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Role of TNF- α and the NF- κ B pathway in drug-induced organ injuries

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Citation

Benedetti, G. O. E. (2013, May 7). *Role of TNF- α and the NF- κ B pathway in drug-induced organ injuries*. Retrieved from <https://hdl.handle.net/1887/20857>

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Title: Role of TNF- α and the NF- κ B pathway in drug-induced organ injuries

Issue Date: 2013-05-07



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A screen for apoptotic synergism between clinical relevant nephrotoxicants and the cytokine TNF- α

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Submitted to In vitro Toxicology

Abstract

Nephrotoxicity remains one of the main reasons for drug withdrawal post-market. Therefore the need for new reliable and sensitive *in vitro* systems for the prediction of nephrotoxicity needs to be addressed. We developed and applied a sensitive fluorescence-based *in vitro* assay for nephrotoxicity screening using immortalized proximal tubular epithelial cells (IM-PTECs), which allows rapid evaluation of toxicant-induced apoptosis and necrosis at both a fixed time point as well as live over a 24-48 hour time-course. Given the importance of the immune system in nephrotoxicity, the most important contributor of nephrotoxicity and main secreted pro-inflammatory cytokine tumor necrosis factor α (TNF- α), was incorporated in our assay. To evaluate our assay, sixteen different nephrotoxics and two control non-nephrotoxics were used. Out of the sixteen nephrotoxics, eight induced cell death, of which five induced apoptosis as well as necrosis. Moreover, four of the apoptosis-inducing toxicants induced a moderate to strong synergistic apoptotic response with TNF- α , including cisplatin, cyclosporine A, tacrolimus and azidothymidine. These toxicants 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 sensitive measurement of synergistic apoptosis between potential nephrotoxics and inflammatory components such as TNF- α and can be used for alternative nephrotoxicity prediction screening.

1. Introduction

Nephrotoxicity is an important reason for drug withdrawal post-market. 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 toxic potential of new chemicals. However, the increase in the number of new chemicals tested and the lack of sensitivity of the traditional animal tests have brought pharmaceutical companies to use *in vitro* cell culture screening systems for toxicity prediction. Several *in vitro* screening systems were developed in order to assess the nephrotoxic potential of compounds (1-5). However, these assays did 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 (6, 7). Loss of cell adhesion also correlates with loss of cell function, pro-apoptotic signalling and cell death (8). During nephrotoxicant-induced ARF, inflammation plays a major role. Several nephrotoxicants have been shown to induce an inflammatory response and attenuation of the inflammation demonstrated renal-protective effects (9-13). 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 aggravate the primary injury induced by the nephrotoxicant (14).

One of the main inflammatory mediators secreted by PTECs (15, 16) as well as by the infiltrating immune cells (17) 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 (18-20). In addition, other nephrotoxicants were shown to up-regulate TNF- α levels in the kidneys, in serum or in macrophages, including adefovir (21), methotrexate (22), carmustine (23) and mitomycin C (24), but a functional role for TNF- α levels 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 (25) and cultured PTECs (26) as well as in murine models (27, 28). 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 (29) and tacrolimus increased TNF- α production by monocytes (30). Despite these contradictory results in the effects of these immunosuppressive drugs on TNF- α production, both are known to induce inflammation after renal transplantation (31-33).

In addition to up-regulation 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 (34) even though it induced inflammation *in vivo* (35). The antiviral azidothymidine reduced TNF- α levels in HIV-infected patients (36, 37) and the analgesic diclofenac decreased mRNA levels of TNF- α in men with overweight (38).

Given the importance of the immune system in nephrotoxicity we set-out to develop an alternative *in vitro* assay for prediction of 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 a fixed time point as well as live over a 24-28 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 presence or absence of TNF- α . In total, 8 nephrotoxicants induced apoptosis of which 4 gave a synergistic apoptotic response with TNF- α , including cisplatin, cyclosporine A, tacrolimus and azidothymidine. Overall, our assay allows sensitive measurement of synergistic apoptosis between potential nephrotoxicants and inflammatory components such as TNF- α and can be used for alternative nephrotoxicity prediction screening.

