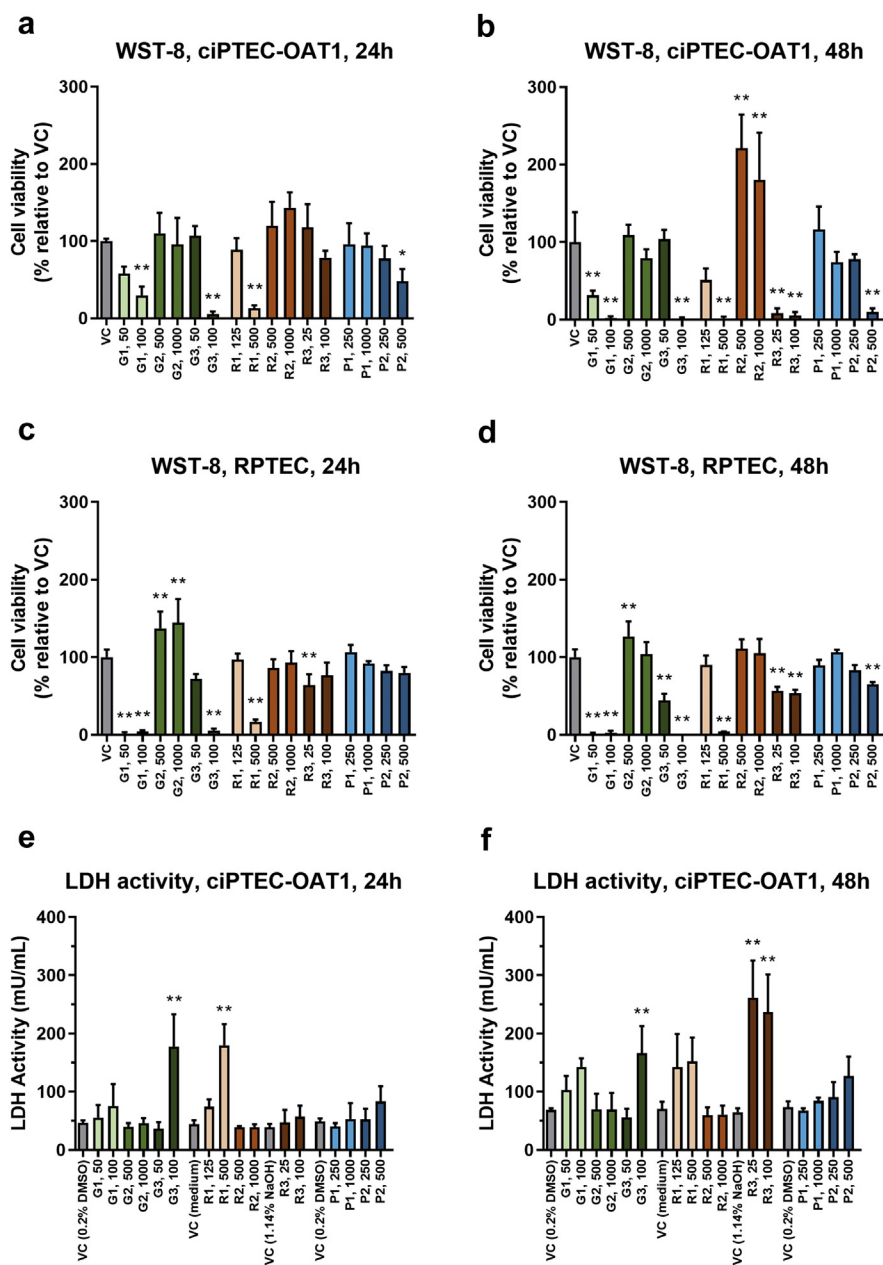


Fig. 3. Cell viability and lactate dehydrogenase (LDH) release in compound screen. (a) Cell viability in ciPTEC-OAT1 after 24 h and (b) 48 h exposure. (c) Cell viability in RPTEC after 24 h and (d) 48 h exposure. (e) Release of LDH, as measure of membrane integrity, in ciPTEC-OAT1 after 24 h or (f) 48 h exposure. (g) LDH release in RPTEC after 24 h or (h) 48 h exposure. (i) N-acetyl- β -D-glucosaminidase (NAG) release by ciPTEC-OAT1 treated with sponsor compounds screen after 24 h (i) and 48 h (j) exposure. Abbreviations: VC, vehicle control; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt; DMSO, Dimethyl sulfoxide; NaOH, Sodium hydroxide. Statistically significant compared to corresponding vehicle: * $p < 0.05$, ** $p < 0.01$.

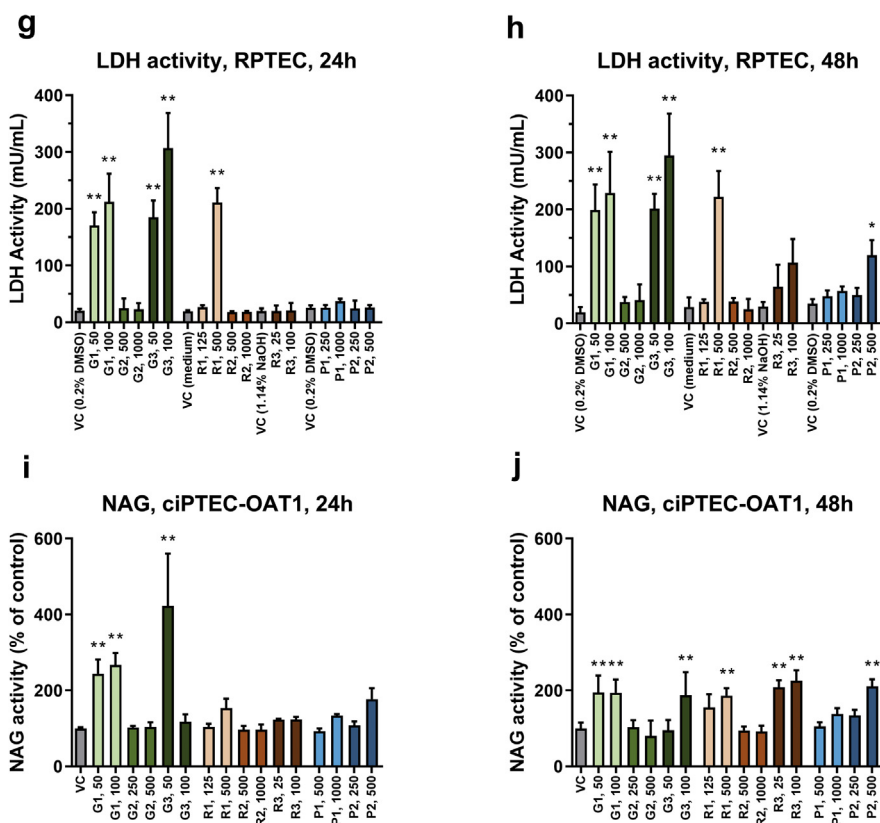


Fig. 3. (continued).

after 24 h exposure. Interestingly, G2 increased cellular production of formazan in RPTECs, while R2 increased this production in ciPTEC-OAT1. The sensitivity of both PTEC cell lines to the treatments was, however, comparable.

