Dissecting drug-induced in vitro cytotoxicity and metabolic dysfunction in conditionally immortalized human proximal tubule cells

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Research Article

Abstract

Acute kidney injury accounts for 20% of all hospitalized adults, and 14 to 26% is drug-induced, emphasizing the importance of proper nephrotoxicity assessment. The 'gold standard' MTT assay is widely used to measure cell viability, but depends on cellular metabolic activity. Consequently, MTT may not be most optimal to assess cytotoxicity, as nephrotoxicity often involves mitochondrial dysfunction. We compared MTT with a direct cell death assay based on a compromised plasma membrane permeability. Mature conditionally immortalized proximal tubule epithelial cells were dose- (0.1-1,000 µM) and time- (0.5, 1, 2, 4, 8 and 24 hours) dependently exposed to a selection of prototypic nephrotoxicants. Dose-dependent reductions in cellular metabolic activity were stronger compared to declines in fluorescence-based cell death, most prominently for cisplatin (1.6 ± 2.0% and 68 ± 4% (mean ± SEM), respectively) and chloroacetaldehyde (2.13 ± 0.05% and 61.0 ± 0.8%). Similar, but more pronounced time-dependent effects were observed, particularly for sanguinarine. We show that assessing cellular metabolic activity by MTT provides a composite readout of cellular metabolic activity and cell death. A nuclear staining approach is preferable when assessing nephrotoxicity of metabolically active compounds. We recommend both assays during drug development to discriminate between metabolically active versus non-active compounds.

Introduction

A steadily increasing number of commonly used drugs as well as various experimental compounds have been associated with acute kidney injury (AKI). AKI is a major disease burden, affecting 20 percent of hospitalized adults worldwide [1,2]. Mortality rates are over 50 percent in severe cases and less severe manifestations are associated with chronic kidney disease [3]. Various prospective cohort studies indicated that drug-induced mechanisms explain 14 to 26 percent of AKI cases, emphasizing the importance of renal toxicity of drugs [4–6]. Early detection of drug-induced toxicity, and particularly renal toxicity, is also an essential part of lead-optimization in drug development, as it influences the success of a candidate compound to proceed in the developmental process. However, lowering the high drug attrition rates in clinical drug development phases remains a key challenge for pharmaceutical companies. Despite the reduction in drug candidate failures due to poor pharmacokinetic profiles, issues regarding efficacy and safety still lead to high attrition rates [7–10]. This has been confirmed by an inventory of the main reasons for drug attrition from four leading pharmaceutical companies, showing a substantial contribution of safety-related (*i.e.*, non-clinical toxicology) causes [10]. Especially nephrotoxicity is often observed in clinical studies that was not detected earlier during non-clinical development [11]. Hence, improved non-clinical testing of therapeutic candidates for their nephrotoxic potential is warranted to reduce attrition rates in clinical development phases [12].

Obtaining accurate and reliable results from *in vitro* cytotoxicity assays is therefore of vital importance. In the 1980s, Mosmann developed a cell viability and proliferation assay, measuring the reductive activity as enzymatic conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) into

dimethyl sulfoxide (DMSO)-soluble formazan crystals by dehydrogenases, which is nowadays often recognized as the 'golden standard' for cytotoxicity assessment [12,13]. However, the MTT assay determines cellular metabolic activity, as it mainly relies on the activity of mitochondrial dehydrogenases, in particular succinate dehydrogenase [14]. Consequently, this method may not be suitable to distinguish drug-induced metabolic suppression from reduced cell viability. This limitation has been described for various known inhibitors of cellular metabolism in three human breast carcinoma cell lines [12]. Similarly, radiation-induced cellular metabolic hyperactivation biases the ability of the MTT assay to assess cell viability [14]. Moreover, MTT reduction can be influenced by other enzymes linked with cellular redox status, like glutathione S-transferases, further warranting cautious interpretation of results obtained with MTT using metabolically active compounds [15]. Finally, chemical reduction of MTT has been found in other organelles such as endoplasmic reticulum and may also explain MTT reduction by flavonoids and polyphenols [12,16–19].

Similar limitations of the MTT assay may also be relevant for the assessment of renal toxicity, as mitochondrial dysfunction is described for a vast number of nephrotoxic drugs [20]. Proximal tubule cells, which are most often affected in renal toxicity, are metabolically very active and have a high mitochondrial content [21]. So far, it has not been investigated whether MTT is the most suitable assay to assess the nephrotoxic potential of drugs. Therefore, we compared the MTT assay with the determination of cell viability by a method independent of cell metabolism, based on cell membrane permeability [22]. This assay combines Yo-Pro-1-iodide that specifically stains apoptotic cells with propidium iodide, which only crosses plasma membranes of necrotic cells, and hoechst to visualize all cells [23–26].

We compared both methods in a renal proximal tubule cell model, ciPTEC-OAT1, exposed to a selection of prototypic nephrotoxic drugs in a dose- and time-dependent manner. We demonstrate that, compared to the fluorescence-based approach, assessment of cellular metabolic activity by means of MTT does not exclusively assess cellular toxicity, but rather provides a composite readout of cell death and decreased metabolic rate.

