Advanced in vitro models for studying drug induced toxicity

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CHAPTER 5

A SCREEN FOR APOPTOTIC SYNERGISM BETWEEN CLINICAL RELEVANT NEPHROTOXICANT AND THE CYTOKINE TNF-α

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ABSTRACT

Nephrotoxicity remains one of the main reasons for post-market drug withdrawal. Tumour necrosis factor α (TNF-α) secretion has been shown to underlie the nephrotoxicity induced by some of these drugs. Yet, there is currently no reliable and sensitive *in vitro* assay available to screen for nephrotoxicants of which toxicity largely depends on TNF-α secretion. Therefore, we developed and applied a sensitive fluorescence-based *in vitro* assay for TNF-α-mediated nephrotoxicity screening using mouse immortalized proximal tubular epithelial cells (IM-PTECs). Our assay allows rapid evaluation of TNF-α-mediated toxicant-induced apoptosis and necrosis using fixed endpoint and live cell measurements. To evaluate our assay, sixteen nephrotoxicants and two control non-nephrotoxicants were used. Out of the sixteen nephrotoxicants, eight induced cell death, of which five induced apoptosis as well as necrosis. Moreover, TNF-α significantly enhanced apoptotic cell death induced by cisplatin, cyclosporine A, tacrolimus and azidothymidine. These nephrotoxicants are known to induce inflammation *in vivo* which has been linked to an enhancement of nephrotoxicity for cisplatin, cyclosporine A and tacrolimus, confirming the functionality of our assay. Overall, our assay allows rapid and sensitive measurement of apoptosis and necrosis induced by a combination of nephrotoxicants and inflammatory components such as TNF-α and can be used as an alternative assay for nephrotoxicity prediction *in vitro*.

INTRODUCTION

Nephrotoxicity is a significant cause of post-market drug withdrawal. This is partly due to inadequate and non-sensitive pre-clinical tests to detect the nephrotoxic potential of new drugs. Acute toxicity testing in animals is traditionally used to evaluate the potential toxicity of new chemicals. However, the increase in the number of new chemicals tested and the lack of sensitivity of the traditional animal tests have prompted pharmaceutical companies to explore *in vitro* cell culture screening systems for toxicity prediction. Several *in vitro* screening systems using different cell lines, such as primary cells, LLC-PK1 (swine), NRK-52E (rat), MDCK (canine), OK (opossum), HK-2 (human) and RPTEC/hTERT1 (human) have been developed to assess the nephrotoxic potential of compounds [1-10]. However, these assays do not take into account the factors contributing to the pathophysiology of nephrotoxicant-induced acute renal failure (ARF) in patients such as mediators of inflammation. Moreover, mostly only one cytotoxicity parameter was taken into account and fixed time-points were used.

It is known that upon a nephrotoxic insult, the proximal tubule is considered the major target. Nephrotoxic injury of proximal tubular epithelial cells (PTECs) is characterized by mitochondrial dysfunction, adenosine triphosphate (ATP) depletion, activation of stress signalling pathways, impaired solute and ion transport, loss of brush border morphology, loss of cell polarity and cytoskeletal disruption [11,12]. Loss of cell adhesion also correlates with loss of cell function, pro-apoptotic signalling and cell death [13]. During nephrotoxicant-induced ARF, inflammation plays a major role. Several nephrotoxicants have been shown to induce an inflammatory response and attenuation of the inflammation can result in renal-protective effects [14-18]. It is believed that during nephrotoxicity, the initial insult by the nephrotoxicant results in changes in vascular endothelial cells and/or in tubular epithelial cells leading to the generation of inflammatory mediators (cytokines and chemokines) by these cells. These inflammatory mediators induce migration and infiltration of leukocytes into the injured kidneys and, importantly, aggravate the primary injury induced by the nephrotoxicant [19].

One the main inflammatory mediators secreted by PTECs [20,21] as well as by the infiltrating immune cells [22] is the pro-inflammatory cytokine tumour necrosis factor α (TNF-α). TNF-α was shown to be up-regulated and directly involved in the pathophysiology of cisplatin- and acetaminophen-induced renal injury [23-25]. In addition, other nephrotoxicants were shown to up-regulate TNF-α levels in the kidneys, in serum or in macrophages, including adefovir [26], methotrexate [27], carmustine [28] and mitomycin C [29], but a functional role for TNF-α in the toxicity induced by these compounds remains unknown. Contradictory results were obtained for the

two immunosuppressive drugs cyclosporine A and tacrolimus. Both cyclosporine A and tacrolimus were shown to inhibit TNF-α production *in vitro* in macrophages [30] and cultured PTECs [31] as well as in murine models [32,33]. However, studies performed on renal transplant recipients treated with cyclosporine A or tacrolimus showed that cyclosporine A did not affect TNF-α production by peripheral blood mononuclear cells [34] and tacrolimus increased TNF-α production by monocytes [35]. Despite these contradictory results in the effects of these immunosuppressive drugs on TNF-α production, both are known to induce inflammation after renal transplantation [36-38].

