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Development of a kidney-on-a-chip model for compound screening and transport studies

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Chapter 4

Drug-Drug Interaction Study on a Proximal-Tubule-on-a-Chip

MK Vormann, L Boot, HL Lanz

Introduction

Renal proximal tubules play a huge role in the elimination of waste products and drugs from the body and are known to be a common target of drug induced toxicity [1]. Tenofovir (TFV) is such an antiretroviral drug of which drug-related nephrotoxicity is widely accepted [2]. TFV is a nucleotide reverse transcriptase inhibitor in the treatment of human immunodeficiency viruses (HIV) and Hepatitis B infected patients [2]. It is transformed by intracellular phosphorylation into the active metabolite tenofovir diphosphate. This metabolite is then incorporated into the viral deoxyribonucleic acid (DNA) where it functions as a DNA-chain terminator [3]. TFV itself is not membrane permeable, thus not suitable for oral administration and needs to be delivered as a prodrug [4] (fig. 1). Since 2012 the prodrug tenofovir disoproxil fumarate (TDF) has been used to treat HIV in combination with the antiretroviral agents elvitegravir, cobicistat, and emtricitabine in the single-tablet regimen Stribild [5]. However, TDF has now been replaced by a newly developed prodrug of TFV: tenofovir alafenamide (TAF). TAF is delivered for the treatment of HIV with the same three antiretroviral agents and available for patients since 2015 under the trade name Genvoya [6]. Measured by revenue Genvoya was the most used HIV medication in 2017 and 2018 [7]. The main reason why TDF was replaced by TAF is the limited risk for nephrotoxicity and reduced loss of bone mineral density [8]. In our recent work we were already able to show the toxicity of TFV on proximal tubule cells, however the prodrugs TDF and TAF were never tested in our model before (chapter 3, [9]).

In this study, it is investigated whether one or both of the TFV prodrugs have a toxic effect on the proximal tubule cells. Also, we wanted to investigate if any of the TFV-combined antiretroviral compounds are nephrotoxic by themselves or amplify the toxic effect while co-incubated with the TFV prodrugs.

TDF is unstable when in contact with blood plasma where it quickly undergoes hydrolysis into TFV [3], [10] (fig. 1). Only a small part of the drug is therefore taken up by the HIV target cells with high levels of TFV remaining in the blood plasma [4]. The newly developed prodrug TAF has a higher stability when in contact with plasma than TDF resulting in a superior efficiency of HIV-target cell delivery. This results in a higher antiviral activity and reduced dosage compared to TDF [4], [11], [12]. When patients switched from Stribild (which contains TDF) to Genvoya (which contains TAF) patients suffered less adverse kidney-related side effects [13].

The elimination of TFV from the blood plasma is mediated via glomerular filtration and secretion via the renal proximal tubule. It is actively transported through the influx transporters organic anion transporter (OAT) 1 and 3, and efflux transporter multi-drug resistance protein (MRP) 4 into the pre-urine [10], [14], [15]. Imbalance of the transporters can result in increased concentrations of TFV in the proximal tubule cells causing a high likeliness for drug-drug-interaction (DDI) [2], [16]. When TFV accumulates in the proximal tubule cells, disruption and inflammation of the

mitochondria in the proximal tubule can be observed [16]. Both prodrugs of TFV are co-administered with three other active compounds, namely elvitegravir, cobicistat, and emtricitabine.

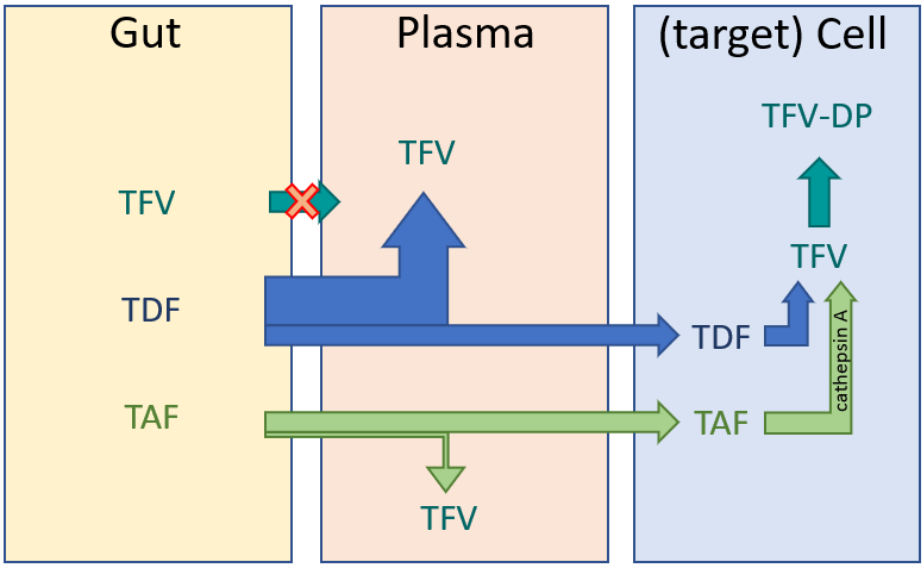

***Figure 1: Comparison of the conversion pathways of tenofovir (TFV)**, tenofovir disoproxil fumarate (TDF), and tenofovir alafenamide (TAF) into the active metabolite tenofovir diphosphate (TFV-DP), a nucleotide reverse transcriptase inhibitor. TFV itself is not membrane permeable and is delivered orally by the prodrugs TDF or TAF. TDF is unstable when in contact with blood plasma where it quickly undergoes hydrolysis into TFV, resulting in a fraction of the delivered TDF entering the target cells. TAF on the contrary is stable and converted mainly intracellularly by cathepsin A into TFV. Adapted from* [3], [4].

Emtricitabine is, similar to TFV, a reverse transcriptase inhibitor which prevents the virus from replicating itself [17]. In combination with TFV it showed a high anti-HIV activity [18]. Elvitegravir reduces the ability of the HIV-1 virus to replicate by preventing the insertion of viral DNA into the host cell [19]. This effect is enhanced by cobicistat, a cytochrome P-450 (CYP) 3A inhibitor, which extends Elvitegravir's time window, requiring one dose per day only [19].

Emtricitabine is thought to be eliminated similarly to TFV via glomerular filtration and active tubular secretion [17]. Although extensive research has been carried out on active tubular secretion of TFV, only a few studies exist which study the renal elimination process of emtricitabine and its possible role in DDI. Experimental data suggest that emtricitabine could be a substrate for multidrug and toxin extrusion protein 1 (Mate 1) [20], one of the efflux transporters of proximal tubule cells.

Around 95% of elvitegravir is recovered in feces and less than 7% in urine, indicating mainly hepatobiliary excretion of elvitegravir [21], [22]. In addition no metabolites of elvitegravir are detected in the urine indicating that elvitegravir is unlikely to cause a nephrotoxic event on its own [22].

Cobicistat, a booster for elvitegravir, was introduced on the market to replace ritonavir, because it is associated with reduced DDI [23]. Administration of cobicistat can lead to increased serum creatinine concentrations which are most probably a result of its inhibition potential of the multidrug and toxin extrusion protein–mediated efflux of creatinine [23], [24]. However, renal elimination of cobicistat happens only to a fraction of the compound, most is eliminated through feces [21].

Until today only a few *in vitro* studies exist which were used to pick up nephrotoxic effects of TFV on proximal tubule cells after individual dosing of TFV [25], [26], and even fewer in combination with elvitegravir, cobicistat, emtricitabine [24]. Considering the low number of studies and models which are described in literature, there is an urgent need for more research for potential test platforms which are able to detect nephrotoxic effects, especially in combinatorial drug exposures. This requires a minimal level of throughput of the testing system. The experimental work presented here further analyzes the toxicity of the two different TFV variants TDF and TAF. In our research we will study the toxicity of all single compounds of both drug combinations as well as potential drug interactions by combining each of the TFV prodrugs with each of the co-administrated compounds.

We will test the five compounds individually and in combination on our recently developed kidney-on-a-chip platform in the OrganoPlate 3-lane system (chapter 2, [27]). We could already assess that this kidney-on-a-chip model can be used as a reliable screening platform which is able to pick up nephrotoxic effects of known and unknown nephrotoxicants (chapter 2, [27] and chapter 3, [9]). In this study we will use renal proximal tubule cells where we could not measure any toxic effect after exposure to mono doses of TFV. Primarily we want to investigate whether one or both of the TFV prodrugs do have a toxic effect on these proximal tubule cells indicating a potential other elimination pathway than TFV. Furthermore, the three compounds co-administrated with the TFV prodrugs are not known to be eliminated via the anion pathway and therefore could show a toxic effect to the cells, as the cation pathway (if used by the compounds) was shown to be present in our model including the elimination via efflux transporter P-glycoprotein. Moreover, we wanted to investigate if any of the compounds does lead to damage in the proximal tubules or potentially amplifies the toxic effect when co-incubated with the TFV prodrugs.