Results

MTT assay shows a stronger dose-dependent decrease upon exposure to nephrotoxic compounds compared to fluorescence-based cell death assessment

Comparison of nephrotoxicity detected by MTT and our fluorescence microscopy method was performed using four compounds, which previously have shown to induce nephrotoxic effects associated with mitochondrial dysfunction. These compounds cover a range of different drug classes and include the antineoplastic drug cisplatin, the antiretroviral drug tenofovir, the Na^+/K^+-ATPase inhibitor sanguinarine, and chloroacetaldehyde, a cytotoxic metabolite of the antineoplastic drug ifosfamide. ciPTEC expressing the organic anion transporter 1 (OAT1) cells were exposed for 24 hours to a dose-range of all four compounds. Cisplatin reduced cellular metabolic activity to 1.6 ± 2.0% whereas fluorescence-based cell

death only lowered viable cells to 68 ± 4% (100 µM; Fig. 1A), suggesting that it substantially impedes cellular metabolic activity in addition to cytotoxicity ($p_{assay} < 0.0001$). For tenofovir a reduction to 32 ± 6% and 43 ± 9% in viable cells was observed using MTT and fluorescence microscopy, respectively (1,000 µM), which did not significantly differ between both assays ($p_{assay} = 0.56$; Fig. 1B). Sanguinarine reduced cell viability to 1.4 ± 0.1% and 20 ± 10% (10 µM; Fig. 1C) using the MTT or fluorescence-based cell death analysis, respectively, with a statistically significant difference between both assays ($p_{assay} < 0.0001$). At increasing chloroacetaldehyde concentrations cell viability significantly differed when measured by MTT compared to our fluorescence-based cell death assay ($p_{assay} < 0.0001$; 2.13 ± 0.05% and 61.0 ± 0.8%, respectively; 1,000 µM; Fig. 1D). These observations imply that previously described toxic effects determined using the MTT assay of at least cisplatin, sanguinarine and chloroacetaldehyde, could have been influenced by compound interference with the metabolizing capacity of the cell. Consequently, it emphasizes the essential difference in readout between both assays described and warrants careful interpretation of results.

Nephrotoxic compounds time-dependently interfere with cellular metabolic activity and fluorescence-based cell death

Remarkably, no differences between both assays could be observed for the prototypic nephrotoxic drug tenofovir. It has, however, been described that drugs can decrease cellular metabolic activity before cellular viability is compromised [27]. Consequently, we investigated whether at earlier timepoints differences between both methods could show different results for tenofovir and all other compounds. These time-dependent effects were measured after 0.5, 1, 2, 4, 8 and 24 hours. To this end, ciPTEC-OAT1 cells were exposed to IC_{70} and IC_{90} drug concentrations (calculated from dose-dependent effects on cellular metabolic capacity, see Table 1), as the commonly used IC_{50} concentration is likely subjected to variation due to its position in the steepest part of the curve. All prototypic nephrotoxicants showed a significant time-dependent reduction in cell viability as measure of cellular metabolic activity and fluorescence-based cell death (Fig. 2). For cisplatin and tenofovir (Fig. 2A and 2B, respectively), this effect was only observed 24 hours after incubation. Also, both assays significantly differed 24 hours after incubation for cisplatin ($p < 0.01$; Fig. 2A) and 8 hours after incubation for tenofovir ($p < 0.05$; Fig. 2B). The most prominent difference was noted for sanguinarine (Fig. 2C). A significant reduction in cell viability was observed 2 hours after incubation ($p < 0.0001$), whereas a significantly lower cell viability as determined by fluorescence microscopy occurred only 4 hours after incubation ($p < 0.05$). Overall, cellular metabolic activity showed a stronger reduction compared to the fluorescence-based approach ($p_{assay} < 0.0001$), indicating that sanguinarine primarily interferes with metabolic capacity, while cell death originates secondary to a reduced metabolic activity. Remarkably, 8 hours after compound exposure, cellular metabolic activity was very low (1.7 ± 0.2%), whereas the nuclear staining approach showed a residual viability of 72 ± 3%, which could suggest activation of other compensatory cellular metabolic pathways that maintain cells viable. Chloroacetaldehyde showed a reduction in cell viability as measure

of both cellular metabolic activity and fluorescence-based cell death, starting 8 hours after compound incubation (Fig. 2D). After 24 hours of incubation, cellular metabolic activity was reduced to a viability of 29 ± 5% (p < 0.0001), whereas according to the fluorescence-based assay, cell death decreased to only 72 ± 9% of control (p < 0.01). Similar, but less prominent effects of compound exposure on cellular metabolic activity and fluorescence-based cell death could be observed for exposure to IC_{70}-concentrations (Fig. 3), at which all four compounds significantly decreased cellular viability.

Table 1

Overview of prototypic nephrotoxicants with calculated IC_{70}- and IC_{90}-concentrations. IC_{70}- and IC_{90}-concentrations are defined as inhibitory concentrations that result in 70% and 90% of the maximal inhibitory effect on cellular metabolic activity, respectively. IC_{70} and IC_{90} concentrations were based on and calculated using IC_{50}-values of mean cellular metabolic activity dose-response curve fittings (Fig. 1). 95% confidence bands were plotted in curve fitting graphs and used to calculate confidence intervals for IC_{70} and IC_{90}-concentrations.

compound	IC_{70} (µM) (95% - CI)	IC_{90} (µM) (95% - CI)
chloroacetaldehyde	22 (17–28)	36 (25–59)
cisplatin	28 (18–38)	43 (28 - ∞)
sanguinarine	4 (3–5)	11 (6 - ∞)
tenofovir	231 (135–427)	422 (182 - ∞)

Discussion

Drug-induced kidney injury is one of the most frequently observed adverse effects and often leads to high attrition rates during clinical drug development stages [1,11]. This warrants the use of proper *in vitro* strategies to assess potential toxic drug effects early in the development of new drugs. Importantly, many drugs are known to disturb mitochondrial function [28], which may bias the interpretation of results obtained by commonly used tetrazolium salt-based methods that rely on enzymatic activity, such as the MTT assay. Here, we compared the toxicity of four nephrotoxic metabolically active drugs by the MTT

assay and a fluorescence-based method, which is not dependent on cellular metabolism. Differences in dose- and time-dependent toxicity patterns were observed between both assays, in which the selected drugs showed a stronger and earlier interference with cellular metabolic capacity.