In addition to upregulation of TNF-α, some nephrotoxicants clearly inhibit TNF-α production. Gentamicin was shown to inhibit lipopolysaccharide (LPS)-induced TNF-α production in human and mouse PTECs [39] even though it induced inflammation *in vivo* [40]. The antiviral azidothymidine reduced TNF-α levels in HIV-infected patients [41,42] and the analgesic diclofenac decreased mRNA levels of TNF-α in obese men [43].

Given the importance of the immune system in nephrotoxicity we set-out to develop an alternative *in vitro* assay for prediction of TNF-α-mediated drug-induced nephrotoxicity. Using immortalized proximal tubular epithelial cells (IM-PTECs) we developed and characterized a fluorescence-based *in vitro* assay for nephrotoxicity screening, which allows apoptosis and necrosis measurements at both at a fixed time point as well as live over a 24-48 hour time-course in the presence of the cytokine TNF-α. IM-PTECs were exposed to 16 different nephrotoxicants and two control non-nephrotoxicants in the presence or absence of TNF-α. In total, 8 nephrotoxicants induced apoptosis of which cisplatin, cyclosporine A, tacrolimus and azidothymidine-induced cell death was enhanced by TNF-α. Overall, our cell model for inflammation-related nephrotoxicity combined with semi high-throughput fluorescence-based measurements allows rapid and sensitive measurement of cell death and can be used as an alternative assay for nephrotoxicity prediction *in vitro*.

MATERIALS AND METHODS

Reagents

Cisplatin (Cis-PtCL2(NH3)2)) was provided by the pharmacy unit of University Hospital in Leiden (The Netherlands). Adefovir was acquired from Shanghai PI chemicals (Shanghai, China). All the other nephrotoxic and non-nephrotoxic compounds were acquired from Sigma-Aldrich (Zwijndrecht, The Netherlands). Mouse recombinant TNF-α was acquired from R&D Systems (Abingdon, UK). AnnexinV-Alexa488 was made as previously described [44]. Propidium iodide was provided by Sigma-Aldrich.

Cell culture

Mouse immortalized proximal tubular cells (IM-PTECs) [45] were cultured at 33°C in DMEM/F12 medium (Invitrogen, Breda, The Netherlands) with 10% fetal bovine serum (Invitrogen, Breda, The Netherlands), 5 µg/ml insulin and transferrin, 5 ng/ml sodium selenite (Roche, Almere, The Netherlands), 20 ng/ml triiodo-thyronine (Sigma-Aldrich), 50 ng/ml hydrocortisone (Sigma-Aldrich), and 5 ng/ml prostaglandin E1 (Sigma-Aldrich) with L-glutamine and antibiotics (both from Invitrogen) and mouse interferon-γ (IFN-γ) (1 ng/ml; R&D Systems) in 5% CO2 and 95% air between passage 3 and 20. Prior to each experiment, the cells were differentiated into proximal tubular cells by culturing them for 4 days in restrictive conditions (at 37°C in the absence of IFN-γ). The cells were then plated in 96-well plates and cultured for 2 more days. In total, IMPTECs were cultured in restrictive conditions for 6 days, allowing the disappearance of SV40 activity and completion of differentiation [45].

Exposure of the cells

Compound concentrations used in the study were based on previously published *in vitro* studies or were extrapolated from the plasma levels in patients. Compound stock solutions were prepared freshly in DMSO, water, NaCl or NaOH depending on the compounds. Stock solutions were diluted with complete medium to obtain 100X the final testing concentration. The final concentration of DMSO was maximum 0.1% IM-PTECs were exposed to 16 different nephrotoxic compounds and 2 control non-nephrotoxic compounds at two different concentrations in combination or not with TNF-α (8 ng/ml). Cisplatin was used as a positive control and the two non-nephrotoxic compounds valacyclovir and bisphenol A were used as negative controls. Proper vehicle controls were used (DMSO, water, NaCl or NaOH) according to the compounds. After 24 hours, the cells were re-exposed only to the compounds that are known to be administered in patients daily. The compound concentrations and exposure schedules are indicated in Table 1.

Cell death measurement

Apoptosis and necrosis were measured simultaneously as described in Fig. 1. Apoptosis was measured using a live cell apoptosis assay previously described [44]. Briefly, binding of annexin V-Alexa488 conjugate to phosphatidyl serine present on the membranes of apoptotic cells was measured at 24 and 48 hours or was followed over time by imaging the cells every hour after drug ±TNF-α exposure with BD Pathway 855 imager (Becton Dickinson, Erembodegem, Belgium). The total area of annexin V-Alexa488 fluorescence per image was quantified using Image Pro (Media Cybernetics, Bethesda, MD). Necrosis was measured by incubating the cells with propidium iodide followed by imaging at 24 and 48 hours with a BD Pathway 855

imager (Becton Dickinson, Erembodegem, Belgium). The number of cells stained by propidium iodide was quantified using Image Pro (Media Cybernetics, Bethesda, MD).

