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

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

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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 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, 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 nephrotoxicants 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 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 µg/ml insulin and transferrin, 5 ng/ml sodium selenite (Roche, Almere, The Netherlands), 20 ng/ml triiodo-thyrionine (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 ± 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 ± 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 µM	0 and 24h
Bisphenol A	5 µM	0 and 24h
Cyclosporine A	10 and 20 µM	0 and 24h
Tacrolimus	10 and 20 µM	0 and 24h
Cephaloridin	200 and 500 µM	0 and 24h
Cephalothin	200 and 500 µM	0 and 24h
Gentamicin	200 and 1000 µM	0 and 24h
Neomycin	40 and 400 µM	0 and 24h
Methotrexate	30 and 300 µM	0h
Carmustine	100 and 300 µM	0h
Mitomycin C	100 and 150 µM	0h
Cisplatin	10 µM	0h
Azidothymidine	200 and 400 µM	0 and 24h
Adefovir	4 and 5 µM	0 and 24h
Foscarnet	250 and 1000 µM	0h
Phenacetin	30 and 100 µM	0 and 24h
Acetaminophen	750 and 1000 µM	0 and 24h
Diclofenac	500 and 1000 µM	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 nephrotoxixc compounds induced apoptosis at 24 or

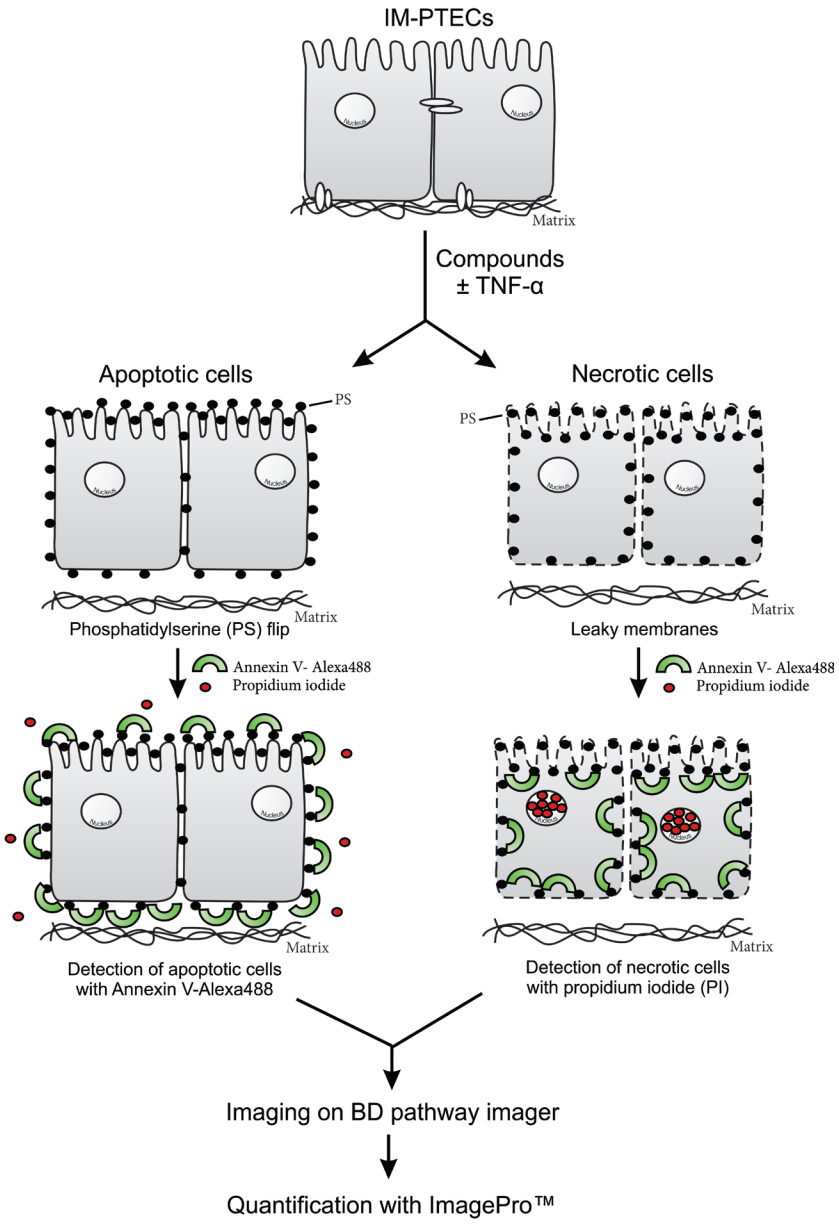

Figure 1. Flowchart of the in vitro screen procedure. Following exposure of the IM-PTECs to the compounds in the presence or not of TNF-α, the amount of apoptotic and necrotic cell death was determined in a fluorescent-based manner at 24 and 48 hours or followed over time. Apoptosis was measured with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