The observed strong inhibitory effect of cisplatin on cellular metabolic activity has previously been related to apoptosis through mitochondrial pathways, as generation of (mitochondrial) oxidative stress appeared critical in its mechanism of cytotoxicity [29–33]. A similar strong reduction in the MTT assay has been reported before [34], and a weaker inhibitory effect using our fluorescence-based cell death assay is in line with the observed cisplatin-induced mitochondrial dysfunction. For tenofovir, a comparable time-dependent effect on cellular metabolic capacity and cytotoxicity was detected. The slightly stronger metabolic response compared to fluorescence-based cell death confirms previous observations that tenofovir primarily targets mitochondria (*e.g.*, ultrastructural defects and mtDNA depletion) of the renal proximal tubule [35–38]. The consequent disruption of mitochondrial biogenesis would explain reduced abundance of mitochondrial enzymes, including dehydrogenases. Sanguinarine also demonstrated a strong reduction in cellular metabolic activity, whereas fluorescence-based cell death was less severely affected. Serious toxicity of sanguinarine has been recognized before [39] and time-dependent differences between both assays align with previously observed mitochondrial effects, including depolarization and inhibition of respiration [40,41]. Finally, the strong metabolic effects of the ifosfamide metabolite chloroacetaldehyde is in agreement with previously observed toxicity in rabbit renal proximal tubule cells, in which ATP levels were dose-dependently reduced [42]. Moreover, chloroacetaldehyde seems to inhibit hexokinase [43], which further explains the strong cellular metabolic effects observed in our study. Overall, our results are in accordance with previously reported cytotoxicity of the drugs studied, and differences between both assays can be explained by effects of the various nephrotoxicants on cellular metabolism.

The observed strong dose- and time-dependent patterns in our study are, however, expected not to be specific to the MTT assay. Similar tetrazolium-based assays, including XTT, WST-1/8 and CCK-8, all rely on enzymatic conversion of a substrate by metabolic capacity of the cell [13,44], these are therefore expected to show similar patterns after exposure to metabolically active drugs. Consequently, these enzymatic assays are not preferred in assessing cellular effects of metabolically active compounds, as they provide a composite readout consisting of cytotoxic and metabolic effects. Besides our fluorescence-based method, other non-metabolism-dependent approaches, including Annexin-V and TUNEL assays have extensively been applied to examine cell death, also in high-throughput settings [45,46]. Apoptosis detection by both methods is, however, limited as compared to Yo-Pro-1, which makes the latter a more sensitive approach to identify cytotoxicity in drug development [47,48].

Our observation that cellular metabolic capacity is time-dependently affected highlights the importance of differentiating between metabolically-dependent and -independent cytotoxicity assays. These observations are especially important for drugs with a metabolic target, as such primary mitochondrial toxicants are expected to decrease mitochondrial dysfunction before cell viability is affected, as we observed for sanguinarine. Interestingly, time-dependent comparison of (nephrotoxic) compounds on

cellular toxicity, could provide an ideal experimental setup to differentiate between primary metabolic active drugs and drugs that inhibit cellular metabolism via indirect mechanisms (*i.e.*, secondary mitochondrial toxicants), including inhibitors of other metabolic pathways. Consequently, such a combination of assays may be instrumental to gain insight into the mechanisms underlying renal adverse drug events.

To conclude, methods measuring cellular metabolic activity, like the MTT assay, do not exclusively assess cellular toxicity. They rather provide a composite readout of cell death and decreased metabolic rate, as opposed to cytotoxicity assessed using assays that do not rely on cellular metabolism. Consequently, the latter cell death assay is suitable for assessing toxicity of metabolically active compounds. A combination of assays would be preferable for initial toxicity screening in drug development to discriminate between metabolically active and non-active drugs. Selection of the proper cell viability assessment methods in early drug development stages or when assessing toxicity of existing drugs is vital, and will contribute to the development of drugs with low nephrotoxic potential and improved safety profile.

Materials And Methods

Compounds

Cisplatin and chloroacetaldehyde solution were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Sanguinarine (chloride) was from Cayman Chemical (Ann Arbor, MI, USA) and tenofovir was obtained from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA, USA).

Cell culture

Human conditionally immortalized proximal tubule epithelial cells expressing organic anion transporter 1 (ciPTEC-OAT1, RRID:CVCL_LI01) were obtained as previously described [49,50]. Proliferating cells were cultured at 33°C and 5% (*v/v*) CO_2. Medium, consisting of 1:1 Dulbecco's modified Eagle's medium and nutrient mixture F-12 without phenol red (DMEM Ham's F-12, Life Technologies, Paisley, UK), supplemented with 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenium, 36 ng/mL hydrocortisone, 10 ng/mL human epidermal growth factor (EGF), 40 pg/mL trio-iodothyrine (all purchased from Sigma-Aldrich), 1% (*v/v*) penicillin/streptomycin (Life Technologies) and 10% (*v/v*) fetal bovine serum (FBS, Greiner Bio-One, Alpen a/d Rijn, The Netherlands), further referred to as PTEC complete medium, was refreshed every 2–3 days. Cells used for experiments varied from passage numbers 49 to 58. ciPTEC-OAT1 was seeded in black or transparent/clear flat bottom 96-wells plates at a density of 63,000 cells/cm^2. Cells proliferated for 1 day at 33°C and 5% (*v/v*) CO_2 in PTEC complete medium, followed by 7 days maturation at 37°C, 5% (*v/v*) CO_2 in PTEC complete medium without antibiotics to differentiate into an epithelial monolayer. Mature ciPTEC-OAT1 were exposed to prototypic nephrotoxic compounds, including cisplatin, tenofovir, sanguinarine and chloroacetaldehyde in a serial √10-dilution (0.1-1,000 µM, unless stated otherwise, dissolved in PTEC complete medium or DMSO for chloroacetaldehyde), for 0.5,

1, 2, 4, 8, or 24 hours at 37°C and 5% (v/v) CO_2, after which cellular metabolic activity or fluorescence-based cell death were assessed. DMSO concentrations did not exceed 0.1% (v/v). Determination of IC_{70} and IC_{90} concentrations (Table 1) for time-dependent compound exposure was based on dose-response curves of cellular metabolic activity (MTT), which were normalized to unexposed ciPTEC-OAT1.