2. Material and methods

2.1. Reagents

Adefovir was acquired from Shanghai PI chemicals (Shanghai, China) and cisplatin (Cis-PtCL₂(NH₃)₂) was provided by the pharmacy unit of University Hospital in Leiden (The Netherlands). 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 described (39). Propidium iodide was provided by Sigma-Aldrich.

2.2. Cell culture

Mouse immortalized proximal tubular cells (IM-PTECs) described previously (40) were cultured at 33°C in HK2 medium (DMEM/F12 medium (Invitrogen, Breda, The Netherlands) with 10% fetal bovine serum (Hyclone, Etten-Leur, The Netherlands), 5 μ g/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 (40).

2.3. Exposures

Compound concentrations were obtained from previous 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 serum to obtain 100 X the final testing concentration. The final concentration of DMSO was 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. 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.

2.4. Cell death measurements

Apoptosis and necrosis were measured simultaneously as described in Fig. 1. Apoptosis was measured using a live cell apoptosis assay previously described (39). 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 $\pm$ TNF- α exposure with a 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).

2.5. Statistical procedures

All data are expressed as mean $\pm$ 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.

Table 1. Compound concentrations and exposure schedules.

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

3. Results

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

To identify nephrotoxic compounds that induced synergistic cell death with 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 immunosuppressants, 4 antibiotics, 4 chemotherapeutics, 3 antivirals and 3 analgesics. First, we tested whether these known nephrotoxicants could induce apoptosis in the IM-PTECs and whether TNF- α potentiated an apoptotic response as has been observed *in vivo* for cisplatin (19, 20). Apoptosis was measured on the BDpathway[®] using annexin V-alexa 488. 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