In addition to cytotoxicity measurements, the impact of treatments on the functionality of the tubular epithelial layer (i.e. barrier integrity in RPTEC in the OrganoPlate) was determined by assessing the apparent permeability to the low-molecular weight marker dextran-TRITC (4.4 kDa) and high-molecular weight marker dextran-FITC (155 kDa). Impaired barrier function was observed after exposure to tobramycin, and five test substances (G1, G3, R1, R3 and P2). The latter were the same five compounds that caused a decrease in cell viability. The effects were generally more marked after 48 h than after 24 h exposure (Fig. 4).

Drug-Transporter Interactions

Drug-transporter interaction with P-gp and MRP2/4 is another important feature that can result in high intracellular concentrations in PTEC leading to renal toxicity of substances. Drug-transporter interactions were determined in ciPTEC-OAT1 as stable expression of these drug transporters has been previously confirmed in this model.¹⁶ For MRP2/4, interactions were found for cyclosporin A, G2, G3 and P2 (Fig. 5a and c). Cyclosporin A, G3, P1 and P2 resulted in interactions for P-gp (Fig. 5b and d). It is interesting to note that the compound G2 clearly impaired MRP2/4 transport but did not cause any cytotoxicity based on other assays performed, suggesting competition for transport solely.

miRNA-Release

Released miRNAs can act as biomarkers of toxicity that are detectable in cell culture medium before other toxicity

biomarkers. The medium of the cells treated with the eight blinded compounds was collected after 24 h of exposure and levels of four selected miRNAs determined as described in materials and methods. Most tested compounds led to increases in the miRNA panel at both tested concentrations (Fig. 6a–d). Exceptions to this assessment were compounds G2 and R2 that did not cause significantly increased release of any of the tested miRNA into the medium. Interestingly, compound R3 led to an increase in all four miRNAs at the lowest tested concentration (25 μ M) while the cells treated with 100 μ M did not show changes in miRNA release.

Specific Exposures to Compounds R1 and R2

Due to the lack of toxicity observed in most parameters with compound R2 and to the high concentrations required to elicit a response with R1, tailor-made subsequent experiments were planned in discussion with the sponsors, who knew the identity of the compounds. To this end, ciPTEC-OAT1 in the OrganoPlate were exposed to several concentrations (ranging between 15 and 1000 μ M) of R1 and R2 for 48 h and 11 days, respectively. At 48 h, compound R1 showed a dose dependent loss in viability assessed with the WST-8 assay with an EC₅₀ of 214.5 and 277.3 μ M (results from two independent experiments). This toxicity was accompanied by a dose dependent induction of HMOX1 (Fig. 7a and b). The results confirm that concentrations \geq ~100 μ M lead to toxicity with an EC₅₀ 214 and 277 μ M (two independent experiments), whereas HMOX1 expression levels increased in a concentration-dependent manner from a concentration of 62.5 μ M (\geq 2-fold induction; Fig. 7). R1 did not lead to any change in expression of NGAL or in NAG released into the medium (Fig. 7b and c).

Based on information provided by the sponsors, compound R2 was additionally tested after long-term (11 days) of