Statistical procedures

All data are expressed as mean ± standard error of the mean (S.E.M.). Statistical significance was determined by GraphPad Prism using an unpaired two-tailed t-test. The level of confidence is represented by p-values indicated in the figures. The apoptotic synergism between nephrotoxicant and TNF-α was quantified with the Chou-Talalay method [46] for the live cell death measurements.

Compounds	Concentrations	Exposure times
Valacyclovir	300 μM	0 and 24 h
Bisphenol A	5 μM	0 and 24 h
Cyclosporine A	10 and 20 μM	0 and 24 h
Tacrolimus	10 and 20 μM	0 and 24 h
Cephaloridin	200 and 500 μM	0 and 24 h
Cephalothin	200 and 500 μM	0 and 24 h
Gentamicin	200 and 1000 μM	0 and 24 h
Neomycin	40 and 400 μM	0 and 24 h
Methotrexate	30 and 300 μM	0 h
Carmustine	100 and 300 μM	0 h
Mitomycin C	100 and 150 μM	0 h
Cisplatin	10 μM	0 h
Azidothymidine	200 and 400 μM	0 and 24 h
Adefovir	4 and 5 μM	0 and 24 h
Foscarnet	250 and 1000 μM	0 h
Phenacetin	30 and 100 μM	0 and 24 h
Acetaminophen	750 and 1000 μM	0 and 24 h
Diclofenac	500 and 1000 μM	0 and 24 h

Table 1. Compound concentrations and exposure schedules

RESULTS

TNF-α enhanced the apoptotic response of renal cells exposed to cyclosporine A, tacrolimus, cisplatin and azidothymidine

To identify nephrotoxic compounds of which nephrotoxicity was enhanced by the cytokine TNF-α we set-up a sensitive fluorescence-based *in vitro* assay for nephrotoxicity screening using immortalized proximal tubular epithelial cells (IM-PTECs). To test the predictive value of this assay, we used a panel of nephrotoxicants, including 2 immuno-suppressants, 4 antibiotics, 4 chemotherapeutics, 3 antivirals and 3 analgesics. None of the compounds significantly affected endogenous TNF-α secretion as determined by ELISA (data not shown), indicating that endogenous TNF-α secretion did not influence the assessment of the tested compounds. To determine whether TNF-α potentiated an apoptotic response as has been observed *in vivo* for cisplatin [24,25], we exposed the IM-PTECS to the panel of nephrotoxicants in combination with exogenous TNF-α. Apoptosis was measured on BD Pathway 855 imager using annexin V-alexa 488 at 24 and 48 hours. While no apoptosis was measured in the two control compounds valacyclovir and bisphenol A, 8 out of 16 nephrotoxic compounds induced apoptosis at 24 or 48 hours after treatment. These compounds included the two immunosuppressants cyclosporine A and tacrolimus, the four chemotherapeutics methotrexate, carmustine, mitomycin C and cisplatin, the antiviral azidothymidine and the analgesic diclofenac (Fig. 2). None of the antibiotics resulted in detectable apoptosis at the high concentrations tested - even after 48 hours exposure (Fig. 2). These findings were confirmed using a commercially available ATP-lite assay (data not shown). Of importance, the presence of serum in the exposure medium did not have an effect on drug-induced cell death as no significant difference was observed between cells exposed under low or high serum conditions, suggesting that drug-protein interactions were not an issue for the compounds tested (data not shown).

TNF-α enhanced the apoptotic response of 4 out of these 8 apoptosis-inducing compounds (Fig. 2). These included cisplatin, the two immunosuppressants cyclosporine A and tacrolimus, as well as the antiviral azidothymidine at the highest concentration. Cyclosporine A-induced apoptosis was more strongly enhanced by TNF-α at 24 hours in comparison to the response to tacrolimus and azidothymidine at 48 hours.

Intriguingly, while cisplatin showed a strong synergy with TNF-α, none of the other DNA damaging nephrotoxicant mimicked this response, despite their pro-apoptotic activities. In contrast, methotrexate showed the opposite effect with TNF-α at the highest concentration: a significant decrease in apoptosis was observed when IM-PTECS were exposed to the compound in combination with TNF-α in comparison