Here we describe the use of a kidney-on-a-chip platform to test the effect of single administrated compounds as well as drug combinations. The model which we use enables compound assessment on 40 parallel cultured renal tubules. The platform is combined with a range of assays including the analysis of lactate dehydrogenase (LDH) activity in the medium as a measure of cell death, viability assays, fluorescence-based barrier integrity monitoring, and immunohistochemical staining of DNA damage marker H2AX. The technology can be used for high

throughput DDI studies which can be used to determine the effects or interaction of drugs on the kidney.

Methods

Cell culture

Proximal tubules were cultured in accordance with the protocols developed previously [27]. In short, renal proximal tubule epithelial cells (RPTEC; Kidney PTEC Control Cells, SA7K Clone, Sigma, Germany, MTOX1030, cultured in the supplier's medium) were seeded at passage 3 to the top channel in the OrganoPlate® 3-lane (*4003 400B*, MIMETAS, the Netherlands) against collagen 1 (composed of 4 mg/mL collagen 1 (AMSbio Cultrex 3D Collagen I Rat Tail, Cat. 3447-319 020-01), 100 mM HEPES (Life Technologies, 15630), and 3.7 mg/mL NaHCO3 (Sigma, 320 S5761)). The OrganoPlate was placed on a rocker platform (OrganoFlow®, MI-OFPR-L, Mimetas) in the incubator (37 °C, 5% CO2) at an angle of 7 degrees and a rotation interval of 8 minutes to enable perfusion though the channels.

Toxicant exposure

At day 6 after seeding of RPTEC to the OrganoPlate, medium of the top and the bottom channel was replaced by TOX medium (Minimum Essential Medium Eagle, Sigma-Aldrich, M4526, supplemented with RPTEC Tox Supplement, Sigma Aldrich, MTOXRTSUP) containing the compounds and/or the vehicle control. Cultures were exposed for 48 hours (see Table 1 for all compound information and concentrations). All compounds were dissolved in Dimethyl sulfoxide (DMSO, Sigma-Aldrich, D8418, vehicle control) according to the supplier's (*MedChemExpress*) recommendations.

The highest concentration which was tested for each of the compounds was dependent on the final DMSO concentration in the exposure medium which was set at 0.5%. This made ensured that in the follow up experiments combinations of the compounds could be tested at DMSO concentrations up to 1%. Concentrations higher than 1% DMSO have been shown to influence results strongly (data not shown). Concentrations were used at a quarter-log dilution range with 9 concentrations (including 0 µM). In follow-up experiments, combinations of each of the three compounds were tested in combination with the two prodrugs of TFV. The first combination was chosen at a concentration which did not show a toxic effect on the tubules of the mono-exposures, whereas in the second combination preferably at least one of the two compounds had shown a toxic effect.

Table 1: Compound information. All ordered at MedChemExpress and dissolved in DMSO.

Compound	Order number	Stock concentration (mM)	Highest experimental concentration in concentration curve (µM)
Tenofovir disoproxil fumarate (TDF)	HY-13782	10	50
Tenofovir alafenamide (TAF)	HY-15232A	60	300
Emtricitabine (Em)	HY-17427	40	200
Elvitegravir (El)	HY-14740	10	50
Cobicistat (Co)	HY-10493	35	175

Furthermore, the drugs were tested in the same ratio as used in the prescribed medication, which was not possible for TAF, as the concentration of TAF would have been too low to show any effect. The TAF concentration was kept around the level on which it was just toxic and combined with the other three compounds in a way to not exceed 1 % DMSO. The chosen concentrations of the combinations can be found in Table 2.

Table 2: Compound concentrations used for the combinations of the drugs. TDF: Tenofovir disoproxil fumarate, TAF: Tenofovir alafenamide fumarate, Em: Emtricitabine, El: Elvitegravir, Co: Cobicistat.

Drug brand name	Low concentration combination (µM)	High concentration combination (µM)
Stribild	74.4 TDF & 35.5 Em	92.0 TDF & 44.0 Em
Stribild	18.6 TDF & 8.9 El	27.0 TDF & 31.0 El
Stribild	8.0 TDF & 15.9 Co	27.7 TDF & 55.2 Co
Genvoya	287.6 TAF & 6.0 Em	575.2 TAF & 13.0 Em
Genvoya	176.0 TAF & 8.9 El	456.4 TAF & 23.0 El
Genvoya	314.9 TAF & 15.9 Co	515.4 TAF & 45.0 Co

LDH assay

The lactate dehydrogenase (LDH) activity assay was performed as previously described [27]. In short: For the analysis of the LDH activity, medium from the wells guiding to the apical side of the tubule was analyzed using a Lactate Dehydrogenase Activity Assay kit (Sigma, MAK066). For the study of the concentration curves the LDH assay was analyzed according to the supplier's recommendations, whereas for the combination screen LDH activity was measured at the same time point for all combinations to be able to directly compare the data.

WST-8 assay (Viability)

To analyze the viability of the cells the cell counting kit-8 (Sigma, 96992) was used as previously described [27]. In short: The 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) buffer was diluted 1:11 in medium and added to both perfusion channels of each chip. After a 20-minute incubation on the rocker, absorbance was measured at 450 nm in the in-and outlets with access to the lumen of the proximal tubule and averaged per chip.

BI assay

For the assessment of the apparent permeability index (P_{app}) of the tubules a barrier integrity assay was performed as previously described [27]. In short: Medium containing 0.5 mg/ml TRITC Dextran 4.4 kDa (Sigma, FD20S) and 0.5 mg/ml FITC Dextran 150 kDa (Sigma, T1287) was added to the lumen of the tubule and leakage out of the tubule into the ECM compartment was measured using a fluorescent microscope (Molecular devices) and analyzed using ImageJ [28].

Immunostaining

The barrier integrity assay was followed by a fixation with 3.7% formaldehyde after which an immunostaining was performed as previously described [27]. Markers Rb-a-Phospho-Histone (H2AX, DNA damage, Cell signaling technology, 9718S, 1:200), actin (skeleton of the cells, Life technologies, R37112, 2 drops/ml buffer), and acetylated tubulin (primary cilia and tubulin structures, Sigma, T6793, 1:4000) in combination with a DNA staining (Hoechst 3342, Invitrogen, H3570, 1:2000) were analyzed.

Statistics

Data analysis was performed using GraphPad Prism (GraphPad Prism, version 9.0.0 (121)). Data were analyzed using one-way ANOVA followed by a Tukey's multiple comparison test which compares each result with each other result. A p value of < 0.05 was considered to be significant.

Results

HIV-patients are commonly treated with Genvoya which replaced Stribild in recent years. Both are tenofovir prodrug-containing medications and are supplied as pills which contain a combination of active, antiretroviral compounds, namely emtricitabine, elvitegravir and cobicistat, and one of the two prodrugs of TFV, TAF or TDF, respectively.

The proximal-tubule-on-a-chip can be used to determine renal toxicity

Proximal tubules were seeded to the top channel of the OrganoPlate 3-lane (fig. 2 A-D). First, collagen 1 was loaded to the middle channel. After polymerization of the collagen 1 proximal tubule, cells were seeded to the top perfusion channel. Next, medium was added to the top wells guiding to the channel system and the OrganoPlate was placed on an interval rocker, enabling perfusion. At day 6 tubules were mature and leak tight and toxicant exposures could be started

[27]. To determine the renal toxicity of the test compounds, medium of both perfusion channels was replaced by medium containing the compounds or vehicle control and the OrganoPlate was placed again for 48 hours on the interval rocker.