Analysis of cellular metabolic activity

To evaluate compound-induced effects on cellular metabolic activity in ciPTEC-OAT1, a colorimetric assay based on tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), was performed [13]. In short, ciPTEC-OAT1 were cultured and exposed as described above in transparent/clear flat bottom 96-wells plates (Corning, Amsterdam, The Netherlands) and compound-exposed ciPTEC-OAT1 were washed three times with serum-free PTEC complete medium (SFM), followed by incubation with 0.5 mg/mL MTT in SFM for 3 hours at 37°C and 5% (v/v) CO_2. Formed formazan crystals were dissolved in DMSO (Merck, Darmstadt, Germany) on a microplate shaker (VWR, Radnor, PA, USA) for 2 hours. Absorption was measured at 560 nm and subtracted from background at 670 nm using Benchmark (Bio-Rad, Veenendaal, The Netherlands). Values were normalized to unexposed ciPTEC-OAT1 control.

Fluorescence-based analysis of cell death

ciPTEC-OAT1 were seeded in black/clear flat bottom 96-wells plates (Fisher Scientific, Landsmeer, The Netherlands), cultured and dose- and time-dependently exposed as described above. Nuclei were stained using hoechst 33342 (20 µg/mL, Life Technologies), Yo-Pro-1-iodide (2 µM, Life Technologies) and propidium iodide (1 µg/mL, Sigma-Aldrich) to differentiate all, early apoptotic, and necrotic cells, respectively, as previously described [26,51]. Cells were incubated with the various dyes for 30 minutes at 37°C. Fluorescence was imaged at a total 100x magnification using Becton Dickinson (BD) Pathway 855 high-throughput microscope (BD Bioscience, Breda, The Netherlands). Obtained images were analyzed for viable and non-viable cells using CellProfiler™ – 3.0.0 [52–54].

Statistical analysis

Curve-fitting and statistical data analysis were performed using GraphPad prism v5.03 (GraphPad Software Inc., San Diego, CA). Data were normalized to untreated (vehicle) control ciPTEC-OAT1. Results were plotted after background subtraction using nonlinear regression with four parameters and variable slope, constraining the bottom to greater than 0.0 on log-transformed x-values. Statistical significance of dose- and time-dependent data was determined by one-way ANOVA, followed by Dunnett's *post hoc* analysis to correct for multiple comparison, and considered being significant when $p < 0.05$. Differences between assay types (p_{assay}) were statistically analyzed using two-way ANOVA and corrected for multiple comparison by Bonferroni's *post hoc* analysis. All data is presented as mean ± SEM of at least three independent experiments (n = 3), performed with five or six experimental replicates.

Declarations

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by C.A.H. The first draft of the manuscript was written by C.A.H. and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

COMPETING INTEREST

F.G.M.R. is co-inventor 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 has conflict of interest through commercialization of ciPTEC models via Cell4Pharma. J.A.M.S. is the founding CEO of Khondrion BV, a Radboud University Medical Center spin-out company founded by J.A.M.S. Other authors do not have any conflict of interest.

DATA AVAILABILITY

The data generated and analyzed during the current study are available from the corresponding authors on reasonable request.

References

1 Siew, E. D. & Davenport, A. The growth of acute kidney injury: a rising tide or just closer attention to detail? *Kidney Int* **87**, 46-61, doi:10.1038/ki.2014.293 (2015).

2 Ishimoto, Y. & Inagi, R. Mitochondria: a therapeutic target in acute kidney injury. *Nephrol Dial Transplant* **31**, 1062-1069, doi:10.1093/ndt/gfv317 (2016).

3 Hoste, E. A. *et al.* Epidemiology of acute kidney injury in critically ill patients: the multinational AKI-EPI study. *Intensive Care Med* **41**, 1411-1423, doi:10.1007/s00134-015-3934-7 (2015).

4 Perazella, M. A. Pharmacology behind Common Drug Nephrotoxicities. *Clin J Am Soc Nephrol* **13**, 1897-1908, doi:10.2215/CJN.00150118 (2018).

5 Moffett, B. S. & Goldstein, S. L. Acute kidney injury and increasing nephrotoxic-medication exposure in noncritically-ill children. *Clin J Am Soc Nephrol* **6**, 856-863, doi:10.2215/CJN.08110910 (2011).

6 Mehta, R. L. *et al.* Spectrum of acute renal failure in the intensive care unit: the PICARD experience. *Kidney Int* **66**, 1613-1621, doi:10.1111/j.1523-1755.2004.00927.x (2004).

7 Hay, M., Thomas, D. W., Craighead, J. L., Economides, C. & Rosenthal, J. Clinical development success rates for investigational drugs. *Nat Biotechnol* **32**, 40-51, doi:10.1038/nbt.2786 (2014).

8 Bunnage, M. E. Getting pharmaceutical R&D back on target. *Nat Chem Biol* **7**, 335-339, doi:10.1038/nchembio.581 (2011).

9 Kola, I. & Landis, J. Can the pharmaceutical industry reduce attrition rates? *Nat Rev Drug Discov* **3**, 711-715, doi:10.1038/nrd1470 (2004).

10 Waring, M. J. *et al.* An analysis of the attrition of drug candidates from four major pharmaceutical companies. *Nat Rev Drug Discov* **14**, 475-486, doi:10.1038/nrd4609 (2015).

11 Troth, S. P., Simutis, F., Friedman, G. S., Todd, S. & Sistare, F. D. Kidney Safety Assessment: Current Practices in Drug Development. *Semin Nephrol* **39**, 120-131, doi:10.1016/j.semnephrol.2018.12.002 (2019).