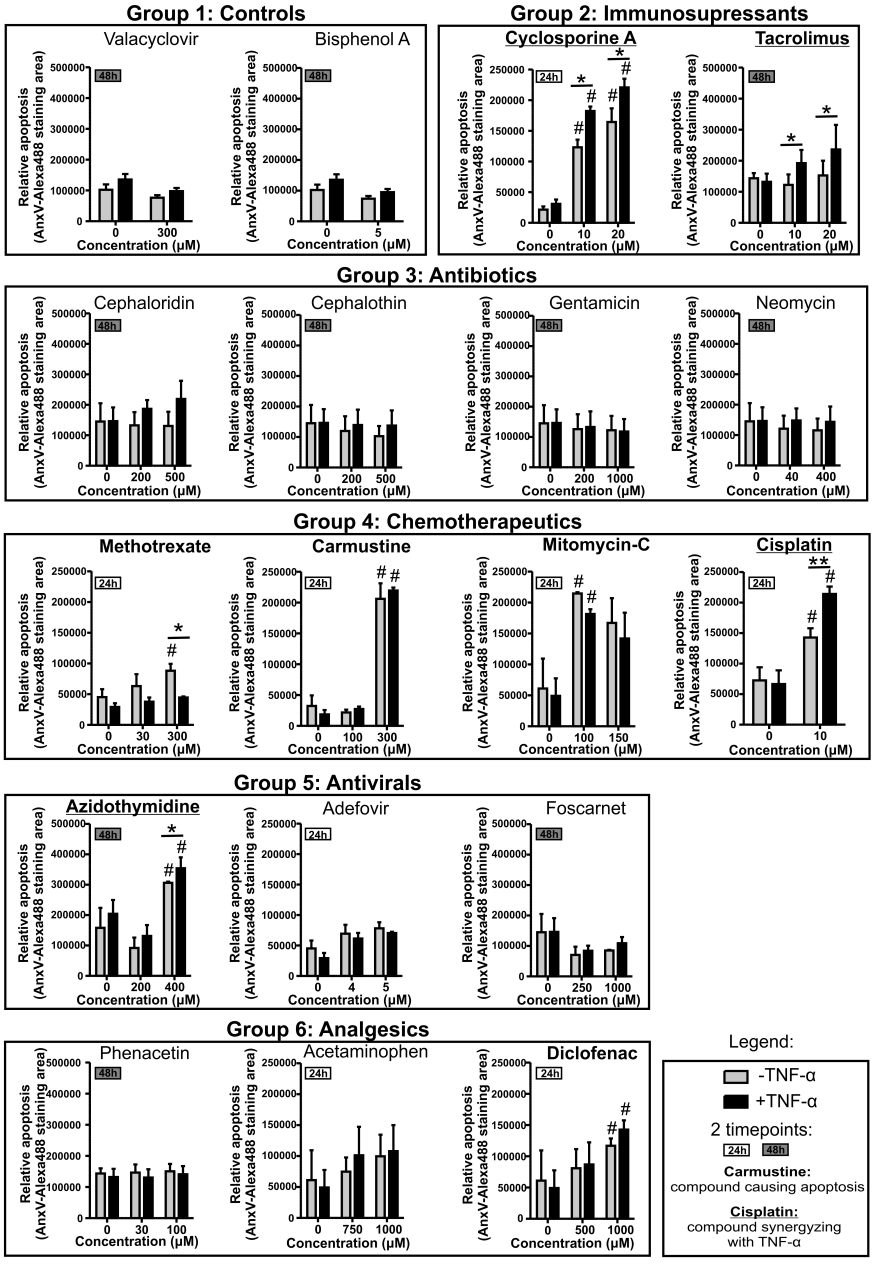

Figure 2. TNF-α enhanced the apoptotic response of renal cells exposed to cyclosporine A, tacrolimus, cisplatin and azidothymidine. IM-PTECs were exposed to two different concentrations of 16 different nephrotoxicants and two non-nephrotoxicants in combination or not of TNF-α (8 ng/ml) and apoptosis 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 ≤ 0.05, ** P ≤ 0.01 and # P ≤ 0 .05 compared to vehicle-treated cells.