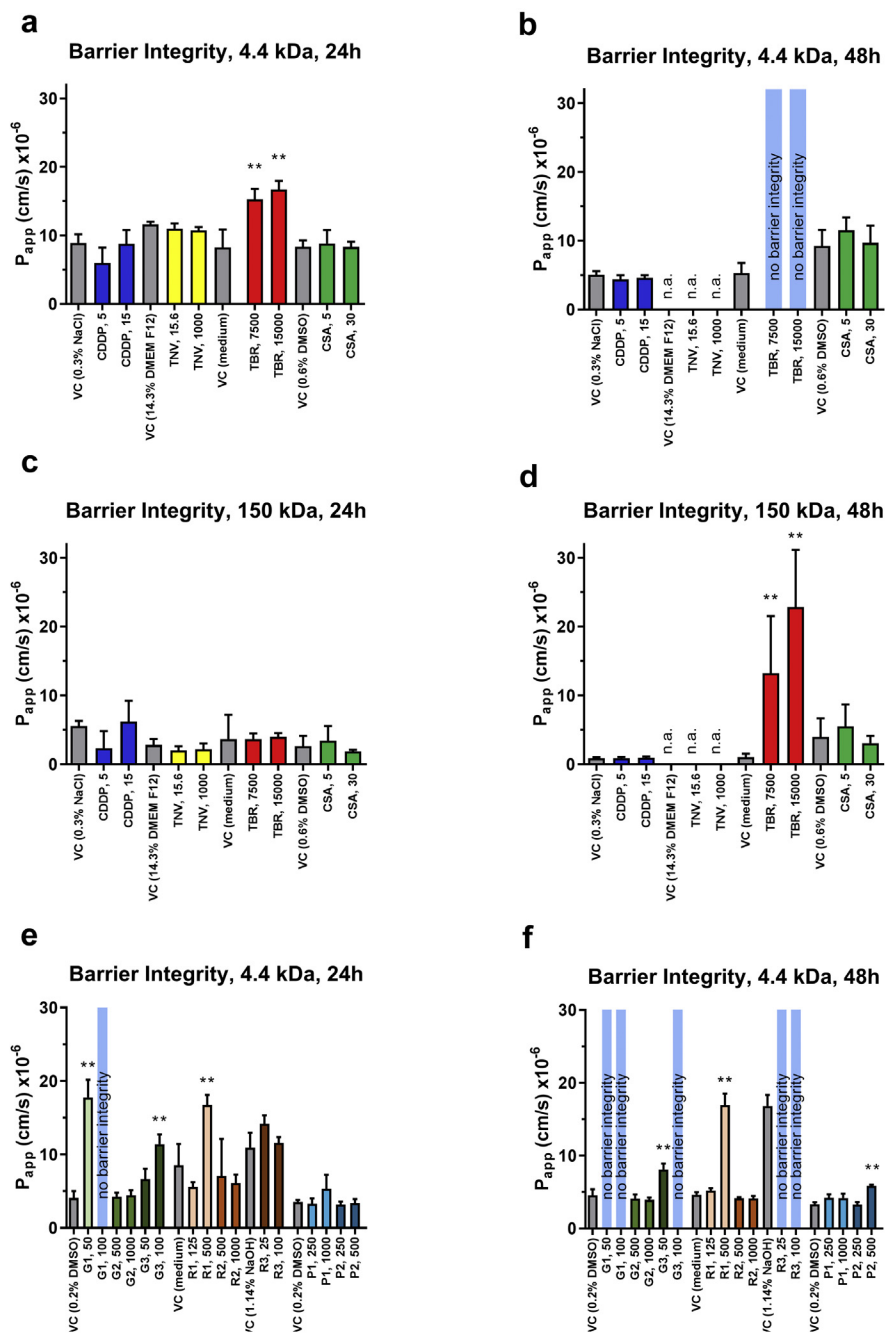


Fig. 4. Barrier integrity of RPTEC tubules. (a) Leakage of high-weight molecular marker (155 kDa) fluorescein isothiocyanate (FITC)-dextran after 24 h or (b) 48 h of exposure to model nephrotoxicants. (c) Leakage of low-weight molecular marker (4.4 kDa) tetramethylrhodamine (TRITC)-dextran after 24 h or (d) 48 h of exposure to model nephrotoxicants cisplatin (CDDP), tenofovir (TNV), tobramycin (TBR) and cyclosporin A (CSA). (e) Leakage of FITC-dextran after 24 h and (f) 48 h of exposure to unknown nephrotoxicants. (g) Leakage of TRITC-dextran after 24 h or (h) 48 h of exposure to unknown nephrotoxicants. Abbreviations: D-F12, Dulbecco's modified Eagle's medium and nutrient mixture F-12; VC, vehicle control; P_{app}, apparent permeability; DMSO, Dimethyl sulfoxide; NaOH, Sodium hydroxide; kDa, kilodaltons; n.a., not available. Statistically significant compared to corresponding vehicle: **p* < 0.05, ***p* < 0.01.

incubation. Cytotoxicity assay at 11 days showed a decrease in cell viability with an EC₅₀ of 367.5 and 267.4 μ M (results from two independent experiments) (Fig. 8a). As with compound R1, HMOX1 expression increased in a dose dependent manner from a concentration of 30.25 μ M (\geq 2-fold induction; Fig. 8b). The compound, however, did not cause an increase in NGAL expression or led to a release of NAG release into the medium at any of the tested time points (48 h or 11 days of exposure, Fig. 7b and c).

Discussion

We demonstrated the use of Nephroscreen, a proximal tubule-on-a-chip platform for the screening of nephrotoxicity and drug-transporter interactions. The robustness and transferability of this platform, a key requirement for broad implementation in industry, was established by the highly comparable results obtained at three different sites as depicted in Fig. 2. Two human renal cell lines, ciPTEC-OAT1 and RPTEC, were exposed to two concentrations of

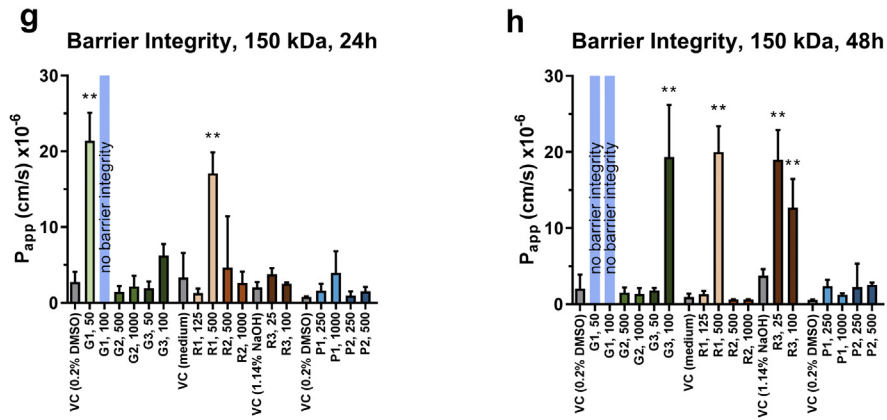


Fig. 4. (continued).

each substance (four model nephrotoxics and eight blinded compounds) at two exposure times (24 and 48 h). Subsequently, tailor-made assays were performed for two of the compounds (R1, colistin and R2, cefepime), exposing ciPTEC-OAT1. Experimental procedures were carried out in three different laboratories as depicted in Fig. 1, following established standard operating procedures (SOPs).

Concordant with the goal of the study, Nephroscreen was specifically designed to fulfill requirements of pharmaceutical companies. In pre-clinical toxicity studies, multiple drug candidates,

typically up to 200 compounds, are assessed *in vitro* and/or *in vivo* in animal experimentation.²² In this study, we implemented well-characterized PTEC lines, considered relevant for nephrotoxicity and drug-transporter interaction studies, as potential alternatives to animal experimentation.^{14–18,23,24} Supply and reproducibility (low batch-to-batch variability) of these commercially available cells are guaranteed. The PTECs were cultured in the OrganoPlate, generating a proximal tubule-on-a-chip consisting of 40 chips on a 384-well microtiter plate format.¹⁵ This model enables the culture of the cells as a tubular structure kept under flow, without the use