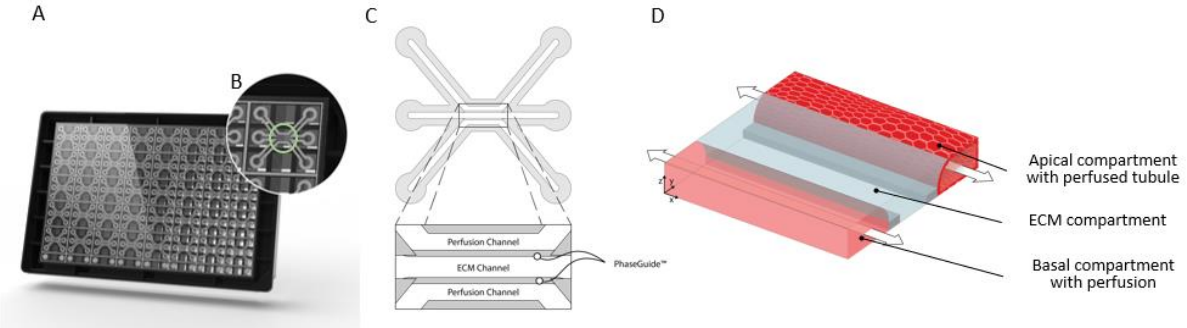

Figure 2: Proximal tubule-on-a-chip model in the OrganoPlate 3-lane platform. A *The top part of the OrganoPlate is a standard 384-well microtiter plate with 40 microfluidic chips embedded into a modified glass bottom.* ***B*** *Zoom-in on one chip with three channels convene in the center of the chip (green circle).* ***C*** *Schematic of the OrganoPlate 3-lane channel system. The three channels are 400 µm wide and 200 µm high and divided by 50 µm high phaseguides which function as liquid pressure barriers.* ***D*** *3D artist impression of one chip. The chip was loaded with collagen 1 (light blue) to the ECM channel and proximal tubule cells (red) were seeded to the top perfusion channel. After cell attachment medium (light red) was added to both perfusion channels and perfusion was started (indicated by white arrows). For exposures medium of both perfusion channels was replaced by medium containing the compounds or vehicle control.*

Exposure to the TFV prodrugs TDF and TAF

The toxicity of each TFV prodrug was tested by exposing the tubules to a dose range of a quarter-log concentration curve. The starting concentrations can be found in Table 1. The effect of the drugs on the proximal tubules was measured in three different ways in a multiplexed fashion: 1) the loss of tubular barrier integrity (P_{app}), 2) the loss of cellular membrane integrity (LDH), and 3) the reduction in enzymatic activity (viability).

The integrity of the tubular barrier after the exposure can be measured through determining the apparent permeability (P_{app}). Increasing values indicate a loss of the barrier function [27], [29]. Both TDF and TAF did not show damage of the cell connections after the exposure (fig. 3 A,B). The viability assay used for this study determines dehydrogenase activity as a measure for cell number [30] and the LDH activity assay measures the content of the enzyme LDH that is released into the cell culture medium upon damage to the plasma membrane [31]. In line with the barrier integrity assay no effect of TDF could be measured in both assays (fig. 3 C-F). TAF did not have an influence on the viability either, however a significant increase of LDH activity in the medium to 180% of the control could be measured.

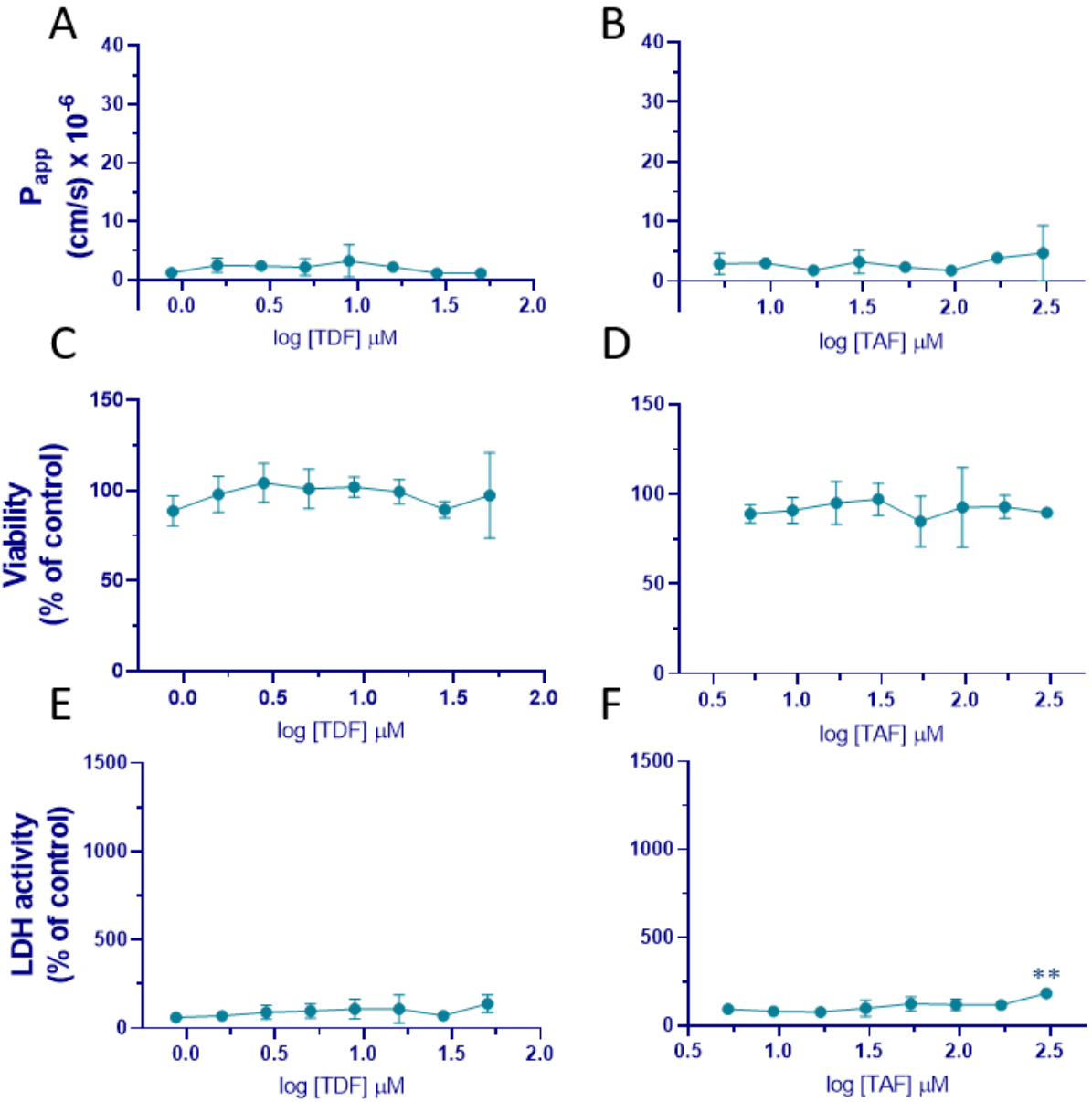

Figure 3: Exposure of RPTEC for 48 hours to concentration curves of the tenofovir prodrugs TDF (A, C, E) and TAF (B, D, F). *Data represent the P_{app} of a 150 kDa dextran (A, B),), the cell viability measured using a WST-8 assay (C, D), and the LDH activity in the medium (E, F). Abbreviations: P_{app}: apparent permeability; LDH: Lactate dehydrogenase; TDF: tenofovir disoproxil fumarate; TAF: tenofovir alafenamide fumarate. 3-5 chips per measure point. Data represent the mean with standard deviation. Data were compared to the lowest concentration of the corresponding compounds, *: p<0.05; **p<0.01.*

High concentrations of elvitegravir and cobicistat caused damage to the proximal tubules

Next to the TFV prodrugs TDF and TAF the same readouts were performed on the three co-administrated compounds. Emtricitabine had no effect on the barrier at the concentrations tested (fig. 4 A). For both elvitegravir and cobicistat a loss in barrier integrity could be measured for the two highest concentrations, 28 µM, 50 µM and 98 µM, 175 µM, respectively (fig. 4 B,C). The barrier loss was highest for cobicistat, at 30 x 10^{-6} cm/s compared to 23 x 10^{-6} cm/s for elvitegravir.

No loss of viability could be measured when cells were exposed to emtricitabine (fig. 4 D). When exposed to elvitegravir only the highest concentration (50 µM) showed a rapid loss in viability (fig. 4 E), whereas the pattern of cobicistat was more comparable to the barrier integrity assay with the last two concentrations showing a rapid loss in viability (fig. 4 F).