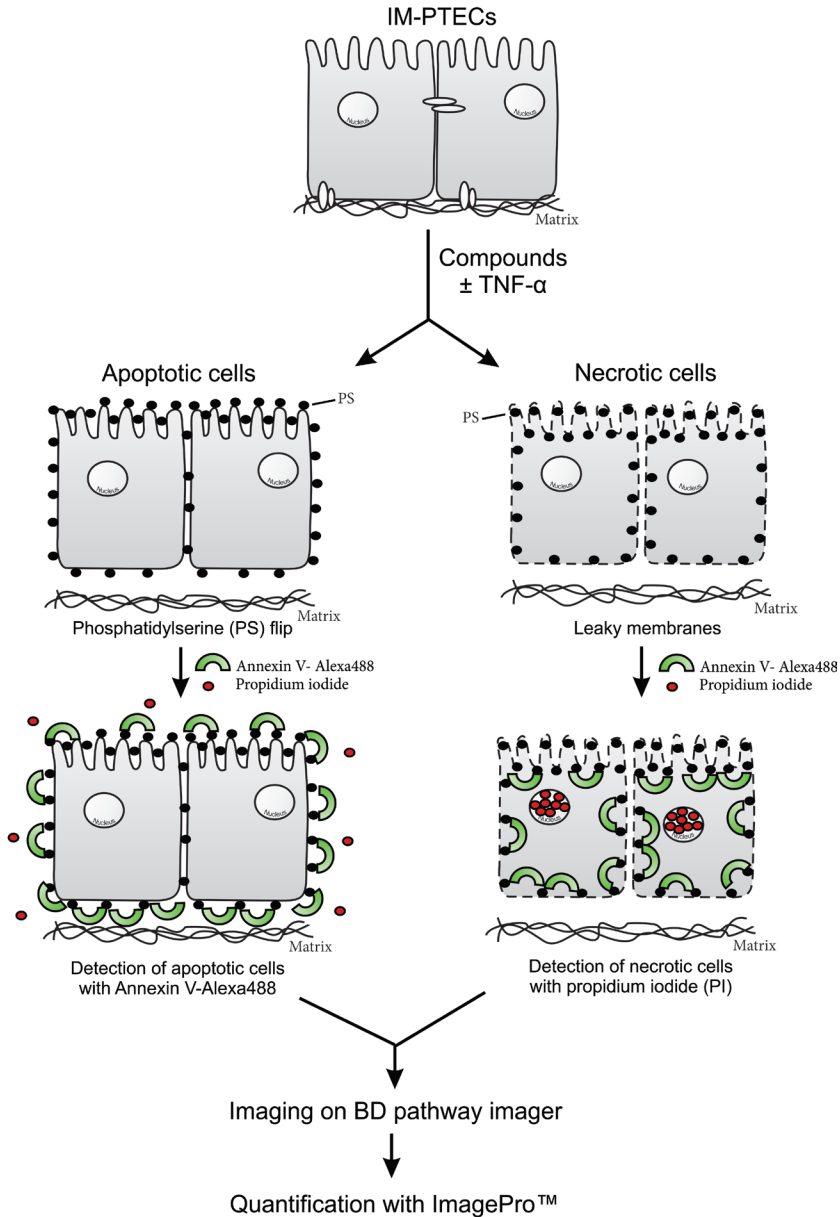


Figure 1. Flowchart of the in vitro screen procedure. Following exposure of the IM-PTECs to the compounds in the presence or not of TNF- α , the amount of apoptotic and necrotic cell death was determined in a fluorescent-based manner at 24 and 48 hours or followed over time. Apoptosis was measured with 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.

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). Exposure to any of the antibiotics did, even after 48 hours, not result in the onset of detectable apoptosis at the high concentrations tested (Fig. 2).

Out of the 8 apoptosis-inducing compounds, 4 showed a synergistic apoptotic response with TNF- α (Fig. 2). These included cisplatin, the two immunosuppressants cyclosporine A and tacrolimus, as well as the antiviral azidothymidine at the highest concentration. Cyclosporine A gave a stronger synergistic apoptotic response 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 nephrotoxics 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 to exposure to the compound alone (Fig. 2).

3.2. 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 nephrotoxics and 2 control non-nephrotoxics in the presence or absence of TNF- α . Five out of the 16 nephrotoxics showed staining for the necrosis marker 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 even after 48 hours (Fig. 3).

Out of the 5 compounds inducing necrosis, only two gave a synergistic necrotic response with TNF- α : cyclosporine A and our positive control 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.