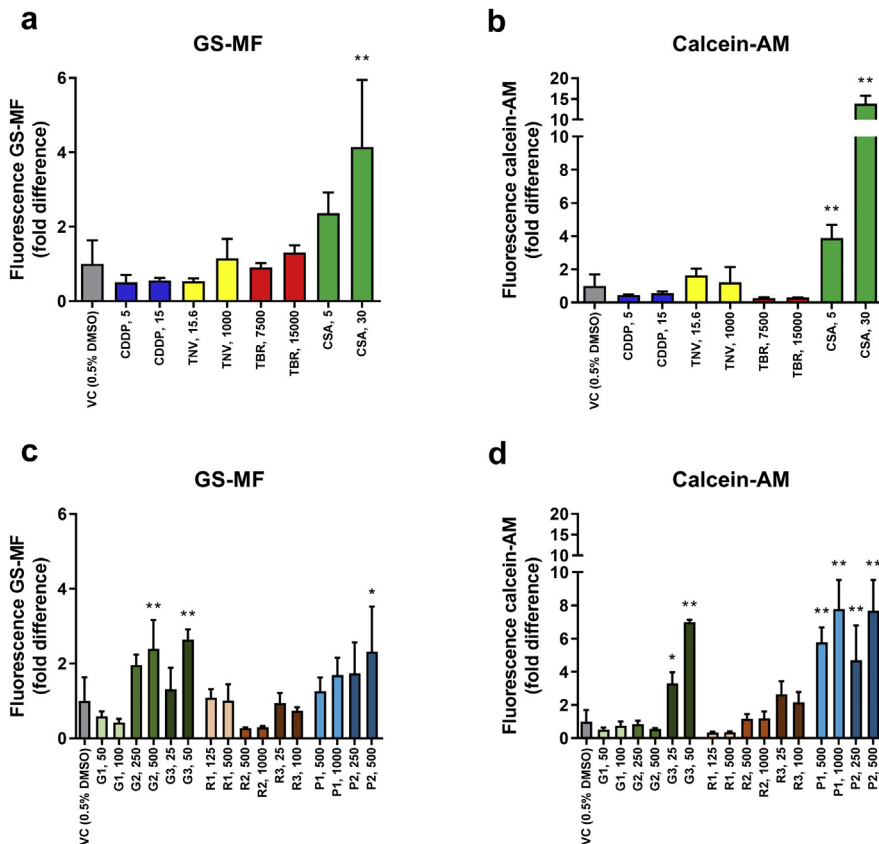


Fig. 5. Drug-transporter interaction at multidrug resistance protein 2/4 (MRP2/4) and P-glycoprotein (P-gp) in ciPTEC-OAT1 after co-incubation with compounds at 37 °C for 1 h followed by arresting of efflux with PSC833, MK571 and KO143 (all 10 μM). For model nephrotoxics cisplatin (CDDP), tenofovir (TNV), tobramycin (TBR) and cyclosporin A (CSA) (a) interactions at MRP2/4 and (b) P-gp and for unknown nephrotoxics (c) interactions at MRP2/4 and (d) P-gp. Abbreviations: GS-MF, Glutathione methylfluorescein; AM, acetoxymethyl; VC, vehicle control; DMSO, Dimethyl sulfoxide. Statistically significant compared to corresponding vehicle: * $p < 0.05$, ** $p < 0.01$.

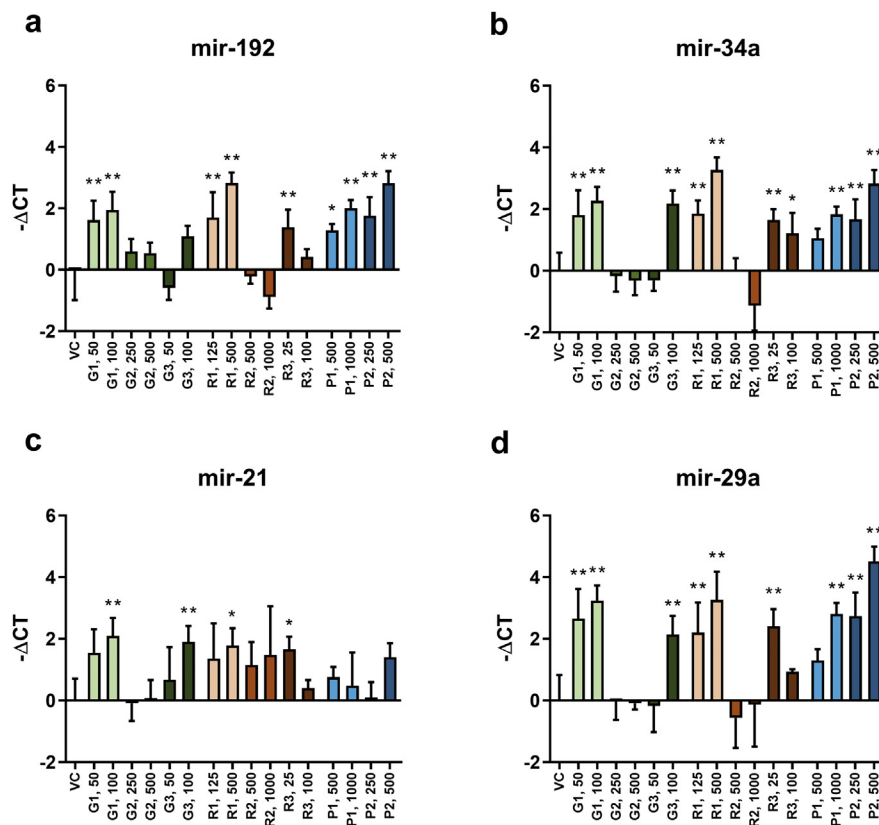


Fig. 6. Release into the medium of mir-192 (a), mir-34a (b), mir-21 (c) and mir-29a (d) after 24 h exposure of ciPTEC-OAT1 to sponsor compounds. Data are represented as Delta CT values in comparison with the vehicle control (VC). Statistically significant compared to corresponding vehicle: * $p < 0.05$, ** $p < 0.01$.