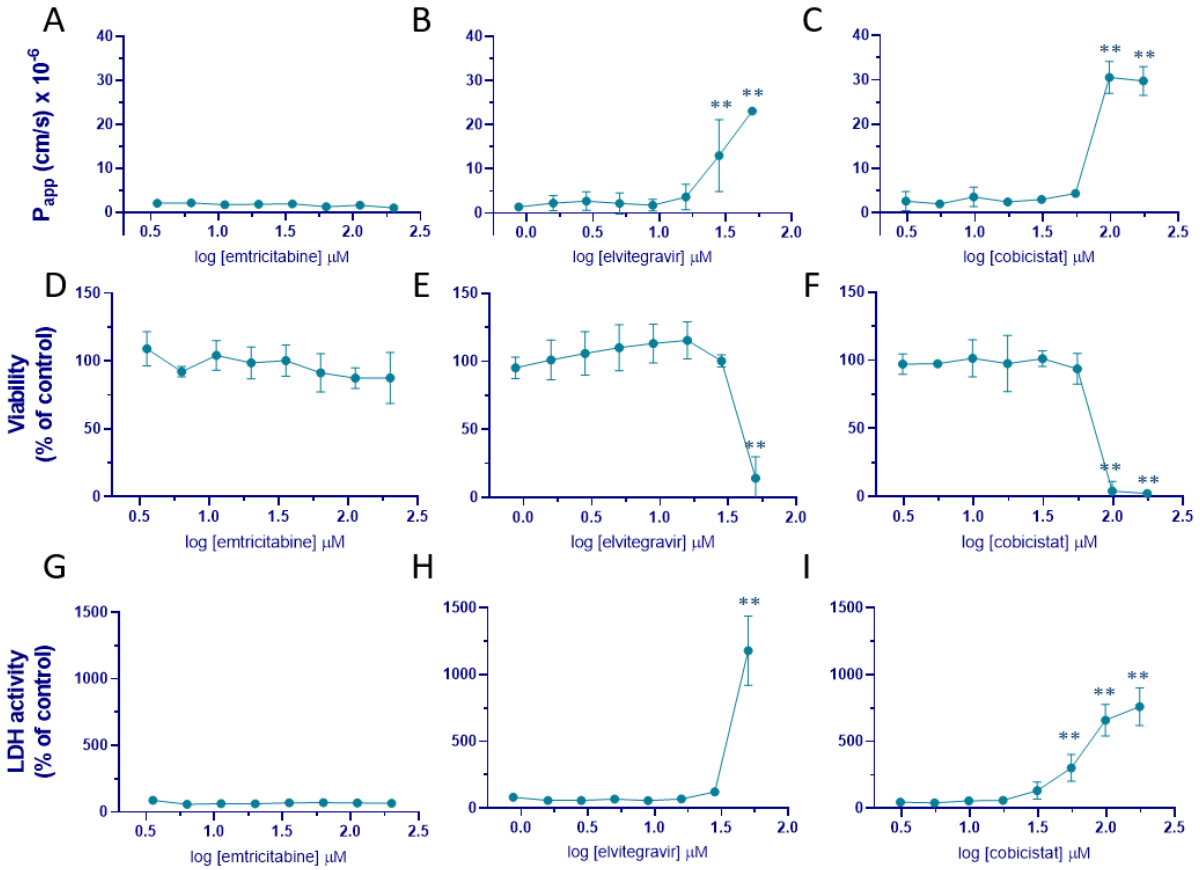

Figure 4: Exposure of RPTEC tubules for 48 hours to dose ranges of emtricitabine (A, D, G), elvitegravir (B, E, H), and cobicistat (C, F, I). *Data represent the P_{app} of a 150 kDa fitc dextran (A-C), the cell viability measured using a WST-8 assay (D-F), and the LDH activity in the medium (G-I). Abbreviations: P_{app}: apparent permeability; LDH: Lactate dehydrogenase; n= 3-5 chips per measure point. Data represent the mean with standard deviation. Data were compared to the lowest concentration of the corresponding compounds, *: p<0.05; **p<0.01.*

In line with the barrier integrity and the viability assay, exposure to emtricitabine did not result in any release of LDH, whereas the highest concentration of elvitegravir, 50 µM, led to a sudden increase of more than 1000 % release of LDH into the culture medium, similar to the measured effect in the viability assay where also only the highest concentration showed an effect. For cobicistat, LDH activity rose more gradually from a concentration starting at 55 µM to about 750% at the highest concentration of 175 µM (fig. 4 D-F).

Interestingly, the damaging effects of elvitegravir and cobicistat were measured for the lowest active concentrations in different assays. While the initial damage of elvitegravir was detected

with the barrier assay, cobicistat caused first release of LDH into the medium before an effect was detected by other assays.

DDIs can be picked up by the proximal-tubule-on-a-chip model

The highest concentration of the quarter-log dilution range was set to a DMSO concentration of 0.5%. In the drug interaction study a DMSO concentration of up to 1% was tolerated as this was the highest concentration which did not influence the behavior of RPTEC during a 48-hour exposure in previously performed experiments (data not shown). The first combination was chosen at a concentration which did not show a toxic effect on the tubules, whereas in the second combination preferably at least one of the two compounds did show a toxic effect. To not exceed 1% DMSO some of the compounds could not be tested at the highest possible concentration, since all drugs were tested at the same ratio as used in the prescribed medication. The chosen concentrations of the drug combinations can be found in Table 2.

From each drug combination the compounds were also tested alone and in combination, while the vehicle concentration was kept the same. The same readouts as for the dose analysis were chosen and in addition a fourth readout was added, which analyzed the occurrence DNA damage by immunostaining for a DNA damage marker H2AX [32].

Figure 5 provides the experimental data of TDF combined with each of the compounds emtricitabine (fig. 5 A,D,G,K), elvitegravir (fig. 5 B,E,i,L), and cobicistat (fig. 5 C,F,J,M).

Emtricitabine did not have a damaging effect on the proximal tubules in any of the assays: neither alone nor in co-incubation with TDF.

An effect of elvitegravir could be detected at its highest concentration (31.0 µM) in all assays, though no drug interaction with TDF (27 µM) could be measured, except a slight increase of DNA damage when dosed together.

Cobicistat caused a damaging effect on the apparent permeability (a 5-fold change compared to vehicle, fig. 5 C), the viability (reduced to 70%, fig. 5 F), the LDH release (a 8-fold change, fig. 5J), and the DNA damage staining (increase to around 350%, fig. 5M) when exposed to the higher concentration (55.2 µM). When co-administered with TDF an enhanced damaging effect could be measured in the barrier integrity assay, leading to complete loss of the barrier function, a reduction to 20% of the viability and an increased signal of the fluorescent intensity to 2000% compared to the vehicle control. A moderate effect of TDF addition could be measured in the LDH assay where the effect on LDH release was 1.3 times higher compared to an exposure to cobicistat only.