12 van Tonder, A., Joubert, A. M. & Cromarty, A. D. Limitations of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays. *BMC Res Notes* **8**, 47, doi:10.1186/s13104-015-1000-8 (2015).

13 Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**, 55-63, doi:10.1016/0022-1759(83)90303-4 (1983).

14 Rai, Y. *et al.* Mitochondrial biogenesis and metabolic hyperactivation limits the application of MTT assay in the estimation of radiation induced growth inhibition. *Sci Rep* **8**, 1531, doi:10.1038/s41598-018-19930-w (2018).

15 York, J. L., Maddox, L. C., Zimniak, P., McHugh, T. E. & Grant, D. F. Reduction of MTT by glutathione S-transferase. *Biotechniques* **25**, 622-624, 626-628, doi:10.2144/98254st03 (1998).

16 Stockert, J. C., Blazquez-Castro, A., Canete, M., Horobin, R. W. & Villanueva, A. MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. *Acta Histochem* **114**, 785-796, doi:10.1016/j.acthis.2012.01.006 (2012).

17 Pagliacci, M. C. *et al.* Genistein inhibits tumour cell growth in vitro but enhances mitochondrial reduction of tetrazolium salts: a further pitfall in the use of the MTT assay for evaluating cell growth and survival. *Eur J Cancer* **29A**, 1573-1577, doi:10.1016/0959-8049(93)90297-s (1993).

18 Wang, P., Henning, S. M. & Heber, D. Limitations of MTT and MTS-based assays for measurement of antiproliferative activity of green tea polyphenols. *PLoS One* **5**, e10202, doi:10.1371/journal.pone.0010202 (2010).

19 Peng, L., Wang, B. & Ren, P. Reduction of MTT by flavonoids in the absence of cells. *Colloids Surf B Biointerfaces* **45**, 108-111, doi:10.1016/j.colsurfb.2005.07.014 (2005).

20 Sanz, A. B., Sanchez-Niño, M. D., Ramos, A. M. & Ortiz, A. in *Mitochondrial Dysfunction Caused by Drugs and Environmental Toxicants* (ed Y. Will, Dykens, J.A.) 169-184 (2018).

21 Bhargava, P. & Schnellmann, R. G. Mitochondrial energetics in the kidney. *Nat Rev Nephrol* **13**, 629-646, doi:10.1038/nrneph.2017.107 (2017).

22 Gawlitta, D., Oomens, C. W., Baaijens, F. P. & Bouten, C. V. Evaluation of a continuous quantification method of apoptosis and necrosis in tissue cultures. *Cytotechnology* **46**, 139-150, doi:10.1007/s10616-005-2551-7 (2004).

23 Boffa, D. J. *et al.* Measurement of apoptosis of intact human islets by confocal optical sectioning and stereologic analysis of YO-PRO-1-stained islets. *Transplantation* **79**, 842-845, doi:10.1097/01.tp.0000155175.24802.73 (2005).

24 Idziorek, T., Estaquier, J., De Bels, F. & Ameisen, J. C. YOPRO-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interfering with cell viability. *J Immunol Methods* **185**, 249-258, doi:10.1016/0022-1759(95)00172-7 (1995).

25 Liu, X., Van Vleet, T. & Schnellmann, R. G. The role of calpain in oncotic cell death. *Annu Rev Pharmacol Toxicol* **44**, 349-370, doi:10.1146/annurev.pharmtox.44.101802.121804 (2004).

26 Schirris, T. J. *et al.* Statin-Induced Myopathy Is Associated with Mitochondrial Complex III Inhibition. *Cell Metab* **22**, 399-407, doi:10.1016/j.cmet.2015.08.002 (2015).

27 Verma, N., Pink, M., Kersch, C., Rettenmeier, A. W. & Schmitz-Spanke, S. Benzo[a]pyrene mediated time- and dose-dependent alteration in cellular metabolism of primary pig bladder cells with emphasis on proline cycling. *Arch Toxicol* **93**, 2593-2602, doi:10.1007/s00204-019-02521-7 (2019).

28 Cez, A. *et al.* Decreased expression of megalin and cubilin and altered mitochondrial activity in tenofovir nephrotoxicity. *Hum Pathol* **73**, 89-101, doi:10.1016/j.humpath.2017.12.018 (2018).

29 Fuertes, M. A., Castilla, J., Alonso, C. & Perez, J. M. Cisplatin biochemical mechanism of action: from cytotoxicity to induction of cell death through interconnections between apoptotic and necrotic pathways. *Curr Med Chem* **10**, 257-266, doi:10.2174/0929867033368484 (2003).

30 Cullen, K. J., Yang, Z. J., Schumaker, L. & Guo, Z. M. Mitochondria as a critical target of the chemotheraputic agent cisplatin in head and neck cancer. *J Bioenerg Biomembr* **39**, 43-50, doi:10.1007/s10863-006-9059-5 (2007).

31 Yu, W. G. *et al.* Cisplatin generates oxidative stress which is accompanied by rapid shifts in central carbon metabolism. *Sci Rep-Uk* **8**, doi:ARTN 4306

10.1038/s41598-018-22640-y (2018).

32 Choi, Y. M. *et al.* Mechanism of Cisplatin-Induced Cytotoxicity Is Correlated to Impaired Metabolism Due to Mitochondrial ROS Generation. *Plos One***10**, doi:ARTN e0135083

10.1371/journal.pone.0135083 (2015).

33 Marullo, R. *et al.* Cisplatin Induces a Mitochondrial-ROS Response That Contributes to Cytotoxicity Depending on Mitochondrial Redox Status and Bioenergetic Functions. *Plos One***8**, doi:ARTN e81162

10.1371/journal.pone.0081162 (2013).