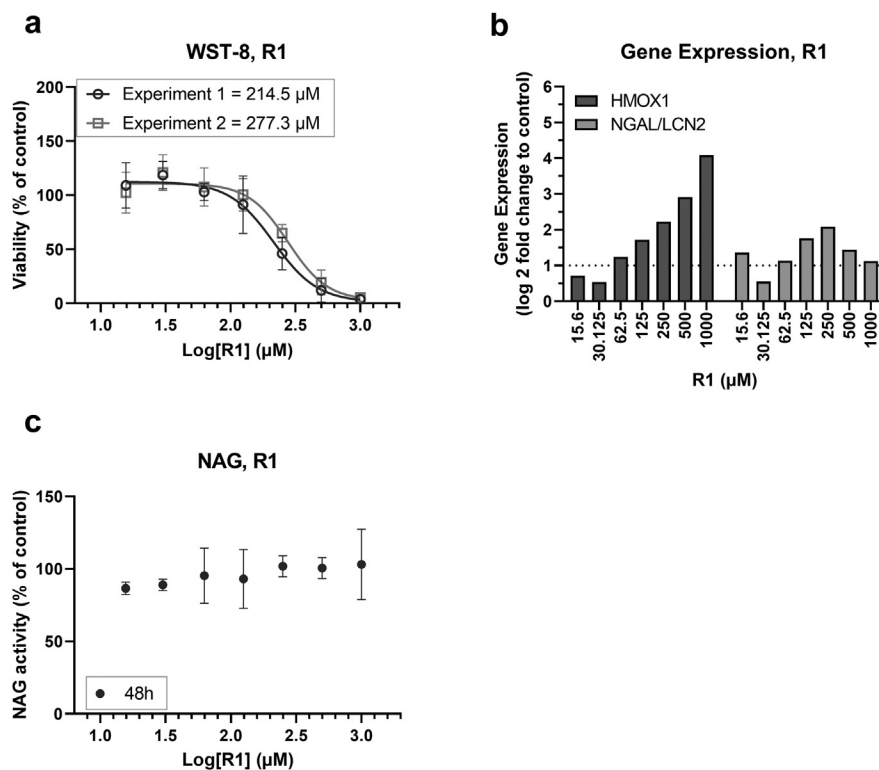


Fig. 7. Exposure of ciPTEC-OAT1 to R1 (Colistin). Data represent cell viability with the IC₅₀ value of each experiment. (a), Gene expression of toxicity markers (b) and release of NAG into the medium after 48 h (c). Abbreviations: HMOX1, heme oxygenase (decycling) 1; LCN2, Lipocalin-2 (LCN2); NGAL, oncogene 24p3 or neutrophil gelatinase-associated lipocalin; NAG, N-acetyl- β -D-glucosaminidase.

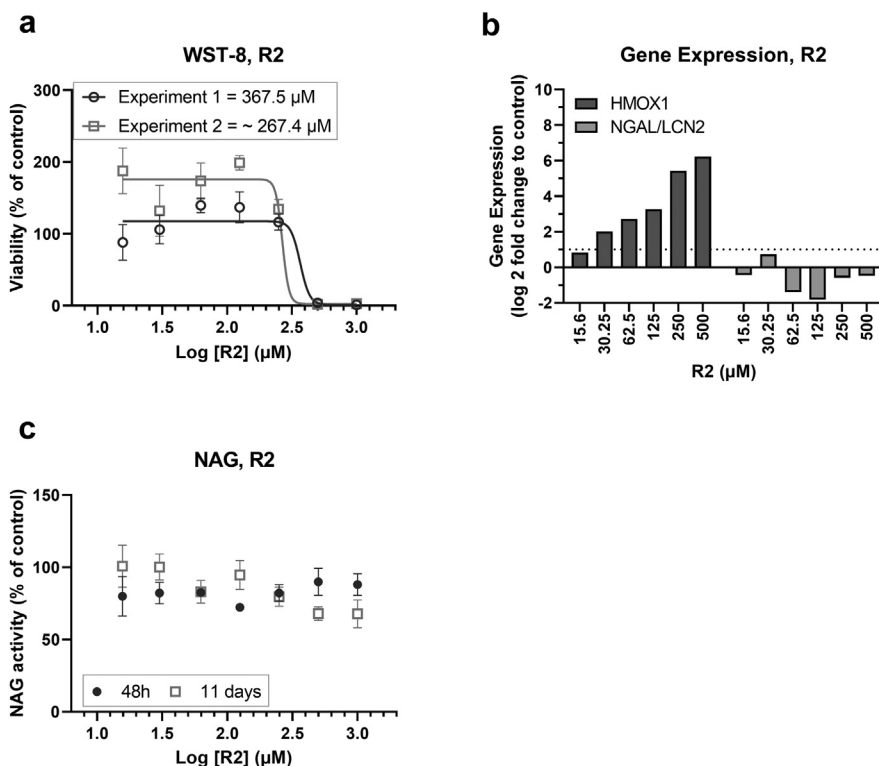


Fig. 8. Exposure of ciPTEC-OAT1 to R2 (Cefepime) for 48 h and 11 days. Data represent cell viability. (a), Gene expression of toxicity markers (b) and release of NAG into the medium after 48 h (circles) and 11 days (squares) (c). Abbreviations: HMOX1, heme oxygenase (decycling) 1; LCN2, Lipocalin-2 (LCN2); NGAL, oncogene 24p3 or neutrophil gelatinase-associated lipocalin; NAG, N-acetyl- β -D-glucosaminidase.

of support membranes. Perfusion was generated by passive leveling resulting in a bidirectional, oscillating flow allowing significant levels of shear stress circumventing the use of pumps.^{15,25} The diameter of the tubule (approximately 400 μ m) is larger than in the human proximal tubule. However, it represents a useful model that allows the long-term culture of polarized cell layers against the ECM with significant barrier function.¹⁵

Effect of the treatments on the PTECs was determined by measuring functional and biochemical parameters. Functional parameters focused on the capacity of compounds to interact with transporters and to impair barrier function. Membrane drug transporters are an important characteristic of PTECs and expression and activity of transporters have been demonstrated previously.^{6,17,18,24} Differences in response towards toxicant exposures between ciPTEC-OAT1 and RPTEC could be explained by different expression levels of drug transporters. For instance, ciPTEC-OAT1, but not RPTEC, functionally express OAT1. This explains the lack of sensitivity of RPTEC towards tenofovir, as this substance requires the OAT1 transporter for cellular uptake.^{18,24} Epithelial barrier function is also a key functional parameter, as a leaky epithelium is often an indicator of impaired kidney function, previously demonstrated in this model.¹⁵

Regarding biochemical parameters, cell viability, LDH release and gene expression of toxicity markers were determined on both PTEC lines to assess the effect of the compounds. For ciPTEC-OAT1, assays for drug-transporter interaction, NAG release and miRNA secretion were also performed,^{14,16} while RPTEC tubules were more suitable for the assessment of barrier integrity.¹⁵ Cell viability, as measured by enzymatic production of formazan (WST-8 assay), and cell membrane integrity, determined by LDH release into the medium, performed well as *in vitro* markers for cytotoxicity, although with different kinetic profiles (Table 1, Figure S2). In ciPTECs,

leakage of NAG into the medium displayed a slightly higher sensitivity in detecting the toxicity caused by compounds G1 and P2 than LDH release (Fig. 3), but a larger data set would be necessary to corroborate this claim. Decreased barrier integrity was almost always coupled with decreased cell viability and increased LDH release. Thus, we assume that the loss of barrier integrity was directly associated with increased cytotoxicity (Table 1).