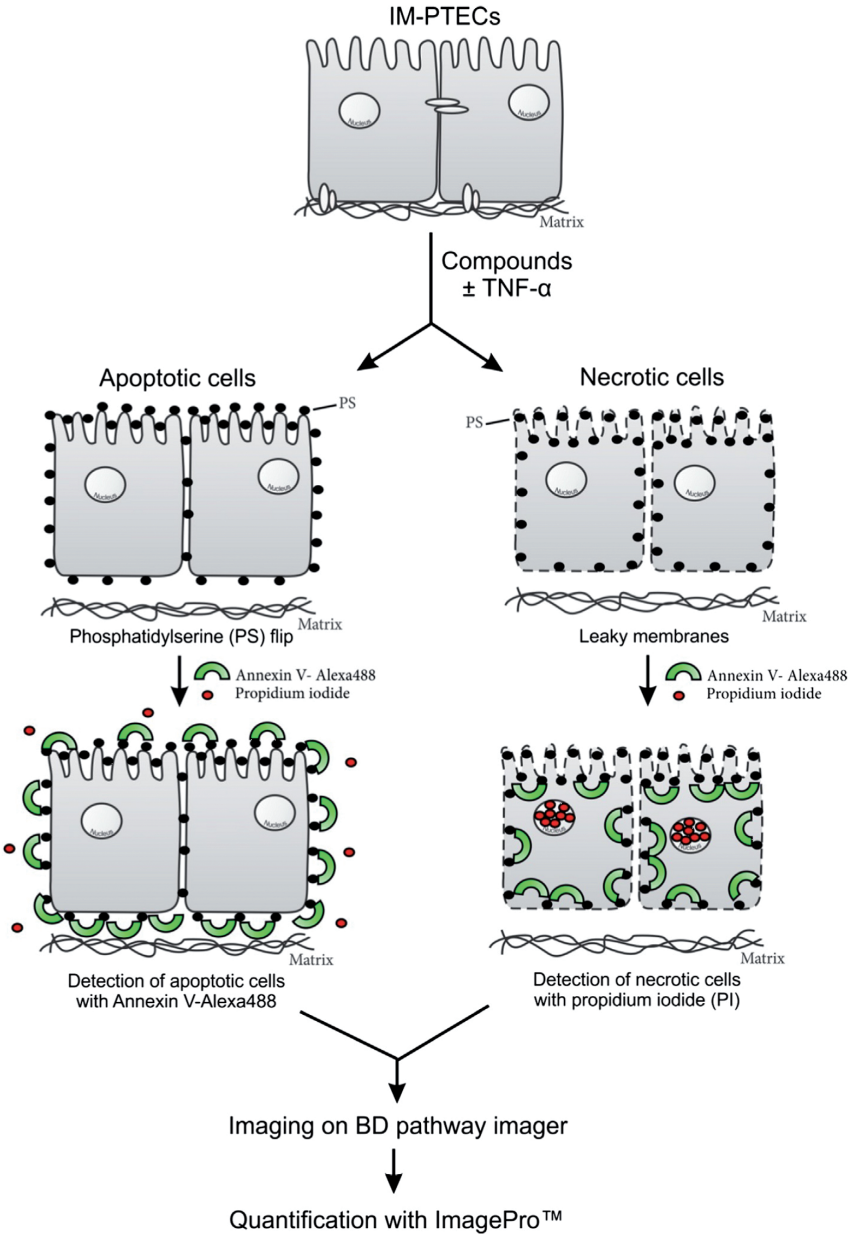

Figure 1. *Flowchart of the in vitro screen procedure.* Following exposure of the IM-PTECs to the compounds in the presence or not of TNF-α, the amount of apoptotic and necrotic cell death was determined in a fluorescent-based manner at 24 and 48 hours or followed over time. Apoptosis was measured with Annexin-V-Alexa488 conjugate, which binds the phosphatidyl serine that flips on the outer membrane in apoptotic cells, and necrosis was measured with propidium iodide, which binds to the nucleus only when the membrane of the cells is partly disrupted. The amount of apoptotic and necrotic cells was then quantified using ImagePro.