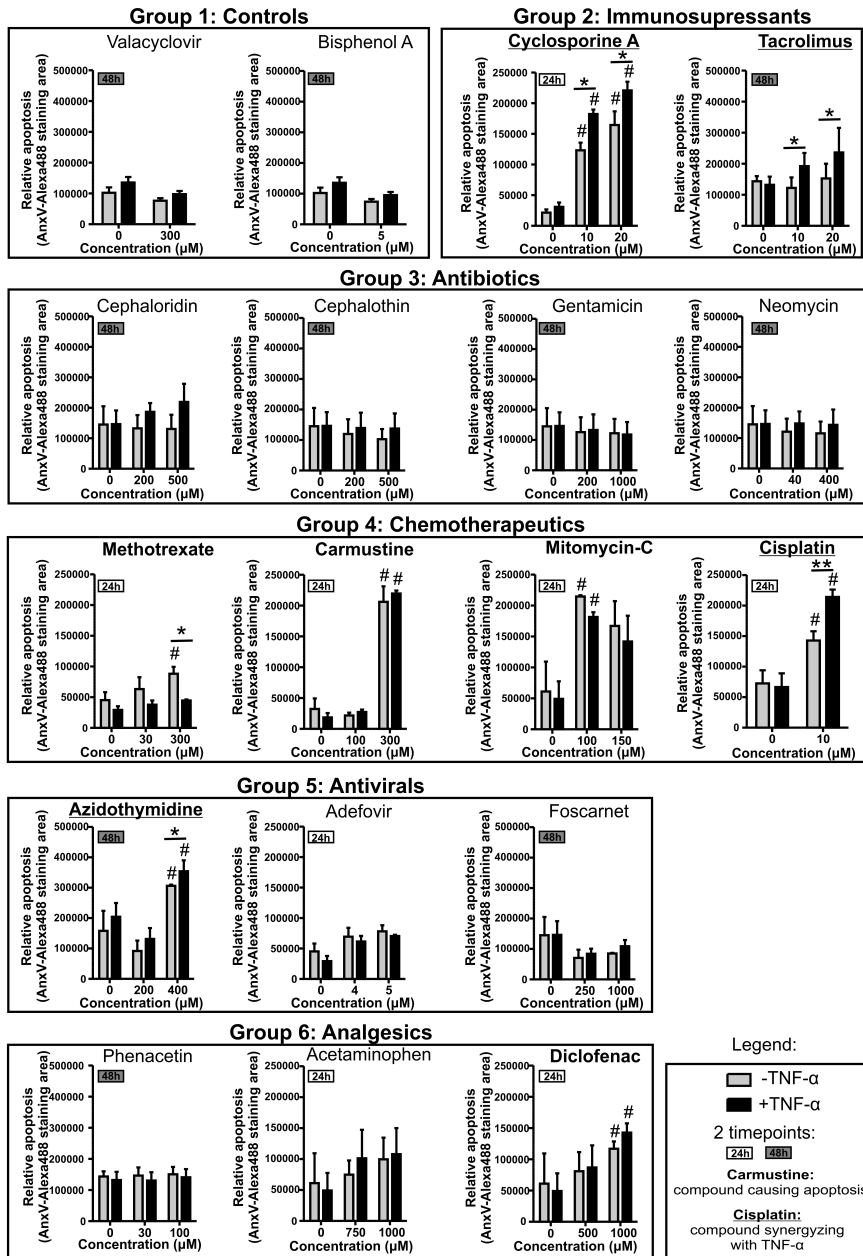


Figure 2. TNF- α enhanced the apoptotic response of renal cells exposed to cyclosporine A, tacrolimus, cisplatin and azidothymidine. IM-PTCs were exposed to two different concentrations of 16 different nephrotoxicants and two non-nephrotoxicants in combination or not of TNF- α (8 ng/ml) and apoptosis 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 $\pm$ S.E.M. * $P \leq 0.05$, ** $P \leq 0.01$ and # $P \leq 0.05$ compared to vehicle-treated cells.