The four model nephrotoxicants (cisplatin, tenofovir, tobramycin, cyclosporin A) assessed in this study elicited a response in at least four of the end-point read-outs (Figure S2, Table 1), in agreement with previous results.^{26–30} Furthermore, our data show that cyclosporin A interacts with P-gp, corroborating that Nephroscreen results reflect its known P-gp inhibition.³¹ Nephrotoxicity of tobramycin, on the other hand, could only be observed at concentrations that were up to 1500-fold higher than plasma concentrations generally found in patients (0.01 mM).³⁰ This is probably due to the low expression of cubilin and megalin receptors in ciPTEC-OAT1,^{17,32,33} a limitation that needs to be taken into consideration for compounds known to be dependent on this cellular uptake mechanism.

During the second phase of the study, eight substances selected and provided by the sponsors were assessed in a blinded manner.

Preliminary knowledge such as clinical data (for R1, R2, R3) or *in vivo* toxicity data were provided by sponsors after the Nephroscreen data collection and analysis had been finalized. The compound concentrations selected and applied to Nephroscreen unveiled nephrotoxic potential at concentrations that are considered relevant for toxicological assessment (Table S2). For R1, R2, and R3 there is clinical data available; these compounds are used at very high concentrations and administered parenterally, so that a high systemic exposure is reached in patients. For the other blinded compounds, there is no clinical data but preclinical data on at least

Table 1
Overview Read-Outs Nephrotoxicity and Renal Drug-Transporter Interactions Assessed in This Nephrotoxicant Screening.

| | Conc. (μM) | ciPTEC-OAT1 | | | | RPTEC | | |
|------|-------------------------|----------------|-------------|------------------------------|--------------|-----------------------------------|----------------|---------------------------------|
| | | Cell Viability | LDH Release | Drug-Transporter Interaction | NAG Activity | miRNA Release | Cell Viability | LDH Release |
| CDDP | 5 | | | | | | | |
| | 15 | ↓ 48 h | | | | | | |
| TNV | 15.6 | ↑ 48 h | | | | | | |
| | 1000 | ↓ 48 h | | | | | | |
| TBR | 7500 | | | | | | | |
| | 15000 | | ↑ 24 h | | | | ↑ 48 h | 4.4 kDa: ↓ 24 h 150 kDa: ↓ 48 h |
| CSA | 5 | | | P-gp | | | ↑ 48 h | 4.4 kDa: ↓ 24 h 150 kDa: ↓ 48 h |
| | 30 | ↓ 48 h | ↑ 24 h | MRP2/4, P-gp | | | | |
| G1 | 50 | ↓ 48 h | | | ↑ 24 h | mir-34a, mir-29a, mir-192 | ↓ 24 h | ↑ 24 h |
| | 100 | ↓ 24 h | | | ↑ 24 h | mir-21, mir-34a, mir-29a, mir-192 | ↓ 24 h | ↑ 24 h |
| G2 | 250 | | | | | | | 4.4 kDa: ↓ 24 h 150 kDa: ↓ 24 h |
| | 500 | | | MRP2/4 | | | ↑ 24 h | |
| | 1000 | | | | | | ↑ 24 h | |
| G3 | 25 | | | P-gp | | | | |
| | 50 | | | MRP2/4, P-gp | ↑ 24 h | | ↓ 48 h | ↑ 24 h |
| | 100 | ↓ 24 h | ↑ 24 h | | ↑ 48 h | mir-21, mir-34a, mir-29a | ↓ 24 h | ↑ 24 h |
| R1 | 125 | | | | | mir-34a, mir-29a, mir-192 | | 4.4 kDa: ↓ 24 h 150 kDa: ↓ 48 h |
| | 500 | ↓ 24 h | ↑ 24 h | | ↑ 48 h | mir-21, mir-34a, mir-29a, mir-192 | ↓ 24 h | ↑ 24 h |
| R2 | 500 | ↑ 48 h | | | | | | |
| | 1000 | ↑ 48 h | | | | | | |
| R3 | 25 | ↓ 48 h | ↑ 48 h | | ↑ 48 h | mir-21, mir-34a, mir-29a, mir-192 | ↓ 24 h | 4.4 kDa: ↓ 48 h 150 kDa: ↓ 48 h |
| | 100 | ↓ 48 h | ↑ 48 h | | ↑ 48 h | mir-34a | ↓ 48 h | 4.4 kDa: ↓ 48 h 150 kDa: ↓ 48 h |
| P1 | 250 | | | | | mir-192 | | |
| | 500 | | | P-gp | | | | |
| | 1000 | | | P-gp | | mir-34a, mir-29a, mir-192 | | |
| P2 | 250 | | | P-gp | | mir-34a, mir-29a, mir-192 | ↓ 48 h | ↑ 48 h |
| | 500 | ↓ 24 h | | MRP2/4, P-gp | ↑ 48 h | mir-34a, mir-29a, mir-192 | | 4.4 kDa: ↓ 48 h |

For cell viability, LDH release, drug-transporter interaction, and barrier integrity: ↑ represents significant increased effect observed compared to corresponding vehicle, ↓ represents significant decreased effect observed compared to corresponding vehicle. CDDP, cisplatin; *CLDN2*, claudin-2; Conc., concentration; CSA, cyclosporin A; FITC, fluorescein isothiocyanate; LDH, lactate dehydrogenase; MRP2/4, multidrug resistance-associated proteins 2 and 4; P-gp, P-glycoprotein; TBR, tobramycin; TNV, tenofovir; TRITC, tetramethylrhodamine.