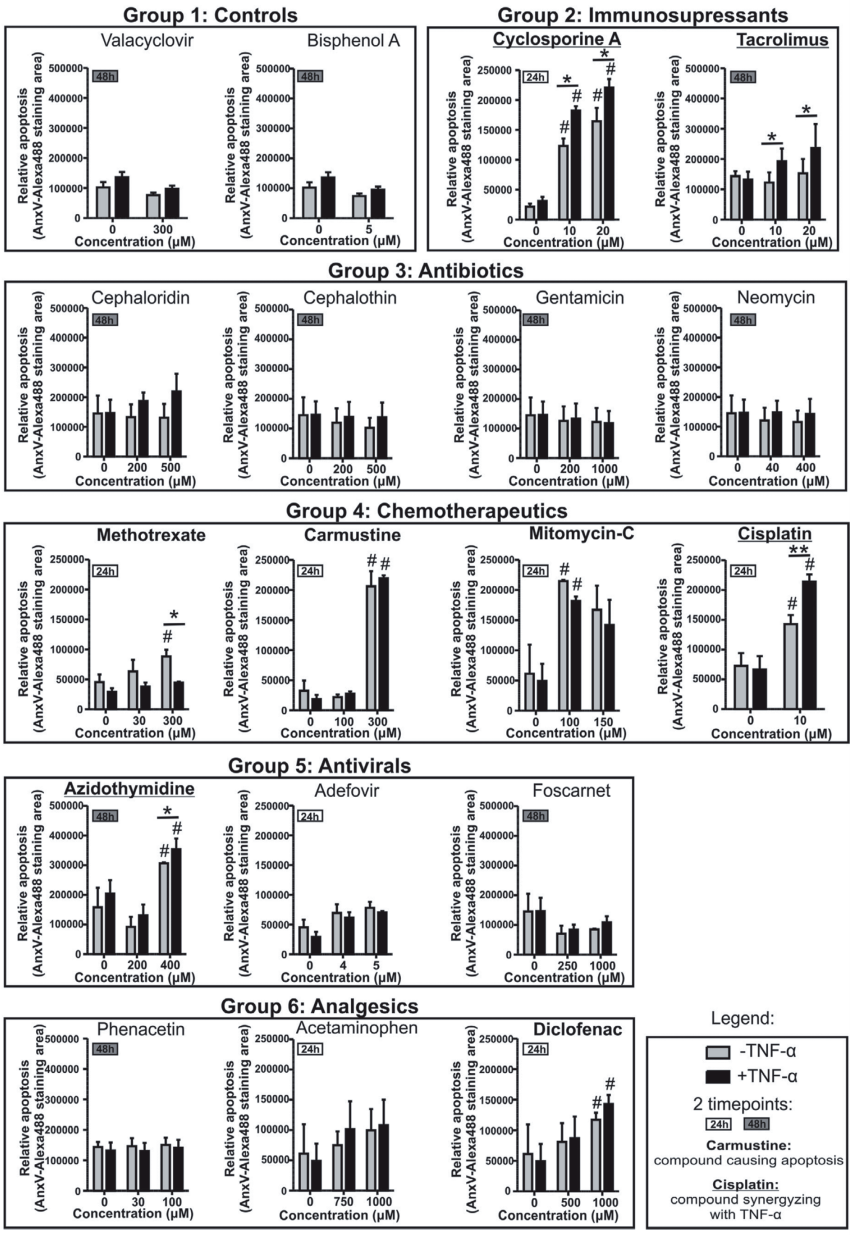

Figure 2. *TNF-α enhanced the apoptotic response of renal cells exposed to cyclosporine A, tacrolimus, cisplatin and azidothymidine*. IM-PTECs were exposed to two different concentrations of 16 different nephrotoxicants and two non-nephrotoxicants in combination or not of TNF-α (8 ng/ml) and apoptosis of the cells was measured at 24 and 48 hours. The compounds are grouped by pharmacological properties. The compounds inducing apoptosis are highlighted in bold and the compounds inducing synergistic apoptosis with TNF-α are highlighted in bold and are underlined. The data are represented as means of three independent experiments ± S.E.M. * $P \leq 0.05$, ** $P \leq 0.01$ and # $P \leq 0.05$ compared to vehicle-treated cells.

to exposure to the compound alone (Fig. 2).

TNF-α enhanced the necrotic response of renal cells exposed to cyclosporine A and cisplatin

In addition to apoptosis, necrosis was measured in our IM-PTECs after exposure to the 16 different nephrotoxicants and 2 control non-nephrotoxicants in the presence or absence of TNF-α. Five out of the 16 nephrotoxicants showed staining for the necrosis maker propidium iodide. These compounds included the immunosuppressant cyclosporine A, the three chemotherapeutics carmustine, mitomycin C and cisplatin and the analgesic diclofenac (Fig. 3). The other three nephrotoxic compounds shown to induce apoptosis (Fig. 2) did not induce any necrosis - even after 48 hours (Fig. 3). As expected, the two negative controls also did not induce any necrotic response (Fig. 3).

Out of the 5 compounds inducing necrosis, TNF-α enhanced the response of two: cyclosporine A and cisplatin (Fig. 3). Although apoptosis induced by these compounds could result in secondary necrosis, the strong necrotic response observed after cyclosporine A treatment, suggests that the necrosis observed for this compound was not secondary necrosis.

The TNF-α-mediated enhancement of apoptosis was confirmed with live apoptosis

To confirm the hits obtained in our primary compound screen, IM-PTECs were exposed to the four synergistic apoptosis-inducing compounds cisplatin, cyclosporine A, tacrolimus, and azidothymidine. For all of these four compounds, the enhanced apoptotic response towards TNF-α addition was confirmed (Fig. 4A and Fig. 5). Using the Chou-Talalay statistical test for synergy we could confirm confirmed the synergistic effect of TNF-α with cisplatin (CI of 0.99) and cyclosporine A (CI of 0.74 for 10μM and CI of 0.84 for 20μM).

More importantly, while cyclosporine A and cisplatin exposure alone resulted in an increase in apoptosis, a significant increase in apoptosis after tacrolimus and azidothymidine exposure was only observed in combination with TNF-α, indicating a synergistic response for combined treatment of TNF-α with tacrolimus and azidothymidine. These data indicate that our assay could identify nephrotoxic compounds that would not have been identified by current assays that do not incorporate the immune component.