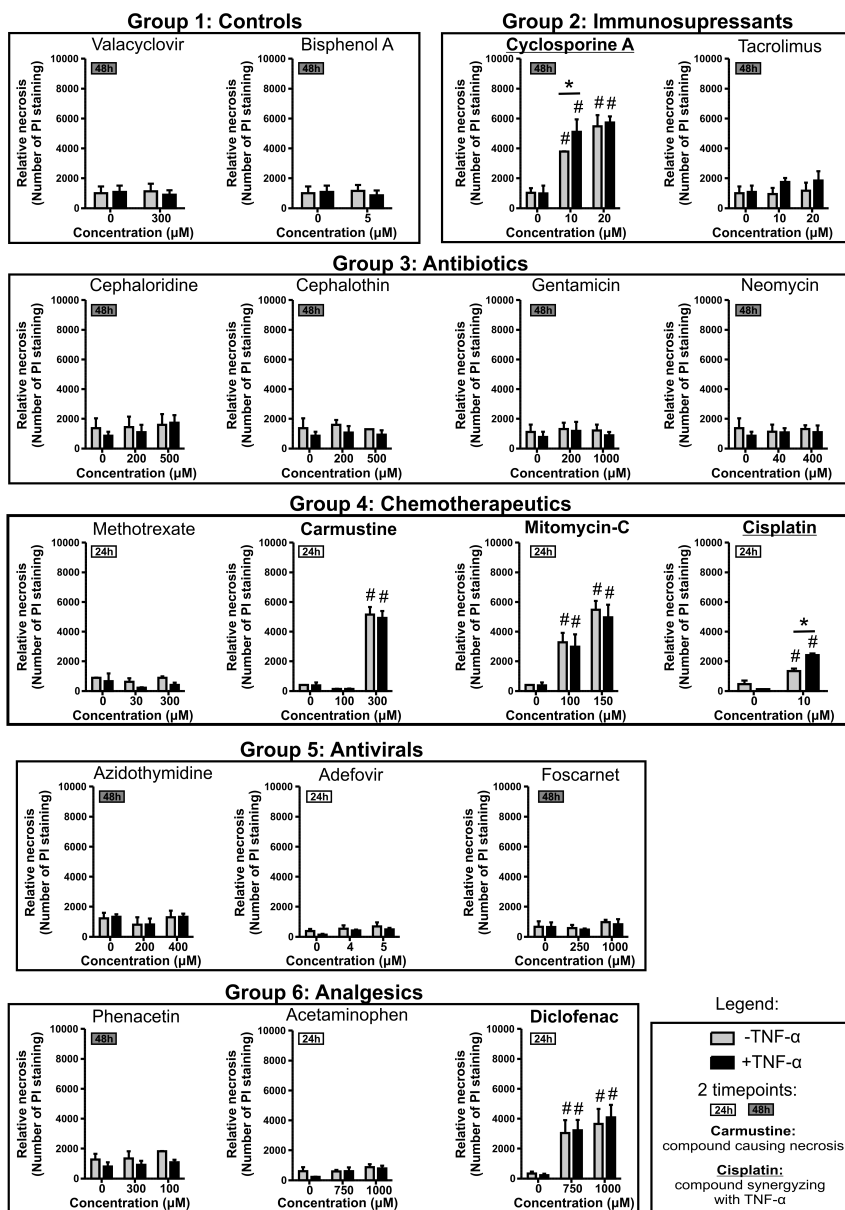


Figure 3. TNF-α enhanced the necrotic response of renal cells exposed to cyclosporine and cisplatin. IM-PTECs were exposed to two different concentrations of 16 different nephrotoxicants and two non-nephrotoxicants in combination or not of TNF-α (8 ng/ml) and necrosis of the cells was measured at 24 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 $\pm$ S.E.M. * $P \leq 0.05$, and # $P \leq 0.05$ compared to vehicle-treated cells.

3.3. The synergistic apoptotic response of renal cells exposed to the four compounds in combination with TNF- α 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 synergistic apoptotic response was confirmed (Fig. 4A and Fig. 5). 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 . These data indicate that our assay could identify nephrotoxic compounds that would not have been identified by current