one animal species show that the exposure (C_{max}) at which nephrotoxicity was observed in a subset of the animals was slightly lower, but in a similar range to that eliciting a positive flag in Nephroscreen. In our experimental set-up, prediction of nephrotoxic liability was highest when combining results of cell viability, LDH release, and miRNA release. Additional functional effects of the compounds were uncovered by the drug-transport interaction measurements. The majority of the unknown nephrotoxics (G1, G3, R1, R2, R3, P1, and P2) resulted in toxicity detected by at least one of the parameters or in drug-transporter interactions. Interestingly, an interaction at MRP2/4 was observed for G2, despite the lack of toxicity. This could be explained by the fact that G2-induced nephrotoxicity was only observed upon long-term exposure *in vivo* (one month, highest tested dose) as compared to short-term (up to 48 h) exposure tested in Nephroscreen. The compound P1 showed a particularly mild effect on Nephroscreen, as it only led to increased levels of miRNAs (mir192, mir29a, and mir34a) in the medium and an interaction at P-gp, without causing cytotoxicity. This is consistent with the existing animal data showing that this vasopressin 1-a antagonist caused tubular degeneration/regeneration only in female rats treated with a high dose (125 mg/kg/day) for two weeks.

In the last phase of this study, tailor-made experiments were performed including expanded concentration ranges of two compounds after their identities had been uncovered: R1 (colistin) and R2 (cefepime). For R1 (colistin) we repeated the cytotoxicity assay and determined gene expression of HMOX-1 and NGAL, as well as the release of NAG in the medium. The EC₅₀ for cytotoxicity obtained in this experiment was in line with the results of the first round of experiments (~200 μM). The *in vitro* cytotoxicity of colistin has been reported by others in the high micromolar range, around 690 μM.³⁴ Preliminary gene expression data, however, uncovered a dose-dependent increase in HMOX-1 from a concentration of 30 μM (>2-fold induction). Transcriptional upregulation of heme oxygenase-1 is a well-known indicator of cellular stress in several cells and tissues, including PTECs and may be a more sensitive biomarker of toxicity.^{19,26} This was also supported by the long-term (11 days) exposure of the Nephroscreen to R2 (cefepime). Cefepime had an EC₅₀-value for cytotoxicity of approximately 300 μM, while the concentration-dependent transcriptional induction of HMOX-1 was observed at concentrations from 62 μM (>2-fold induction). These results also show that a long-term exposure is required for this compound, as the short-term experiment failed to show toxicity up to a concentration of 1000 μM and unpublished *in vitro* results with PTEC toxicity at concentrations >500 μM (Table S2).

The ideal set-up for a screening tool requires short-term incubation in order to generate results quickly. Our results show that for most compounds, exposure during 24 and 48 h suffices to detect compound-induced damage to renal tubular cells. However, as demonstrated with the results obtained with R2, the developed platform can also be implemented as a second tier assay for selected substances of interest that may require subchronic or chronic exposure. The sensitivity and specificity of Nephroscreen cannot be determined as only a small set of test compounds was included. Future testing should expand the number of compounds and include compounds with other toxicity target organs (non-nephrotoxic) as well as non-toxic compounds to further characterize the model. Also, side-by-side comparison with conventional 2D cell cultures may be performed to achieve direct comparison and therefore show the value in this model over more simple models. Other proximal tubule-on-a-chip models have, however, demonstrated that 3D microfluidic models increase *in vivo* physiology and sensitivity of PTECs towards nephrotoxicant exposure.^{11–13} Additional future improvements could include the

corroboration and systematic assessment of HMOX-1 induction as an earlier biomarker and its implementation as a FRET-based assay for HMOX1 expression.¹⁹ The implementation of cell lines expressing more types of functional transporters relevant, such as ciPTEC-OAT1/OAT-3 could also be envisaged.¹⁸ Moreover, data on additional compounds could support the creation of intelligent decision algorithms to evaluate the multiparametric data provided by Nephroscreen in an unbiased manner.

Conclusion

An advanced screening tool is needed to increase speed, reduce costs and animal testing while assessing the potential nephrotoxicity of new drug candidates. Taken together, our results indicate that Nephroscreen, consisting of a microfluidic organ-on-a-chip system coupled with a multiparametric biomarker analysis is able to identify potential nephrotoxics. Nephroscreen is a reliable medium-throughput, standardized, automatable system that proved efficacious in identifying nephrotoxics and provided insights into their mode of toxicity.

Conflicts of Interest

This publication contains original work. The authors Marianne K. Vormann, Henriëtte L. Lanz, Linda Gijzen, Angélique van den Heuvel, Simon Hutter, Jos Joore, Sebastiaan J. Trietsch, and Paul Vulto are or were employees of Mimetas B.V. This affiliation is declared. The OrganoPlate® is a registered trademark of MIMETAS BV. Martijn Wilmer and Frans Russel are co-inventors on patent EP2010/066792 “Novel conditionally immortalized human proximal tubule cell line expressing functional influx and efflux transporters” assigned to Radboud University Medical Center and commercialized through Cell4Pharma.com, for which Martijn Wilmer acts as scientific advisor.

Acknowledgements

Funding: 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), UK.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.xphs.2021.01.028>.

References

1. Soo JY, Jansen J, Masereeuw R, Little MH. Advances in predictive *in vitro* models of drug-induced nephrotoxicity. *Nat Rev Nephrol*. 2018;14(6):378–393.
2. Uchino S, Kellum JA, Bellomo R, et al. Beginning, Ending Supportive Therapy for the Kidney I. Acute renal failure in critically ill patients: a multinational, multicenter study. *JAMA*. 2005;294(7):813–818.
3. Hoste EA, Bagshaw SM, Bellomo R, et al. Epidemiology of acute kidney injury in critically ill patients: the multinational AKI-EPI study. *Intensive Care Med*. 2015;41(8):1411–1423.
4. Mehta RL, Pascual MT, Soroko S, et al. Program to Improve Care in Acute Renal D. Spectrum of acute renal failure in the intensive care unit: the PICARD experience. *Kidney Int*. 2004;66(4):1613–1621.
5. Guengerich FP. Mechanisms of drug toxicity and relevance to pharmaceutical development. *Drug Metabol Pharmacokinet*. 2011;26(1):3–14.
6. Morrissey KM, Stocker SL, Wittwer MB, Xu L, Giacomini KM. Renal transporters in drug development. *Annu Rev Pharmacol Toxicol*. 2013;53:503–529.
7. Varma MV, Feng B, Obach RS, et al. Physicochemical determinants of human renal clearance. *J Med Chem*. 2009;52(15):4844–4852.
8. Ivanyuk A, Livio F, Biollaz J, Buclin T. Renal drug transporters and drug interactions. *Clin Pharmacokinet*. 2017;56(8):825–892.