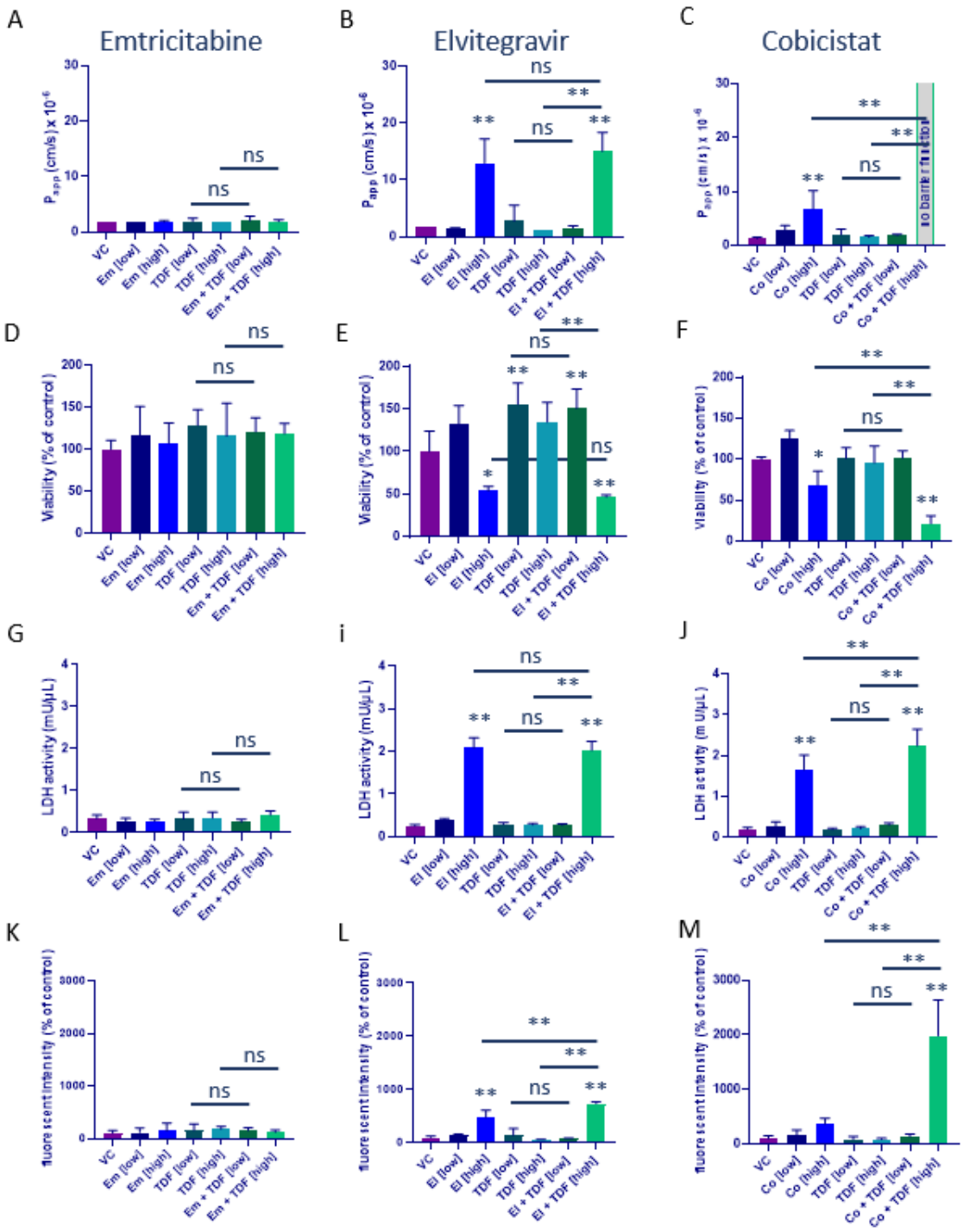

Figure 5: Exposure of RPTEC to combinations of the single components of the drugs co-incubated with TDF. ***A,D,G,K*** *co-incubation of TAF with emtricitabine (em),* ***B,E,i,L*** *Co-incubation of TAF with elvitegravir (el)),* ***C,F.J,M*** *co-incubation of TAF with cobicistat (co).* ***A-C*** *P_{app} measured with a fluorescent barrier integrity assay.* ***D-F*** *Viability measured with a WST-8 assay.* ***G-J*** *Analysis of LDH activity in the medium.* ***K-M*** *Fluorescent intensity of the H2AX immunofluorescence staining which is an antibody which detects DNA damage. Abbreviations: VC: vehicle control; P_{app}: apparent permeability; LDH: Lactate dehydrogenase; TDF: tenofovir disoproxil fumarate; Em: Emtricitabine; El: Elvitegravir; Co: Cobicistat. 3-5 chips per measure point. Data represent the mean with standard deviation. Data were compared to the vehicle control (VC) when not indicated differently by horizontal bars. ns: not significant; *: p<0.05; **p<0.01.*

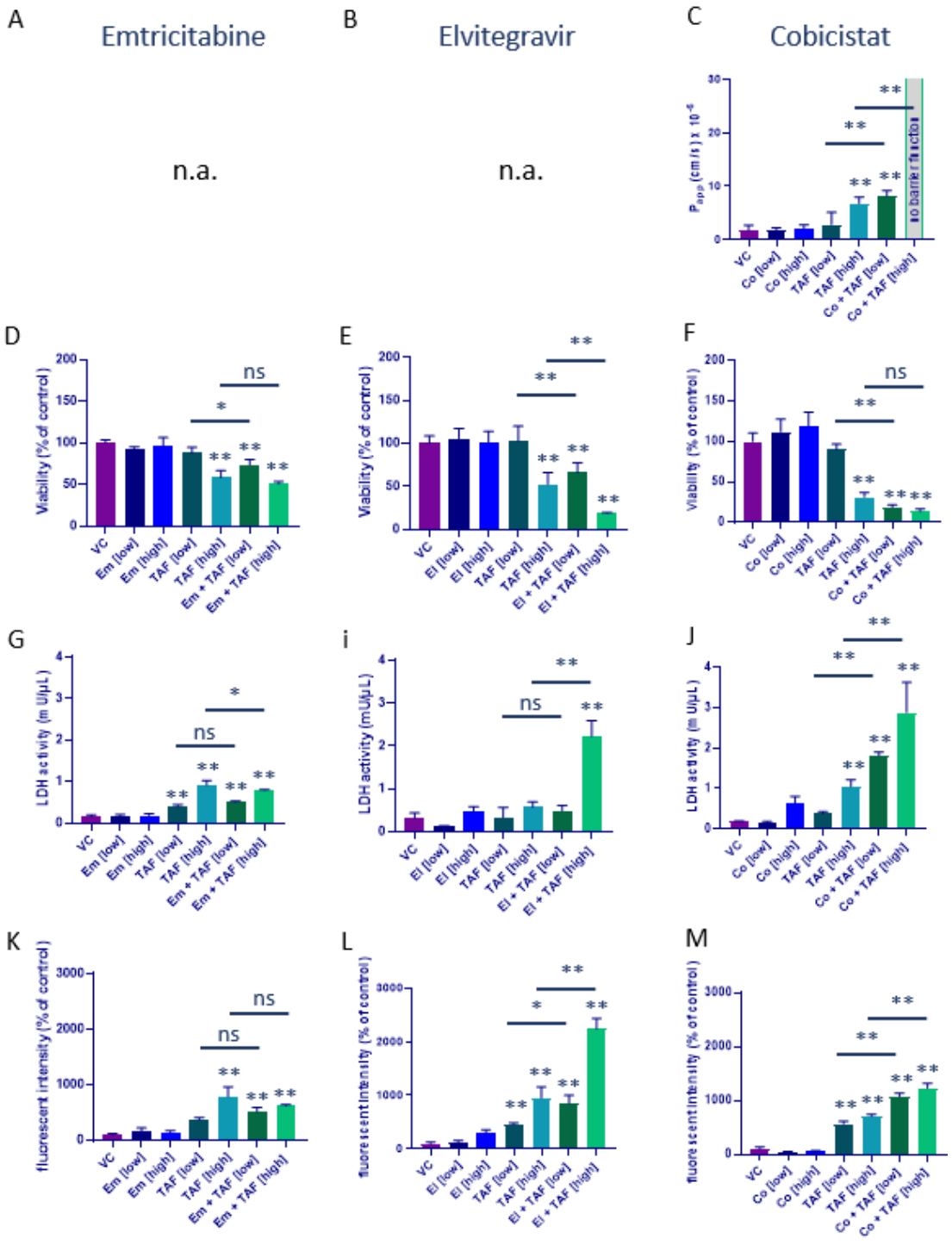

Figure 6: Exposure of RPTEC to combinations of the single components of the drugs co-incubated with TAF. ***A,D,G,K*** *co-incubation of TAF with emtricitabine (em),* ***B,E,i,L*** *Co-incubation of TAF with elvitegravir (el)),* ***C,F.J,M*** *co-incubation of TAF with cobicistat (co).* ***A-C*** *P_{app} measured with a fluorescent barrier integrity assay.* ***D-F*** *Viability measured with a WST-8 assay.* ***G-J*** *Analysis of LDH activity in the medium.* ***K-M*** *Fluorescent intensity of the H2AX immunofluorescence staining which is an antibody which detects DNA damage. Abbreviations: VC: vehicle control; P_{app}: apparent permeability; LDH: Lactate dehydrogenase; TAF: tenofovir alafenamide fumarate; Em: Emtricitabine; El: Elvitegravir; Co: Cobicistat; n.a.: data not available. 3-5 chips per measure point. Data represent the mean with standard deviation. Data were compared to the vehicle control (VC) when not indicated differently by horizontal bars. ns: not significant; *: p<0.05; **p<0.01.*

The experimental data of TAF combined with each of the compounds emtricitabine (fig. 6 A,D,G,K), elvitegravir (fig. 6 B,E,i,L), and cobicistat (fig. 6 C,F,J,M) is provided in figure 6.

When TAF was tested at a higher concentration than tested during the dose range study, TAF showed a toxic effect in all assays when exposed to concentrations above 300 µM (fig. 6). This observation was in line with the results of the dose range study (fig. 3), where a moderate effect of 300 µM TAF was seen in the LDH assay.

Similar to the exposure with TDF, little or no effect of emtricitabine was measured when co-exposed with TAF. In the viability assay a slight decreased viability could be measured during co-exposure of TAF (low) mono dose compared to TAF (low) + emtricitabine (fig. 6 D). This effect was not measured with any of the other assays. However, an opposite mild effect of the combination of TAF (high) + emtricitabine was measured in the LDH activity assay (fig. 6 G). These mild effects indicate that emtricitabine and TAF do not enhance each other in damaging the proximal tubules.

The combination of elvitegravir and TAF did enhance the damage on the tubules when dosed together at both the low (176.0 µM TAF and 8.9 µM elvitegravir) and the high (456.4 µM TAF and 23.0 µM elvitegravir) combinations which could be measured in all readouts with one exception: the combination of TAF 176.0 µM (low) + elvitegravir 8.9 µM did not increase the LDH activity (fig. 6 i), though an effect was detected with the viability assay (fig. 6 E) and the DNA damage staining (fig. 6 L).