Finally, to obtain more detailed information on the time of onset of apoptosis for the four synergistic apoptosis-inducing compounds cyclosporine A, tacrolimus, azidothymidine and cisplatin, a real time fluorescence-based apoptosis assay was used as was developed previously in our laboratory [44]. IM-PTECs were exposed to

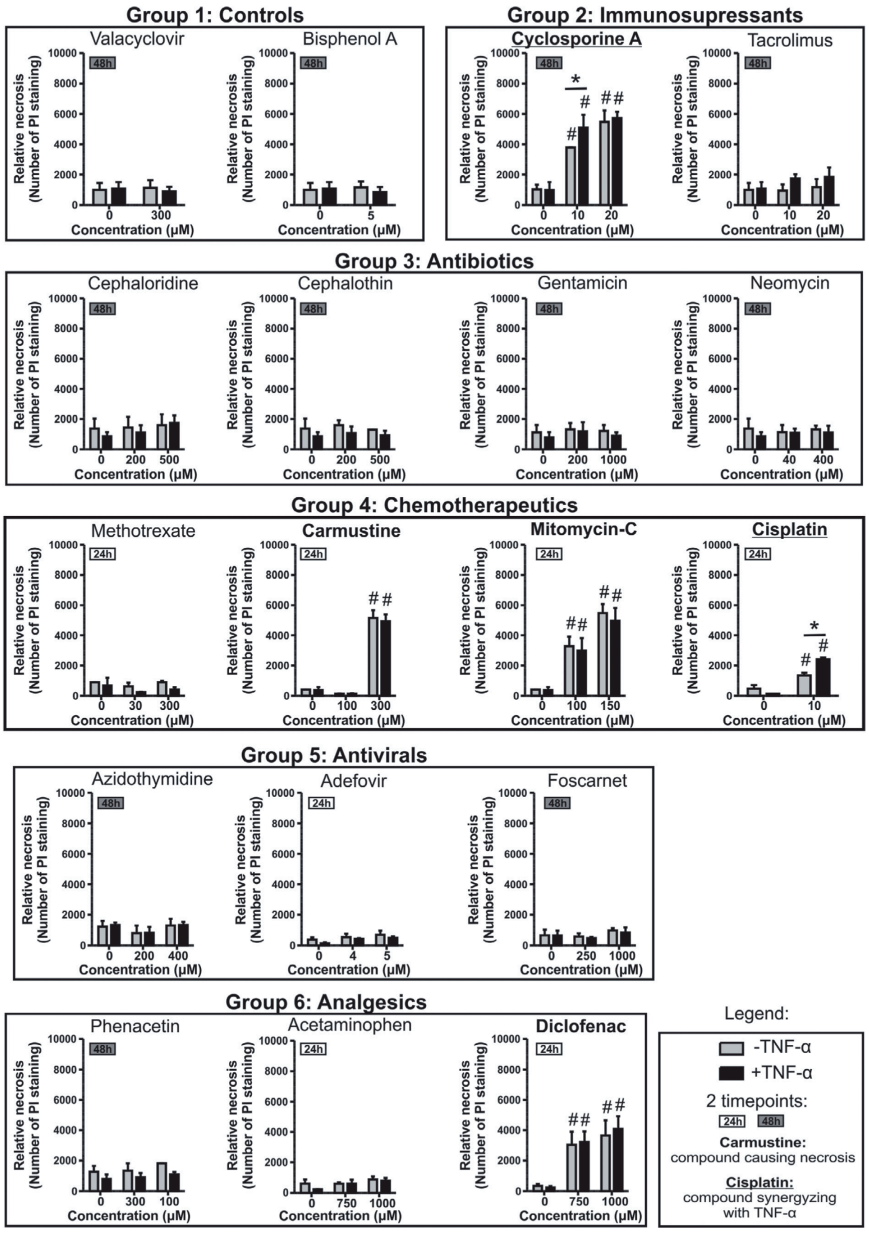

Figure 3. *TNF-α enhanced the necrotic response of renal cells exposed to cyclosporine and cisplatin.* IM-PTECs were exposed to two different concentrations of 16 different nephrotoxicants and two non-nephrotoxicants in combination or not of TNF-α (8 ng/ml) and necrosis of the cells was measured at 24 and 48 hours. The compounds are grouped by pharmacological properties. The compounds inducing necrosis are highlighted in bold and the compounds inducing synergistic necrosis with TNF-α are highlighted in bold and are underlined. The data are represented as means of three independent experiments ± S.E.M. * $P \leq 0.05$, and # $P \leq 0$ .05 compared to vehicle-treated cells.

the four compounds in the presence or absence of TNF-α and the onset of apoptosis was followed over time. While cisplatin and cyclosporine A gave a strong TNF-α-mediated apoptotic response at 24 hours, as predicted, hardly any effect was observed after tacrolimus or azidothymidine treatment at 24 hours (Fig. 4B). Yet, TNF-α significantly enhanced apoptosis of the IM-PTEC cells over a 24-48 hour time-course. Using this live apoptosis assay, we could clearly observe that TNF-α enhanced the onset of apoptosis induced by both cisplatin and cyclosporine A.