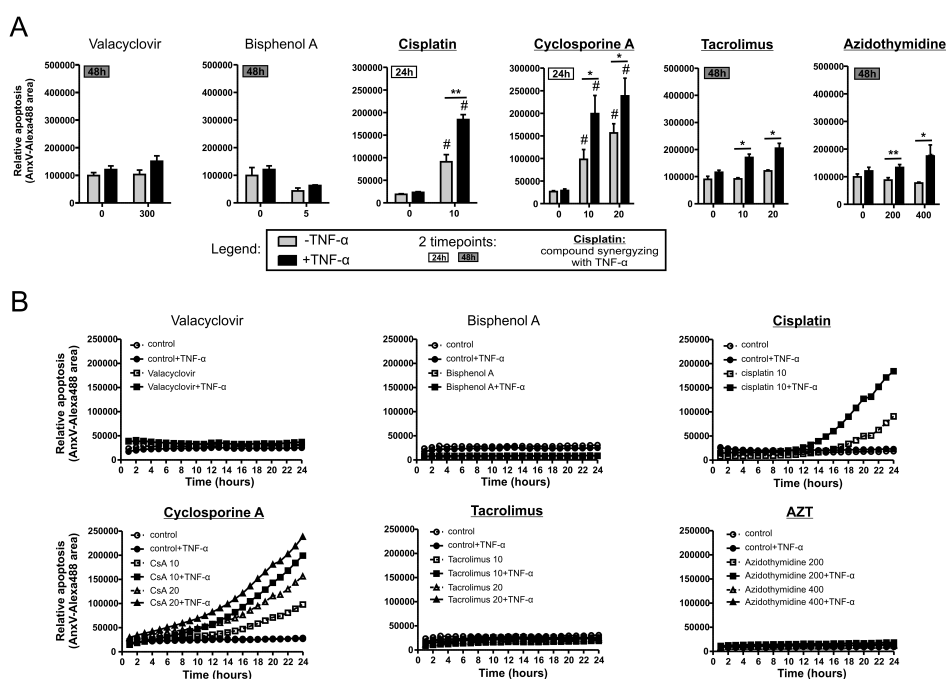


Figure 4. The synergistic apoptotic response of renal cells exposed to the four compounds in combination with TNF- α was confirmed with live apoptosis. IM-PTECs were exposed to two different concentrations of the 4 synergistic nephrotoxicants and the two non-nephrotoxicants in combination or not of TNF- 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 $\pm$ S.E.M. * $P \leq 0.05$, ** $P \leq 0.01$ and # $P \leq 0.05$ compared to vehicle-treated cells.

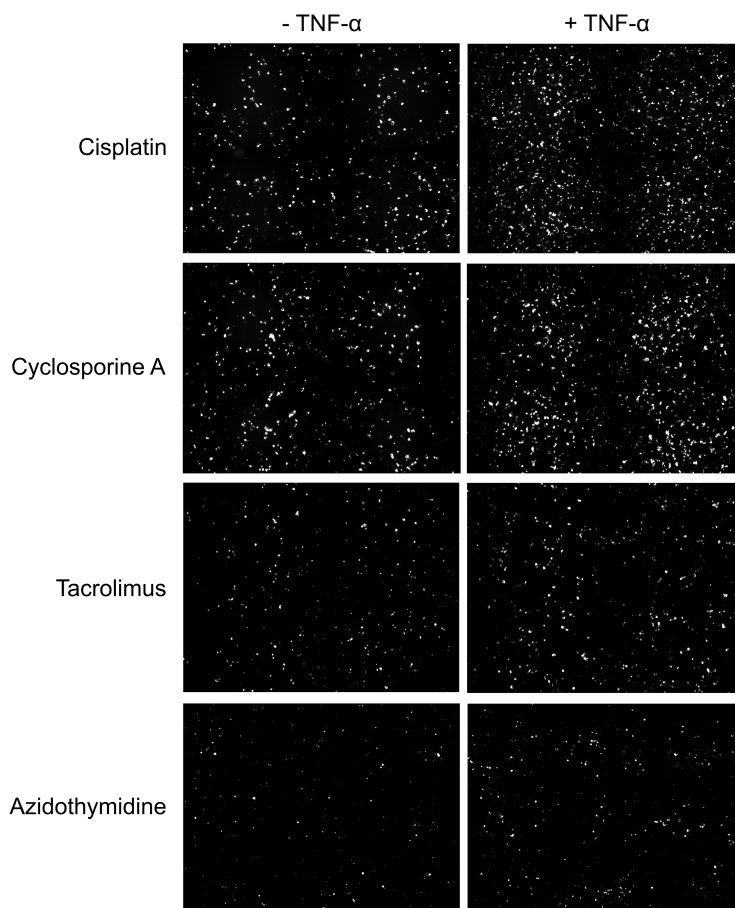


Figure 5. Annexin-V staining of apoptotic cells after exposure to the drugs synergizing with TNF- α . Annexin-V staining of the apoptotic cells was visualized by a BD Pathway 855 imager for IM-PTECS exposed to compounds in combination of not with TNF- α . Increased annexin-V stained cells was demonstrated 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.

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 by our laboratory (39). IM-PTECs were exposed to the four compounds in combination or not with TNF- α and the onset of apoptosis was followed over time. While cisplatin and cyclosporine A gave a strong synergistic apoptotic

response at 24 hours, as predicted, hardly any effect was observed after tacrolimus or azidothymidine treatment at 24 hours (Fig. 4B). Using this live apoptosis assay, we could clearly observe that TNF- α enhanced the onset of apoptosis induced by both cisplatin and cyclosporine A.