Exposure to cobicistat alone did not show any damaging effect to the proximal tubules at both chosen doses of 15.9 µM (low) and 45 µM (high). For TAF 314 µM (low) a damaging effect could only be detected in the DNA staining (fig. 6 M), whereas an effect of the higher dose (515.4 µM) could be measured in all assays (fig. 6 C,F,J,M). When cobicistat and TAF were combined, an enhanced damaging effect could be observed with all assays for the low and the high doses of the combinations, which in the barrier integrity assay led to a complete loss of the barrier function.

In summary, these results show that elvitegravir and cobicistat have a damaging effect on the proximal tubules. When TAF was tested at a higher concentration (above 300 µM) than tested during the dose range study, TAF caused a moderate toxic effect in all assays. Interaction of compounds leading to increased damage could be seen when cobicistat and TDF were exposed in the same cocktail. A strong effect of TAF when exposed together with elvitegravir or cobicistat could be measured with all readout assays.

Discussion

The present study was focused on assessing whether we can use our proximal tubule-on-a-chip model (fig. 2) in a DDI study using a panel of multiplexed assays to examine the toxicity of mono and combinatorial dosages of HIV-targeting drugs. Increased toxicity could be clearly shown when

combining compounds, whereas single compound exposure showed a lower level of toxicity on the proximal tubules.

Mono doses of TDF did not show any damaging effect for a concentration up to 92 µM (fig. 3, 5). In our study we exposed the cells in a medium containing around 1% fetal bovine serum (TOX medium). Callebaut et al. showed that TDF is mostly converted to TFV when in contact with serum [3]. We presume that in our study TDF was converted into TFV as well after dilution in TOX medium. TFV toxicity in literature ranges from 10 µM in OAT1 (over-) expressing cells to no toxic events measurable in human RPTEC at concentrations even above 4 mM [24]–[26] (Table 3).

Table 3: IC50 values after TFV exposure in human proximal tubule cell sources with and without stable transporter expression

Cell source	Stable transporter expression	IC 50 after TFV exposure (µM)	Reference
Human RPTEC		> 1000, > 4000	[9], [24]
ciPTEC-OAT1	OAT1	189	[25]
HEK293T-OAT1	OAT1	10, 78	[26], [24]
HEK293T-OAT1/MRP4	OAT1 and MRP4	299	[24]

Considering that TDF is converted into TFV when in contact with serum it is likely that the damaging molecular concentration of TDF will be similar to the damaging molecular concentration of TFV. In the current study TDF was not tested above 100 µM. Most likely TDF was not tested at its actual toxic concentration. However, we used human RPTEC without OAT transporters which, similarly to the human RPTEC used by Stray et al. [24] and us [9], did not show a cytotoxic response when exposed to TFV. Further research can be undertaken to investigate if there is a damaging effect of TDF measurable after exposure in serum-free medium. Moreover, it is recommended to dissolve the TFV prodrugs in an aqueous solutions (for example (serum free) culture medium) to be able to test the compound at higher concentrations, without exceeding the maximum vehicle concentration of DMSO.

In contrast to TDF, a damaging effect of TAF on the proximal tubules could be detected from values of 300 µM and higher (fig. 3 and 6) for the individual dosing and a value of 176 µM when dosed in combination with elvitegravir. These concentrations fall into the dose ranges which were reported by us (chapter 3, [9]) and are slightly above the concentrations other groups reported (Table 3) when exposing proximal tubules cells to TFV. TAF is thought to be stable when in contact with blood plasma [3] and not a compound for OAT1 transport as it enters cells passively [15]. Our findings support the outcome of these studies, since the exposure to TAF resulted in

nephrotoxic damage at similar concentrations as TFV when exposed to cells expressing anion transporter OAT 1.

In the present study we wanted to examine if, next to the TFV prodrugs, one of the additive compounds had a toxic or enhancing toxic effect on the proximal tubules when combined with the TFV variants. In the following couple of paragraphs the possible toxicity and toxicity-enhancing effects of different compounds are discussed, using the results of this research.

Emtricitabine did not show a toxic effect on the proximal tubules (fig. 4, 5, 6). This is in line with existing literature, where no direct toxic effect of emtricitabine on human RPTEC is found [24]. Even though it is thought that emtricitabine is eliminated similarly to TFV via glomerular filtration and active tubular secretion, we can suggest from our results that the clearance pathways are most probably not the same, because co-incubation of emtricitabine and TFV did not show any interaction in the used readouts.

Elvitegravir did not have an enhancing effect on the cytotoxicity when dosed in combination with TDF (fig. 5). When dosed together with TAF, elvitegravir strongly enhanced the damaging effect (fig. 6). Most interestingly, neither elvitegravir nor TAF increased the LDH activity in the medium individually. When the two were combined at high concentrations, super-additive synergistic effects occurred with increased LDH activity to more than 450%. This result could be somewhat surprising, as elvitegravir is not believed to affect the kidneys but is mainly eliminated via hepatobiliary excretion [22]. It is likely that if this elimination pathway is not available, exposure to high concentrations of elvitegravir does cause damage to the renal cells.

Cobicistat is transported via the cation pathway (influx via OCT2, efflux via mate 1), whereas TFV is transported via the anion pathway (influx via OAT 1/3 and efflux via MRP4) [10], [24], [33]. As we were able to detect damage of drugs transported via the cation pathway previously [27], it is possible that if cobicistat is having a damaging effect on the proximal tubules this effect would be measured. When cobicistat was dosed at concentrations of 55 µM, mild damaging effects were measured. When cobicistat was dosed at 55 µM in combination with 27.7 µM TDF (which by itself did not show any damaging effect) increased damage on the tubules could be measured with all assays. Similar results could be detected when cobicistat was dosed together with TAF (fig. 6). Here, an increase in cytotoxicity was measured for the low dosed combinations as well as the highly concentrated combinations. Therefore, it can be concluded that the combination of TFV and cobicistat at high molecular concentrations leads to enhanced cytotoxicity in the proximal tubule cells.

In the next paragraph the possible interaction of cobicistat with TFV will be discussed. Cobicistat is generally well tolerated by patients and has limited known side effects. In both drug cocktails cobicistat is used as a CYP 3A inhibitor for elvitegravir. Elvitegravir is predominantly metabolized by CYP3A with a short half-life [23]. When elvitegravir is co-formulated with cobicistat the

elimination of elvitegravir is prolonged, allowing an administration once per day [22]. TFV is not known to be an CYP inhibitor/enhancer or being metabolized by CYP3A enzymes [34] as TFV is eliminated via the urine unchanged [35]. It is likely that the increased damage on the proximal tubules which we observed cannot be attributed to an accumulation of TFV in the cells after a delayed metabolism when dosed together with cobicistat. Another possible explanation may be that at high concentrations of cobicistat, TFV can enter the cells by other pathways than the OAT1 transport which is lacking in RPTEC. In a study by Stray et al. [24] the potential of cobicistat to influence the pharmacology of TFV was assessed. They found that cobicistat did not interact with the transport of TFV by OAT1, but they could detect a minor inhibitory effect of cobicistat on OAT3 transport. However, they suggested that this effect was unlikely to be clinically relevant as most TFV transport in the proximal tubules is mediated by OAT1. Next to the influx via OAT1 they also assessed the efflux transporter MRP4. In our recent work we were able to confirm the presence of MRP mediated efflux in our model [27]. Stray et al. could show that MRP4 mediated efflux is sensitive to cobicistat in serum-free medium. However, this effect was eliminated when they performed the experiments in serum-containing medium. In our study we used much higher concentrations of both TFV and cobicistat. Therefore, it could be possible that not all cobicistat was eliminated in the presence of serum (1% in the exposure medium) and was able to inhibit MRP4 efflux, resulting in higher concentrations of both TFV and cobicistat in the cells leading to an increase in damage when combining the drugs.

The study we performed does have some important limitations, which actually can guide future research. First, when comparing the concentrations of the compounds in the culture medium to the maximum clinically observed concentrations in plasma, those which showed a toxic effect in our *in vitro* model were higher than reported *in vivo* [36], [37]. Thus, to be able to translate our results to the *in vivo* situation, further research on our *in vitro* model needs to be performed. Starting with pharmacokinetic modeling to determine drug concentrations and combinations to be tested [38], many aspects would need to be optimized and standardized: the culture of the model itself, the assays and the readout methods, exposure concentrations, and exposure duration. Eventually these data can be used in *in silico* models to accurately predict the *in vivo* situation [39], [40].

Another limitation in this study is that a commercially available cell source was used which does not express all crucial transporters which are responsible for (part of) the transport of some of the tested compounds. Nonetheless, we were able to show that TFV delivered in the form of prodrug TAF has a cytotoxic effect on our proximal tubule model at concentrations similar to exposure of OAT1-expressing cells when exposed to TFV. In the combination studies additive or even synergistic damaging effects on the proximal tubules were detected. Using an even broader panel of assays and a second cell source with OAT1 expression would be a fruitful area for further work.