9. Wilmer MJ, Ng CP, Lanz HL, Vulto P, Suter-Dick L, Masereeuw R. Kidney-on-a-Chip technology for drug-induced nephrotoxicity screening. *Trends Biotechnol.* 2016;34(2):156-170.
10. Tiong HY, Huang P, Xiong S, Li Y, Vathsala A, Zink D. Drug-induced nephrotoxicity: clinical impact and preclinical in vitro models. *Mol Pharm.* 2014;11(7):1933-1948.
11. Homan KA, Kolesky DB, Skylar-Scott MA, et al. Bioprinting of 3D convoluted renal proximal tubules on perfusable chips. *Sci Rep.* 2016;6:34845.
12. Jang KJ, Mehr AP, Hamilton GA, et al. Human kidney proximal tubule-on-a-chip for drug transport and nephrotoxicity assessment. *Integr Biol.* 2013;5(9):1119-1129.
13. Sakolish C, Weber EJ, Kelly EJ, et al. Technology transfer of the microphysiological systems: a case study of the human proximal tubule tissue chip. *Sci Rep.* 2018;8(1):14882.
14. Suter-Dick L, Mauch L, Ramp D, et al. Combining extracellular miRNA determination with microfluidic 3D cell cultures for the assessment of nephrotoxicity: a proof of concept study. *AAPS J.* 2018;20(5):86.
15. Vormann MK, Gijzen L, Hutter S, et al. Nephrotoxicity and kidney transport assessment on 3D perfused proximal tubules. *AAPS J.* 2018;20(5):90.
16. Vriend J, Nieskens TTG, Vormann MK, et al. Screening of drug-transporter interactions in a 3D microfluidic renal proximal tubule on a chip. *AAPS J.* 2018;20(5):87.
17. Wilmer MJ, Saleem MA, Masereeuw R, et al. Novel conditionally immortalized human proximal tubule cell line expressing functional influx and efflux transporters. *Cell Tissue Res.* 2010;339(2):449-457.
18. Nieskens TT, Peters JG, Schreurs MJ, et al. A human renal proximal tubule cell line with stable organic anion transporter 1 and 3 expression predictive for antiviral-induced toxicity. *AAPS J.* 2016;18(2):465-475.
19. Adler M, Ramm S, Hafner M, et al. A quantitative approach to screen for nephrotoxic compounds in vitro. *J Am Soc Nephrol.* 2016;27(4):1015-1028.
20. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods.* 2012;9(7):676-682.
21. Caetano-Pinto P, Janssen MJ, Gijzen L, Verscheijden L, Wilmer MJ, Masereeuw R. Fluorescence-based transport assays revisited in a human renal proximal tubule cell line. *Mol Pharm.* 2016;13(3):933-944.
22. Sakolish CM, Esch MB, Hickman JJ, Shuler ML, Mahler GJ. Modeling barrier tissues in vitro: methods, achievements, and challenges. *EBioMedicine.* 2016;5:30-39.
23. Fedecostante M, Westphal KGC, Buono MF, et al. Recellularized native kidney scaffolds as a novel tool in nephrotoxicity screening. *Drug Metab Dispos.* 2018;46(9):1338-1350.
24. Li S, Zhao J, Huang R, et al. Development and application of human renal proximal tubule epithelial cells for assessment of compound toxicity. *Curr Chem Genomics Transl Med.* 2017;11:19-30.
25. van Duinen V, van den Heuvel A, Trietsch SJ, et al. 96 perfusable blood vessels to study vascular permeability in vitro. *Sci Rep.* 2017;7(1):18071.
26. Ozkok A, Edelstein CL. Pathophysiology of cisplatin-induced acute kidney injury. *Biomed Res Int.* 2014;2014:967826.
27. Jafari A, Khalili H, Dashti-Khavidaki S. Tenofovir-induced nephrotoxicity: incidence, mechanism, risk factors, prognosis and proposed agents for prevention. *Eur J Clin Pharmacol.* 2014;70(9):1029-1040.
28. Ramamoorthy H, Abraham P, Isaac B. Mitochondrial dysfunction and electron transport chain complex defect in a rat model of tenofovir disoproxil fumarate nephrotoxicity. *J Biochem Mol Toxicol.* 2014;28(6):246-255.
29. Mingeot-Leclercq MP, Tulkens PM. Aminoglycosides: nephrotoxicity. *Antimicrob Agents Chemother.* 1999;43(5):1003-1012.
30. Schentag JJ, Lasezkay G, Cumbo TJ, Plaut ME, Jusko WJ. Accumulation pharmacokinetics of tobramycin. *Antimicrob Agents Chemother.* 1978;13(4):649-656.
31. Polli JW, Wring SA, Humphreys JE, et al. Rational use of in vitro P-glycoprotein assays in drug discovery. *J Pharmacol Exp Ther.* 2001;299(2):620-628.
32. Vriend J, Peters JGP, Nieskens TTG, et al. Flow stimulates drug transport in a human kidney proximal tubule-on-a-chip independent of primary cilia. *Biochim Biophys Acta Gen Subj.* 2020;1864(1):129433.
33. Sun Y, Goes Martini A, Janssen MJ, Garrelds IM, Masereeuw R, Lu X, et al. Megalin: a novel endocytic receptor for prorenin and renin. *Hypertension.* 2020;75(5):1242-1250.
34. Roberts KD, Azad MA, Wang J, et al. Antimicrobial activity and toxicity of the major lipopeptide components of polymyxin B and colistin: last-line antibiotics against multidrug-resistant gram-negative bacteria. *ACS Infect Dis.* 2015;1(11):568-575.