34 Nieskens, T. T. G. *et al.* Expression of Organic Anion Transporter 1 or 3 in Human Kidney Proximal Tubule Cells Reduces Cisplatin Sensitivity. *Drug Metab Dispos***46**, 592-599, doi:10.1124/dmd.117.079384 (2018).

35 Kohler, J. J. *et al.* Tenofovir renal toxicity targets mitochondria of renal proximal tubules. *Lab Invest***89**, 513-519, doi:10.1038/labinvest.2009.14 (2009).

36 Lewis, W., Day, B. J. & Copeland, W. C. Mitochondrial toxicity of NRTI antiviral drugs: an integrated cellular perspective. *Nat Rev Drug Discov***2**, 812-822, doi:10.1038/nrd1201 (2003).

37 Lee, H., Hanes, J. & Johnson, K. A. Toxicity of nucleoside analogues used to treat AIDS and the selectivity of the mitochondrial DNA polymerase. *Biochemistry***42**, 14711-14719, doi:10.1021/bi035596s (2003).

38 Milian, L. *et al.* Tenofovir-induced toxicity in renal proximal tubular epithelial cells: involvement of mitochondria. *AIDS***31**, 1679-1684, doi:10.1097/QAD.0000000000001572 (2017).

39 Gong, X. L. *et al.* Sanguinarine triggers intrinsic apoptosis to suppress colorectal cancer growth through disassociation between STRAP and MELK. *Bmc Cancer***18**, doi:ARTN 578

10.1186/s12885-018-4463-x (2018).

40 Chang, M. C. *et al.* Induction of necrosis and apoptosis to KB cancer cells by sanguinarine is associated with reactive oxygen species production and mitochondrial membrane depolarization. *Toxicol Appl Pharmacol***218**, 143-151, doi:10.1016/j.taap.2006.10.025 (2007).

41 Serafim, T. L. *et al.* Sanguinarine cytotoxicity on mouse melanoma K1735-M2 cells-Nuclear vs. mitochondrial effects. *Biochemical Pharmacology***76**, 1459-1475, doi:10.1016/j.bcp.2008.07.013 (2008).

42 Springate, J., Chan, K., Lu, H., Davies, S. & Taub, M. Toxicity of ifosfamide and its metabolite chloroacetaldehyde in cultured renal tubule cells. *In Vitro Cell Dev Biol Anim***35**, 314-317, doi:10.1007/s11626-999-0080-y (1999).

43 Knouzy, B., Dubourg, L., Baverel, G. & Michoudet, C. Ifosfamide metabolite chloroacetaldehyde inhibits cell proliferation and glucose metabolism without decreasing cellular ATP content in human breast

cancer cells MCF-7. *J Appl Toxicol* **30**, 204-211, doi:10.1002/jat.1485 (2010).

44 Ginouves, M., Carme, B., Couppie, P. & Prevot, G. Comparison of tetrazolium salt assays for evaluation of drug activity against Leishmania spp. *J Clin Microbiol* **52**, 2131-2138, doi:10.1128/JCM.00201-14 (2014).

45 Arifulin, E. A., Bragina, E. E., Kurilo, L. F. & Sheval, E. V. High-throughput analysis of TUNEL-stained sperm using image cytometry. *Cytometry A* **91**, 854-858, doi:10.1002/cyto.a.23164 (2017).

46 Gelles, J. D. & Chipuk, J. E. Robust high-throughput kinetic analysis of apoptosis with real-time high-content live-cell imaging. *Cell Death Dis* **7**, e2493, doi:10.1038/cddis.2016.332 (2016).

47 Fujisawa, S. *et al.* Evaluation of YO-PRO-1 as an early marker of apoptosis following radiofrequency ablation of colon cancer liver metastases. *Cytotechnology* **66**, 259-273, doi:10.1007/s10616-013-9565-3 (2014).

48 Wlodkowic, D., Telford, W., Skommer, J. & Darzynkiewicz, Z. Apoptosis and beyond: cytometry in studies of programmed cell death. *Methods Cell Biol* **103**, 55-98, doi:10.1016/B978-0-12-385493-3.00004-8 (2011).

49 Wilmer, M. J. *et al.* Novel conditionally immortalized human proximal tubule cell line expressing functional influx and efflux transporters. *Cell Tissue Res* **339**, 449-457, doi:10.1007/s00441-009-0882-y (2010).

50 Nieskens, T. T. *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* **18**, 465-475, doi:10.1208/s12248-016-9871-8 (2016).

51 Vriend, J. *et al.* Organic anion transporters 1 and 3 influence cellular energy metabolism in renal proximal tubule cells. *Biol Chem* **400**, 1347-1358, doi:10.1515/hsz-2018-0446 (2019).

52 McQuin, C. *et al.* CellProfiler 3.0: Next-generation image processing for biology. *PLoS Biol* **16**, e2005970, doi:10.1371/journal.pbio.2005970 (2018).

53 Kamentsky, L. *et al.* Improved structure, function and compatibility for CellProfiler: modular high-throughput image analysis software. *Bioinformatics* **27**, 1179-1180, doi:10.1093/bioinformatics/btr095 (2011).