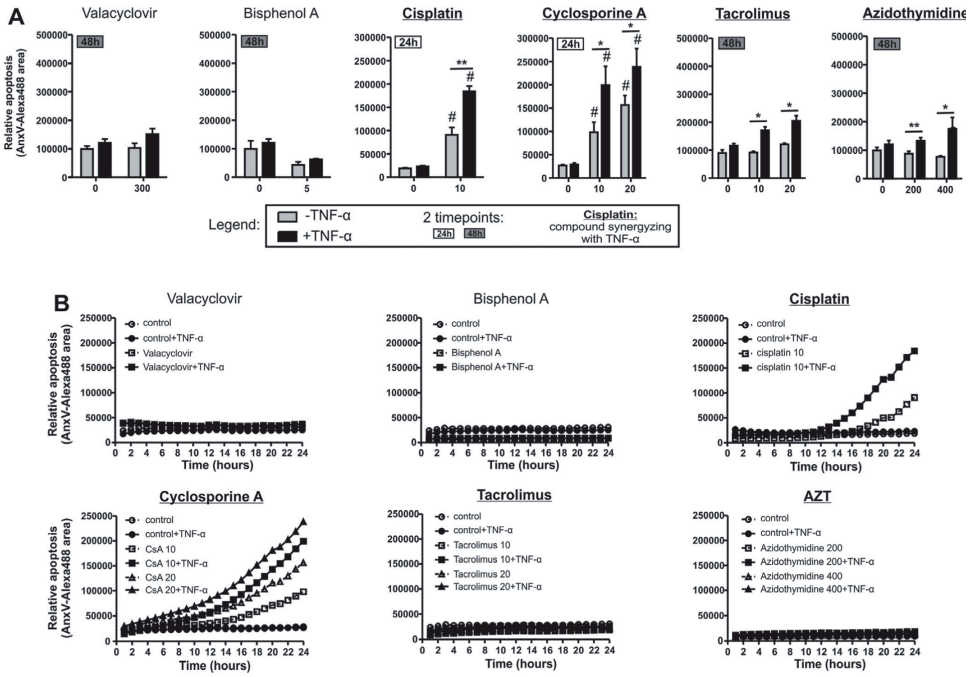

Figure 4. *The synergistic apoptotic response of renal cells exposed to the four compounds in combination with TNF-α was confirmed with live apoptosis*. IM-PTECs were exposed to two different concentrations of the 4 synergistic nephrotoxicants and the two non-nephrotoxicants in combination or not of TNF-α (8 ng/ml) and apoptosis of the cells was measured at 24h and 48h (A) or was followed over time until 24 hours (B). The compounds inducing synergistic apoptosis with TNF-α are highlighted in bold and are underlined. The data are represented as means of three independent experiments ± S.E.M. * $P \leq 0.05$, ** $P \leq 0.01$ and # $P \leq 0.05$ compared to vehicle-treated cells.

DISCUSSION

Recent efforts have been made to develop new *in vitro* screening tests for the prediction of nephrotoxicity of new chemicals. Although, these *in vitro* systems cannot reproduce the complex behaviour of animal cells *in vivo*, they allow toxicity testing

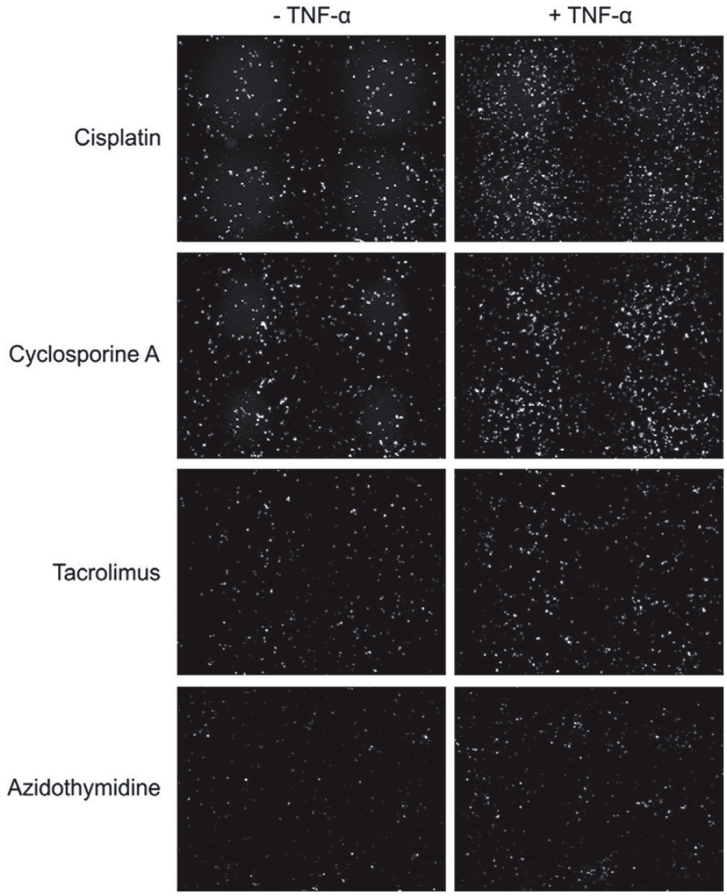

Figure 5. *Annexin-V staining of apoptotic cells after exposure to the drugs synergizing with TNF-α.* Annexin-V staining of the apoptotic cells was visualized by a BD Pathway 855 imager for IM-PTECS exposed to compounds in combination of not with TNF-α. Increased annexin-V stained cells was demonstrated for all 4 compounds inducing a synergistic apoptosis of the cells with TNF-α. The images represented here were obtained with the highest concentration for each compound at 24 hours for cisplatin and cyclosporine A and 48 hours for tacrolimus and azidothymidine and are representative of three independent experiments.