One last limitation in this study which affected the results significantly was that in the combination study TAF was used in much higher concentrations than would have been adequate when trying to resemble the ratio of TAF to co-administered compounds in Genvoya. In Genvoya, the molecular concentration of TAF is only a fraction of the three other compounds. Here it would be interesting to re-design the initial experiments by dissolving the compounds in, if possible, aqueous solutions to be able to test the compounds at the correct ratios.

Although we know that the concentrations used in this study were too high to be physiologically relevant, we could show that the proximal-tubule-on-a-chip platform is a suitable tool that can be used to study the effect of drugs and drug combinations on the kidney.

Conclusion

The present study was designed to determine if the proximal tubule-on-a-chip can be used to study compound panels and toxicity-enhancing effects when compounds are co-administered. To this end different read-out assays were used to study the toxicity of the drugs. We could show that particularly the combination of the assays can lead to a more complete picture of the compound's toxicity. For some compounds, their earliest toxicity can be identified in the barrier integrity assay because they damage the cell connections first, other compounds affect the cell membrane initially, resulting in an increase of LDH activity in the medium. The findings of this investigation complement those of earlier published data (chapter 2, [27] and chapter 3, [9]), where also the combination of assays offered a broader picture on the toxicity mechanism. For future studies it could be of high interest to combine the assays from all studies.

In recent years, Stribild, which contains the tenofovir (TFV) prodrug tenofovir disoproxil fumarate (TDF) was replaced by Genvoya which contains the prodrug tenofovir alafenamide (TAF). TAF is thought to be taken up by cells passively, whereas TDF is converted into TFV in the presence of serum containing medium and then enters cells via anion transporter OAT1. The model used in this research is lacking transporter OAT1 and did not show a response to TFV exposure in previous studies [9]. However, the present results could show that TAF, when tested individually, is cytotoxic at similar molecular concentrations compared to the exposure concentrations of TFV when exposed to cells containing the OAT1 transport mechanism (chapter 3, [9]), while no cytotoxic effect was detected with TDF. From these results we can conclude that TAF enters cells passively, whereas TDF converts into TFV and is dependent on active anion transport to accumulate inside the cells and cause cytotoxicity.

TAF is administered in Genvoya at a fraction of TDF in Stribild (10 mg and 300 mg, respectively, [41], [42]). When TAF was dosed at the correct ratio compared to the toxic concentration of TFV, no toxicity was measured in the present study. We can therefore conclude that our experiments

confirmed that the new drug combination (Genvoya) using TAF instead of TDF does have a decreased potential for adverse kidney-related side effects, supporting the results seen clinically.

In the present study the combination of TAF and the other three antiretroviral agents was not dosed at the correct ratio as used in Genvoya because this would exceed the concentration of solvent DMSO. At correct ratios TAF could have been tested only at a fraction of the concentration found toxic in the mono dose study. An obvious area for future research could start by focusing on testing the drugs used in Genvoya at the concentrations which resemble the ratio of TAF to co-administered compounds in Genvoya. Doing this would grant a better model which can be used to mimic the influence of TAF and its co-administered compounds on the results.

In most research which study the effect of TFV and its prodrugs, they are tested individually. Our results indicate that elvitegravir as well as cobicistat also affected the proximal tubule cells. We suggest that in future studies TFV and its prodrugs should be tested in combination with the drugs with which they will be co-administered. However, during *in vitro* experiments some of the drugs might be incorrectly dosed, as *in vivo* they would be metabolized quickly by the liver or cleared via the gut. To overcome this problem a possible solution could be to connect the present model with a liver-on-the-chip system [43] and/or a gut-on-a-chip system [29], [44]. Or, include pharmacokinetic modeling to choose proper concentrations for experiments.

We were able to show that the proximal-tubule-on-a-chip model can serve as a steppingstone for future DDI studies. Our platform is able to distinguish which processes are affected first by the different drug (combinations) and pick up synergistic (additive and super-additive) effects of co-administered drugs. Moreover, the platform can be potentially used to assess if these synergistic effects are relevant or not when used in combination with pharmacogenetic modeling.

References

[1] S. K. Nigam, W. Wu, K. T. Bush, M. P. Hoenig, R. C. Blantz, and V. Bhatnagar, "Handling of drugs, metabolites, and uremic toxins by kidney proximal tubule drug transporters," *Clinical Journal of the American Society of Nephrology*, vol. 10, no. 11, pp. 2039–2049, 2015, doi: 10.2215/CJN.02440314.

[2] J. J. Kohler *et al.*, "Tenofovir renal proximal tubular toxicity is regulated by OAT1 and MRP4 transporters," *Laboratory Investigation*, vol. 91, no. 6, pp. 852–858, 2011, doi: 10.1038/labinvest.2011.48.

[3] C. Callebaut, G. Stepan, Y. Tian, and M. D. Miller, "*In vitro* virology profile of tenofovir alafenamide, a novel oral prodrug of tenofovir with improved antiviral activity compared to that of tenofovir disoproxil fumarate," *Antimicrobial Agents and Chemotherapy*, vol. 59, no. 10, pp. 5909–5916, 2015, doi: 10.1128/AAC.01152-15.

[4] A. S. Ray, M. W. Fordyce, and M. J. M. Hitchcock, "Tenofovir alafenamide: A novel prodrug of tenofovir for the treatment of Human Immunodeficiency Virus," *Antiviral Research*, vol. 125, pp. 63–70, 2016, doi: 10.1016/j.antiviral.2015.11.009.

[5] U.S. Food & Drug Administration, “Drug Approval Package, Stribild, Fixed Dose,” 2012. https://www.accessdata.fda.gov/drugsatfda_docs/nda/2012/203100Orig1s000TOC.cfm#:~:text=Approval Date%3A 08%2F27%2F2012 (accessed Oct. 29, 2020).

[6] U.S. Food & Drug Administration, “GENVOYA (elvitegravir, cobicistat, emtricitabine, and tenofovir alafenamide) fixed-dose combination tablet.,” 2015. https://www.accessdata.fda.gov/drugsatfda_docs/nda/2015/207561Orig1s000Approv.pdf (accessed Oct. 29, 2020).

[7] Statista, “Selected top HIV/AIDS drugs worldwide based on revenue from 2016 to 2018,” *Statista*, 2019. https://www.statista.com/statistics/273434/revenue-of-the-worlds-most-important-aids-drugs/ (accessed Oct. 29, 2020).

[8] E. De Clercq, “Role of tenofovir alafenamide (TAF) in the treatment and prophylaxis of HIV and HBV infections,” *Biochemical Pharmacology*, vol. 153, pp. 2–11, Jul. 2018, doi: 10.1016/j.bcp.2017.11.023.

[9] M. K. Vormann *et al.*, “Implementation of a Human Renal Proximal Tubule on a Chip for Nephrotoxicity and Drug Interaction Studies,” *Journal of Pharmaceutical Sciences*, pp. 1–14, 2021, doi: 10.1016/j.xphs.2021.01.028.

[10] A. S. Ray *et al.*, “Mechanism of active renal tubular efflux of tenofovir,” *Antimicrobial Agents and Chemotherapy*, vol. 50, no. 10, pp. 3297–3304, 2006, doi: 10.1128/AAC.00251-06.

[11] T. K. Novick, M. J. Choi, A. Z. Rosenberg, B. A. McMahon, D. Fine, and M. G. Atta, “Tenofovir alafenamide nephrotoxicity in an HIV-positive patient,” *Medicine*, vol. 96, no. 36, p. e8046, Sep. 2017, doi: 10.1097/MD.0000000000008046.

[12] X. Liu and G. Pan, Eds., *Drug Transporters in Drug Disposition, Effects and Toxicity*, vol. 1141. Singapore: Springer Singapore, 2019. doi: 10.1007/978-981-13-7647-4.

[13] P. E. Sax *et al.*, “Tenofovir Alafenamide Vs. Tenofovir Disoproxil Fumarate in Single Tablet Regimens for Initial HIV-1 Therapy,” *JAIDS Journal of Acquired Immune Deficiency Syndromes*, vol. 67, no. 1, pp. 52–58, Sep. 2014, doi: 10.1097/QAI.0000000000000225.

[14] Y. Uwai, H. Ida, Y. Tsuji, T. Katsura, and K. I. Inui, “Renal transport of adefovir, cidofovir, and tenofovir by SLC22A family members (hOAT1, hOAT3, and hOCT2),” *Pharmaceutical Research*, vol. 24, no. 4, pp. 811–815, 2007, doi: 10.1007/s11095-006-9196-x.