54 Carpenter, A. E. *et al.* CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol* **7**, R100, doi:10.1186/gb-2006-7-10-r100 (2006).
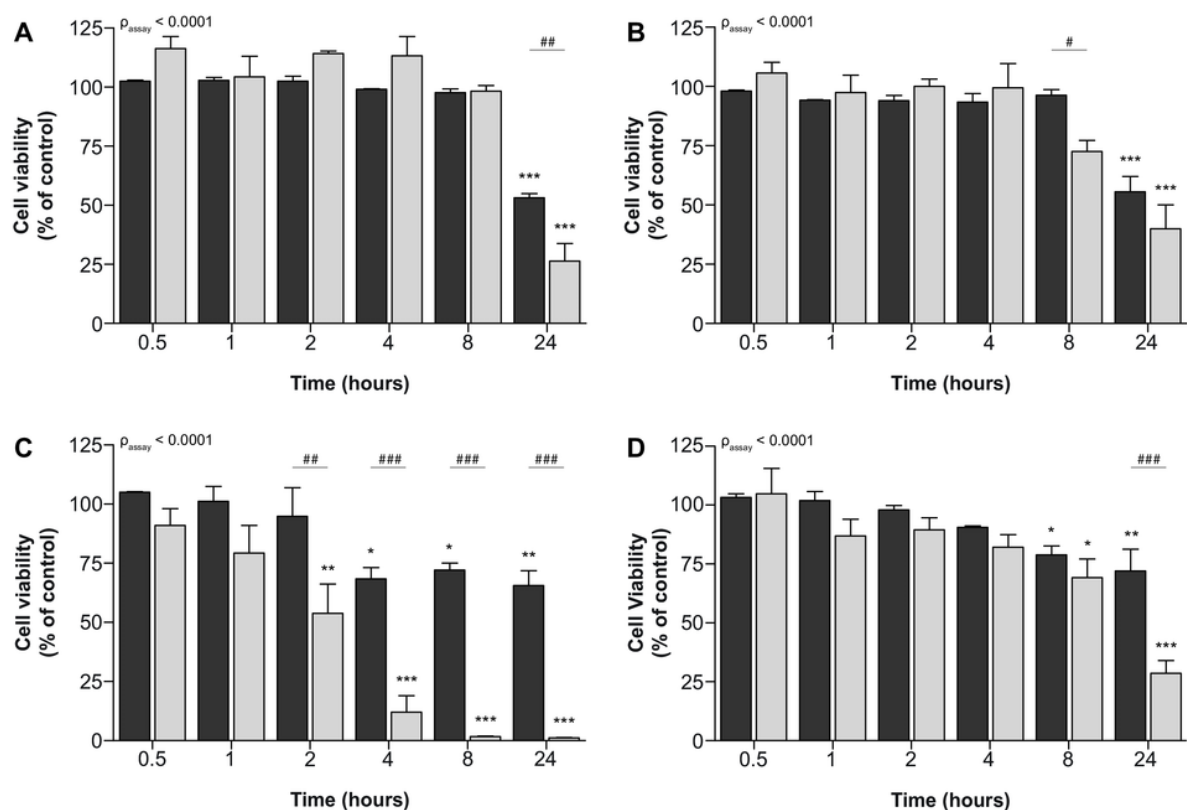
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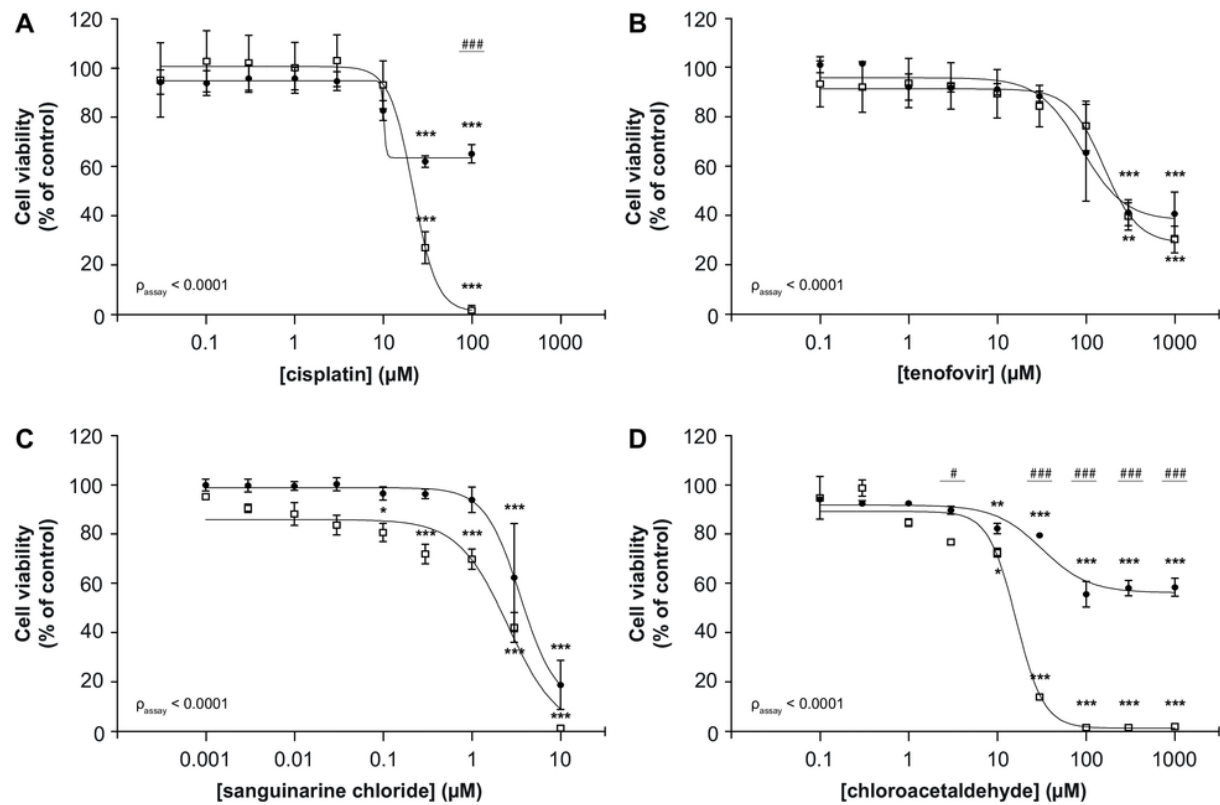
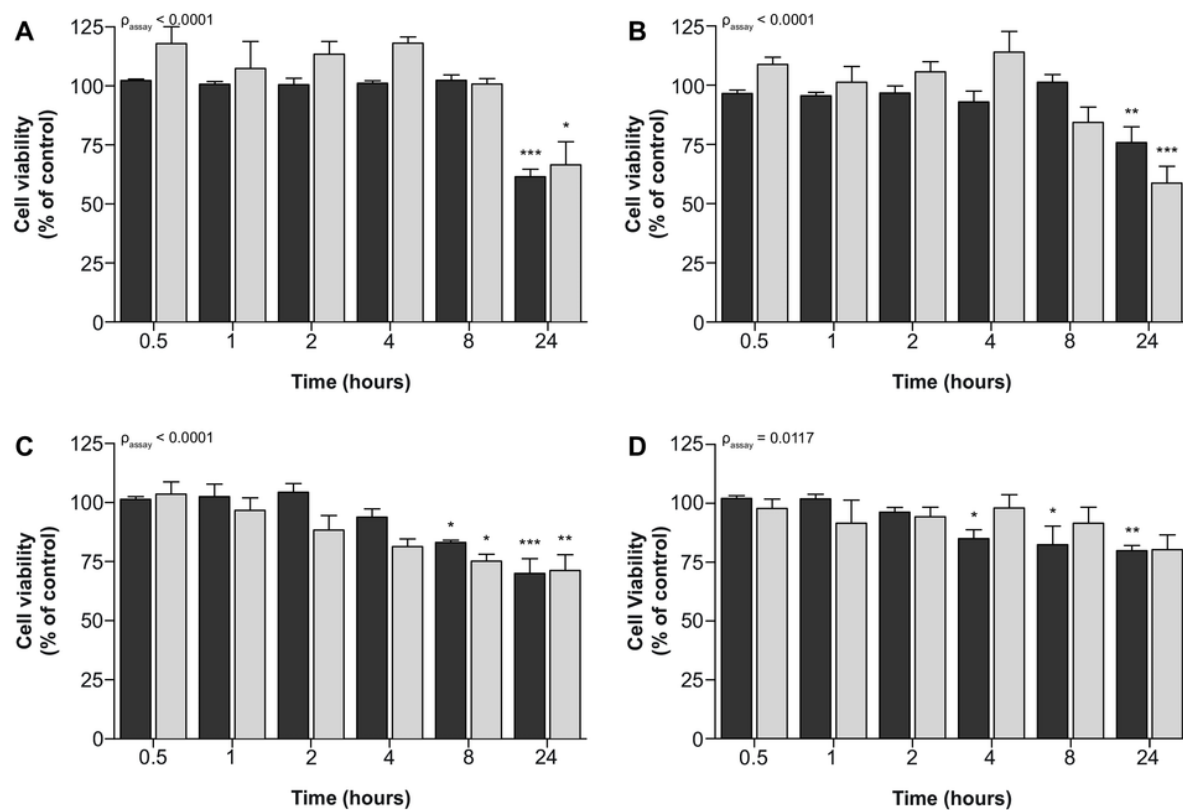Hoogstraten *et al.*, Figure 1