4. Discussion

Recent efforts have been made by pharmaceutical companies and academia in order to develop new *in vitro* screening tests for the prediction of nephrotoxicity of new chemicals. Although, these *in vitro* systems cannot reproduce completely the complex interactions of animal *in vivo*, they allow toxicity testing in a high throughput manner and provide important predictive and mechanistic information. Yet, nephrotoxicity remains a critical reason for drug withdrawal post-market. 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. Apoptosis and necrosis induced by the compounds in the presence or not of TNF- α was assessed by a semi high throughput fluorescence-based method. In total we identified 8 out of 16 compounds that induced cell death, of which 5 induced apoptosis as well as necrosis. Moreover, 4 compounds were able to induce a synergistic apoptotic response with TNF- α , including cisplatin, cyclosporine A, tacrolimus and azidothymidine.

In a recent study, we aimed at identifying a mechanism underlying the synergistic response between cisplatin and TNF- α . This synergistic apoptotic response was due to inhibition of the NF- κ B pathway and consequently increased c-Jun N-terminal kinase (JNK) activation (41). A similar mechanism may be involved in the synergistic 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- κ B activation in renal cells (42). Moreover, cyclosporine A was shown to induce JNK activation in primary human PTECs (43) and tacrolimus induced JNK activation in Madin Darby canine kidney cells (44). Thus far, it remains unknown whether the inhibition of NF- κ B 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- κ B, but studies were performed in Epstein-Barr virus-positive Burkitt lymphoma lines (45). 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. Such TNF-protected effect on methotrexate-induced apoptosis was also observed in macrophages *in vitro* and was shown to be due to enhanced activation of the NF- κ B pathway (46). It could be that in our IM-PTECs methotrexate and TNF- α protected the cells via enhanced activation of the NF- κ B pathway. It is unknown what the role of JNK is in the activation of the NF- κ B pathway.

Using cell death as a read-out for renal cell injury, 50% of the compounds could not be identified as a nephrotoxicant in our assay, while in clinic it is known that the compounds used in our screen all induce nephrotoxicity. Also in other studies and even currently used pre-clinical assays it is difficult to define these nephrotoxicity inducing compounds (4, 5, 34, 47). This lack of cytotoxicity in our assay could still be explained by lack of bioactivation in reactive intermediates in the case of acetaminophen and phenacetin (5, 48), by most likely lack of the membrane binding site in the brush border and basolateral membrane for neomycin and gentamicin (49) or low expression of the transporter organic anion-transporting polypeptide 1 (OAT1) responsible for the uptake of cephaloridine, cephalothin (50) and adefovir (51, 52). Alternatively, our IM-PTEC model may largely depend on glycolysis compared to oxidative phosphorylation for PTEC in the *in vivo* situation. These issues need further investigation.

Despite the fact that the nephrotoxic potential of some compounds was not identified with our assay, the addition of the immune component TNF- α proved to increase the sensitivity of the assay over traditional ones, since the toxicity of the compound tacrolimus was detected only in the presence of TNF- α . This observation correlated well with patient data where cyclosporine A induced more nephrotoxicity and inflammation after kidney transplantation than tacrolimus (32, 53). Furthermore, some of the compounds that failed to induce cytotoxicity in our assays were shown to affect the NF- κ B pathway in renal cells or other cell lines and organs, including acetaminophen (54), gentamycin (55, 56) and neomycin (57, 58). Therefore, by incorporating NF- κ B reporter IM-PTECs in our current fluorescence-based cytotoxicity assay, the sensitivity of our assay could be further enhanced and most likely identify compounds like gentamycin and neomycin as nephrotoxicants in an *in vitro* pre-clinical setting.

To summarize, although not yet suited for all potential nephrotoxicants our *in vitro* screening system consisting of exposing IM-PTECs to compounds in combination with the cytokine TNF- α and measuring apoptosis and necrosis with a high throughput fluorescence-based method provides a new sensitive screening assay for the detection

of the nephrotoxic potential of new chemicals. Given the potential involvement of NF- κ B as a common mechanism for compounds that induce synergistic apoptosis, in the future, other read-outs such as a NF- κ B translocation and activation should be incorporated.

Acknowledgements

This work was performed as part of the Netherlands Toxicogenomics Center (NTC)/Netherlands Genomics Initiative supported by the Dutch Organization for Scientific Research (NWO) grant nr. 050-06-510.

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