[15] R. A. Bam, S. R. Yant, and T. Cihlar, “Tenofovir alafenamide is not a substrate for renal organic anion transporters (OATs) and does not exhibit OAT-dependent cytotoxicity,” *Antiviral Therapy*, vol. 19, no. 7, pp. 687–692, 2014, doi: 10.3851/IMP2770.

[16] J. J. Kohler *et al.*, “Tenofovir renal toxicity targets mitochondria of renal proximal tubules,” *Laboratory Investigation*, vol. 89, no. 5, pp. 513–519, 2009, doi: 10.1038/labinvest.2009.14.

[17] L. M. Bang and L. J. Scott, “Emtricitabine: An Antiretroviral Agent for HIV infection,” *Drugs*, vol. 63, no. 22, pp. 2413–2424, 2003, doi: 10.2165/00003495-200363220-00003.

[18] K. Borroto-Esoda, J. E. Vela, F. Myrick, A. S. Ray, and M. D. Miller, “*In vitro* evaluation of the anti-HIV activity and metabolic interactions of tenofovir and emtricitabine,” *Antiviral Therapy*, vol. 11, no. 3, pp. 377–384, 2006.

[19] J. L. Olin, L. M. Spooner, and O. M. Klibanov, “Elvitegravir/cobicistat/emcitrabine/ténofovir disoproxil (fumarate de) en association dans un seul comprimé pour le traitement de l’infection

par le VIH," *Annals of Pharmacotherapy*, vol. 46, no. 12, pp. 1671–1677, 2012, doi: 10.1345/aph.1R468.

[20] J. Reznicek, M. Ceckova, L. Cerveny, F. Müller, and F. Staud, "Emtricitabine is a substrate of MATE1 but not of OCT1, OCT2, P-gp, BCRP or MRP2 transporters," *Xenobiotica*, vol. 47, no. 1, pp. 77–85, 2017, doi: 10.3109/00498254.2016.1158886.

[21] D. J. Cada, S. Torres, T. L. Levien, and D. E. Baker, "Elvitegravir/Cobicistat/Emtricitabine/Tenofovir disoproxil fumarate tablets," *Hospital Pharmacy*, vol. 48, no. 1, pp. 48–56, 2013, doi: 10.1310/hpj4801-48.

[22] S. Ramanathan, A. A. Mathias, P. German, and B. P. Kearney, "Clinical pharmacokinetic and pharmacodynamic profile of the HIV integrase inhibitor elvitegravir," *Clinical Pharmacokinetics*, vol. 50, no. 4, pp. 229–244, 2011, doi: 10.2165/11584570-000000000-00000.

[23] B. M. Shah, J. J. Schafer, J. Priano, and K. E. Squires, "Cobicistat: A New Boost for the Treatment of Human Immunodeficiency Virus Infection," *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*, vol. 33, no. 10, pp. 1107–1116, Oct. 2013, doi: 10.1002/phar.1237.

[24] K. M. Stray *et al.*, "Evaluation of the effect of cobicistat on the *in vitro* renal transport and cytotoxicity potential of tenofovir," *Antimicrobial Agents and Chemotherapy*, vol. 57, no. 10, pp. 4982–4989, 2013, doi: 10.1128/AAC.00712-13.

[25] T. T. G. Nieskens *et al.*, "A Human Renal Proximal Tubule Cell Line with Stable Organic Anion Transporter 1 and 3 Expression Predictive for Antiviral-Induced Toxicity," *AAPS Journal*, vol. 18, no. 2, pp. 465–475, 2016, doi: 10.1208/s12248-016-9871-8.

[26] X. Zhang, R. Wang, M. Piotrowski, H. Zhang, and K. L. Leach, "Intracellular concentrations determine the cytotoxicity of adefovir, cidofovir and tenofovir," *Toxicology in vitro*, vol. 29, no. 1, pp. 251–258, 2015, doi: 10.1016/j.tiv.2014.10.019.

[27] M. K. Vormann *et al.*, "Nephrotoxicity and Kidney Transport Assessment on 3D Perfused Proximal Tubules," *AAPS Journal*, vol. 20, no. 5, pp. 1–11, 2018, doi: 10.1208/s12248-018-0248-z.

[28] J. Schindelin *et al.*, "Fiji: An open-source platform for biological-image analysis," *Nature Methods*, vol. 9, no. 7, pp. 676–682, 2012, doi: 10.1038/nmeth.2019.

[29] S. J. Trietsch *et al.*, "Membrane-free culture and real-time barrier integrity assessment of perfused intestinal epithelium tubes," *Nature Communications*, vol. 8, no. 1, pp. 1–7, 2017, doi: 10.1038/s41467-017-00259-3.

[30] H. Tominaga *et al.*, "A water-soluble tetrazolium salt useful for colorimetric cell viability assay," *Analytical Communications*, vol. 36, no. 2, pp. 47–50, 1999, doi: 10.1039/a809656b.

[31] C. Legrand *et al.*, "Lactate dehydrogenase (LDH) activity of the number of dead cells in the medium of cultured eukaryotic cells as marker," *Journal of Biotechnology*, vol. 25, no. 3, pp. 231–243, 1992, doi: 10.1016/0168-1656(92)90158-6.

[32] L. J. Kuo and L. X. Yang, "γ-H2AX- A novel biomaker for DNA double-strand breaks," *In vivo*, vol. 22, no. 3, pp. 305–310, 2008.

[33] E. I. Lepist *et al.*, "Contribution of the organic anion transporter OAT2 to the renal active tubular secretion of creatinine and mechanism for serum creatinine elevations caused by cobicistat," *Kidney International*, vol. 86, no. 2, pp. 350–357, 2014, doi: 10.1038/ki.2014.66.

[34] Gilead Sciences, "VIREAD® (tenofovir disoproxil fumarate) Tablets Patient Information," 2005.

[35] B. P. Kearney, J. F. Flaherty, and J. Shah, "Tenofovir disoproxil fumarate: Clinical pharmacology and pharmacokinetics," *Clinical Pharmacokinetics*, vol. 43, no. 9, pp. 595–612, 2004, doi: 10.2165/00003088-200443090-00003.

[36] C. M. Perry, "Elvitegravir/Cobicistat/Emtricitabine/Tenofovir Disoproxil Fumarate Single-Tablet Regimen (Stribild®): A Review of Its Use in the Management of HIV-1 Infection in Adults," *Drugs*, vol. 74, no. 1, pp. 75–97, Jan. 2014, doi: 10.1007/s40265-013-0158-4.

[37] J. M. Custodio *et al.*, "Pharmacokinetics and Safety of Tenofovir Alafenamide in HIV-Uninfected Subjects with Severe Renal Impairment," *Antimicrobial Agents and Chemotherapy*, vol. 60, no. 9, pp. 5135–5140, Sep. 2016, doi: 10.1128/AAC.00005-16.

[38] C. Perry, G. Davis, T. M. Conner, and T. Zhang, "Utilization of Physiologically Based Pharmacokinetic Modeling in Clinical Pharmacology and Therapeutics: an Overview," *Current Pharmacology Reports*, vol. 6, no. 3, pp. 71–84, 2020, doi: 10.1007/s40495-020-00212-x.

[39] B. Feng, J. L. LaPerle, G. Chang, and M. V. Varma, "Renal clearance in drug discovery and development: Molecular descriptors, drug transporters and disease state," *Expert Opinion on Drug Metabolism and Toxicology*, vol. 6, no. 8, pp. 939–952, 2010, doi: 10.1517/17425255.2010.482930.

[40] S. Polak, "*In vitro* to human *in vivo* translation – pharmacokinetics and pharmacodynamics of quinidine," *ALTEX*, vol. 30, no. 3, pp. 309–318, 2013, doi: 10.14573/altex.2013.3.309.

[41] Gilead Sciences, "STRIBILD® (elvitegravir, cobicistat, emtricitabine, and tenofovir disoproxil fumarate) tablets, for oral use - prescribing information," 2012.

[42] Gilead Sciences, "GENVOYA® (elvitegravir, cobicistat, emtricitabine, and tenofovir alafenamide) tablets, for oral use - prescribing information," 2015.

[43] K. M. Bircsak *et al.*, "A 3D microfluidic liver model for high throughput compound toxicity screening in the OrganoPlate®," *Toxicology*, vol. 450, no. August 2020, p. 152667, 2021, doi: 10.1016/j.tox.2020.152667.

[44] E. Naumovska *et al.*, "Direct on-chip differentiation of intestinal tubules from induced pluripotent stem cells," *International Journal of Molecular Sciences*, vol. 21, no. 14, pp. 1–15, 2020, doi: 10.3390/ijms21144964.