Figure 1

Comparison of MTT and fluorescence-based cell death methods to assess dose-dependent reduction of ciPTEC cellular viability. Cellular metabolic activity (□) and fluorescence-based cell death (●) were determined in ciPTEC-OAT1 after 24 hours incubation with a serial $\sqrt{10}$-dilution of nephrotoxic compounds (A) cisplatin, (B) tenofovir, (C) sanguinarine chloride and (D) chloroacetaldehyde. All results were normalized to unexposed controls. Statistical analyses: one-way ANOVA corrected for multiple comparison by Dunnett's post hoc analysis to compare differences between vehicle control and exposed conditions * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; and two-way ANOVA corrected for multiple comparison by Bonferroni's post hoc analysis to compare differences between assays # $p < 0.05$, ### $p < 0.001$. P-value corresponding to overall significance between assays is indicated for each compound. Mean ± SEM; n=3 independent experiments.

Hoogstraten *et al.*, Figure 2

Figure 2

Time-dependent effects upon exposure of ciPTEC-OAT1 to IC90-concentrations of nephrotoxic drugs on cellular metabolic activity and fluorescence-based cell death. Mature ciPTEC-OAT1 were exposed to IC90-concentrations of nephrotoxic drugs for 0.5, 1, 2, 4, 8 or 24 hours, after which fluorescence-based cell death (■) or cellular metabolic activity (■) assays were performed. (A) cisplatin, (B) tenofovir, (C) sanguinarine, (D) chloroacetaldehyde. For IC90-values see table 1. All results were normalized to unexposed controls. Statistical analyses: one-way ANOVA corrected for multiple comparison by Dunnett's post hoc analysis to compare differences between vehicle control and exposed conditions * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; and two-way ANOVA corrected for multiple comparison by Bonferroni's post hoc analysis to compare differences between assays # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$. P-value corresponding to overall significance between assays is indicated for each compound. Mean ± SEM; n=3 independent experiments.

Hoogstraten *et al.*, Figure 3

Figure 3

Time-dependent effects upon exposure of ciPTEC-OAT1 to IC70-concentrations of nephrotoxic drugs on cellular metabolic activity and fluorescence-based cell death. Mature ciPTEC-OAT1 were exposed to IC70 concentrations of nephrotoxic drugs for 0.5, 1, 2, 4, 8 and 24 hours, after which fluorescence-based cell death or (■) cellular metabolic activity (■) assays were performed. (A) cisplatin, (B) tenofovir, (C) sanguinarine, (D) chloroacetaldehyde. For IC70-values see table 1. All results were normalized to unexposed controls. Statistical analyses: one-way ANOVA corrected for multiple comparison by Dunnett's post hoc analysis to compare differences between vehicle control and exposed conditions * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; and two-way ANOVA corrected for multiple comparison by Bonferroni's post hoc analysis to compare differences between assays. P-value corresponding to overall significance between assays is indicated for each compound. Mean ± SEM; n=3 independent experiments.