in a high throughput manner and provide important predictive and mechanistic information. Yet, nephrotoxicity remains a critical cause of drug withdrawal. Here we set-out to extend beyond these current *in vitro* tests by incorporating one of the most important contributors of nephrotoxicity and main secreted pro-inflammatory cytokine TNF-α. To evaluate the predictive potential of our assay a small screen was performed using 16 different nephrotoxicants. Cell death induced by the compounds in the presence or absence of TNF-α was assessed by a semi-high throughput fluorescence-based method. This innovative method allowed the measurement of both apoptosis and necrosis in real-time, which is a clear advantage over current com-

mercially available assays. In total we identified 8 out of 16 compounds that induced cell death, of which 5 induced apoptosis as well as necrosis. Moreover, TNF-α significantly enhanced the nephrotoxic response of cisplatin, cyclosporine A, tacrolimus and azidothymidine.

We recently examined the mechanisms underlying the synergistic response between cisplatin and TNF-α. This synergistic apoptotic response was due to inhibition of the NF-κB pathway and consequently increased c-Jun N-terminal kinase (JNK) activation [47]. A similar mechanism may be involved in the response between TNF-α and the three other nephrotoxicants identified in our screen. Indeed, Du *et al*. showed that both cyclosporine A and tacrolimus inhibited TNF-α-induced NF-κB activation in renal cells [48]. Moreover, cyclosporine A was shown to induce JNK activation in primary human PTECs [49] and tacrolimus induced JNK activation in Madin Darby canine kidney cells [50]. Thus far, it remains unknown whether the inhibition of NF-κB is directly associated with the changes in JNK activation as we observed for cisplatin and TNF-α co-exposure. Also, azidothymidine was reported to inhibit NF-κB, but studies were performed in Epstein-Barr virus-positive Burkitt lymphoma lines [51]. Moreover, to the best of our knowledge, no studies showed that azidothymidine treatment results in activation of JNK.

In contrast to the aforementioned compounds, co-exposure of the chemotherapeutic methotrexate with TNF-α led to a reduction in methotrexate-induced apoptosis. Protective effects of TNF-α on methotrexate-induced apoptosis were previously observed in macrophages *in vitro* and was shown to be due to enhanced activation of the NF-κB pathway [52]. Whether TNF-α reduce methotrexate-induced apoptosis in IM-PTECs via enhanced activation of the NF-κB pathway has also yet to be determined.

Since cell death was used as a read-out for renal cell injury, 50% of the compounds could not be identified as a nephrotoxicant in our assay, despite the fact that all of the compounds used in our screen induce nephrotoxicity in the clinic. One explanation may be that despite being nephrotoxic, many of the compounds are not reported to induce cell death *in vivo*. Indeed, in contemporary pre-clinical assays it is difficult to identify compounds that are nephrotoxic but do not induce cell death [4,5,39,53]. Even so, the lack of acetaminophen- and phenacetin-induced cytotoxicity in our assay could be explained by lack of bioactivation to reactive intermediates [5,54]. The lack of cell death response for neomycin and gentamicin is most likely due to the lack of the membrane binding site in the brush border and basolateral membrane [55] and for cephaloridine, cephalothin, and adefovir could be due to low expression of the transporter organic anion-transporting polypeptide 1 (OAT1) responsible for the uptake of the compounds [56-58]. Alternatively, our IM-PTEC cells, like any other proximal tubular cell line cultured *in vitro* depend on glycolysis com-

pared to oxidative phosphorylation for PTEC in the *in vivo* situation. These issues require further investigation.

Despite the fact that the nephrotoxicity of some compounds was not identified with our assay, the real-time, dynamic measurement of cell death in combination with the addition of the immune component TNF-α allowed identification of compounds that were mainly toxic in the presence of TNF-α, such as tacrolimus. This observation correlated well with patient data where cyclosporine A induced more nephrotoxicity and inflammation after kidney transplantation than tacrolimus [37,59]. Furthermore, some of the compounds that failed to induce cytotoxicity in our assays were shown to affect the NF-κB pathway in renal cells or other cell lines and organs, including acetaminophen [60], gentamycin [61,62] and neomycin [63,64]. Incorporating NF-κB reporter IM-PTECs in our current fluorescence-based cytotoxicity assay may enhance assay sensitivity and identify compounds like gentamycin and neomycin as nephrotoxicants in an *in vitro* pre-clinical setting.

In conclusion, real-time measurements of apoptosis and necrosis in IM-PTECs in the presence of TNF-α, represents a novel method to detect nephrotoxicants that may go undetected in conventional *in vitro* nephrotoxicity screening assays.

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