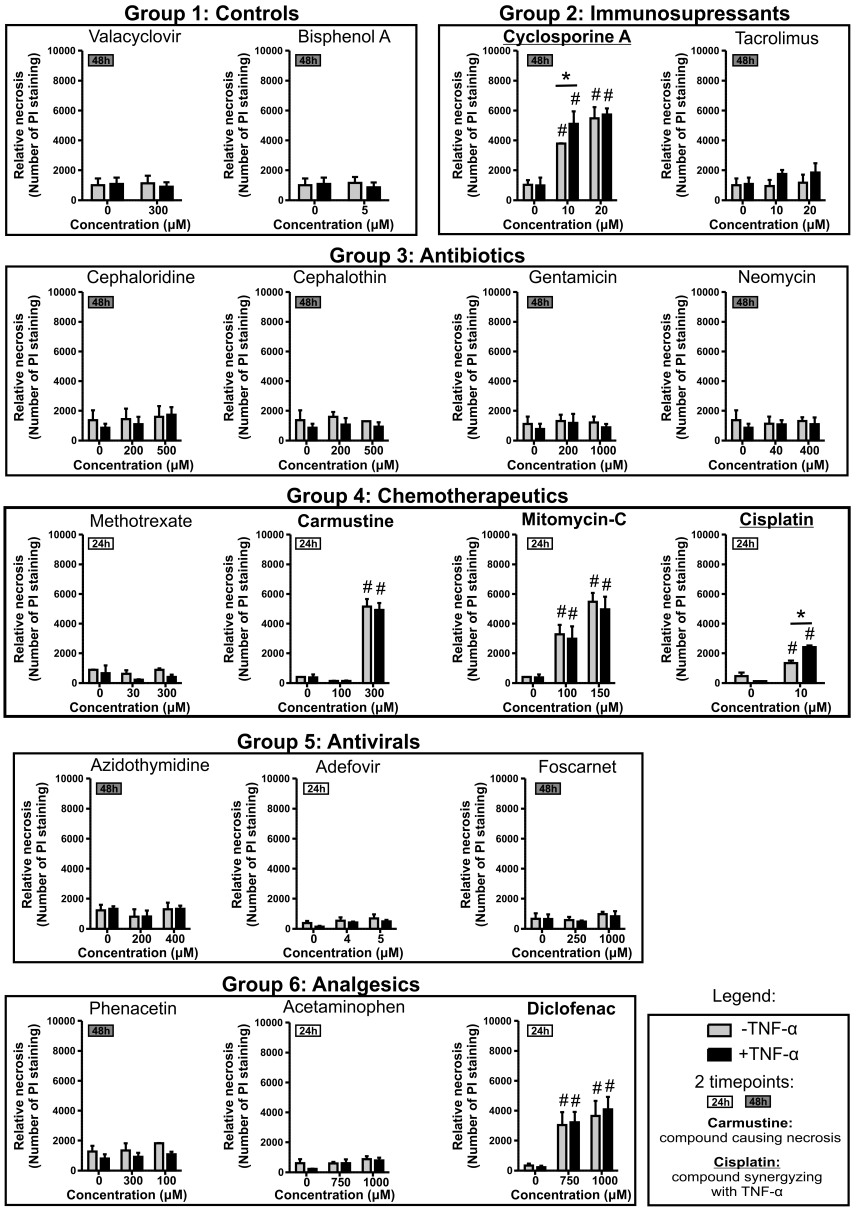

Figure 3. TNF-α enhanced the necrotic response of renal cells exposed to cyclosporine and cisplatin. IM-PTECs were exposed to two different concentrations of 16 different nephrotoxicants and two non-nephrotoxicants in combination or not of TNF-α (8 ng/ml) and necrosis of the cells was measured at 24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

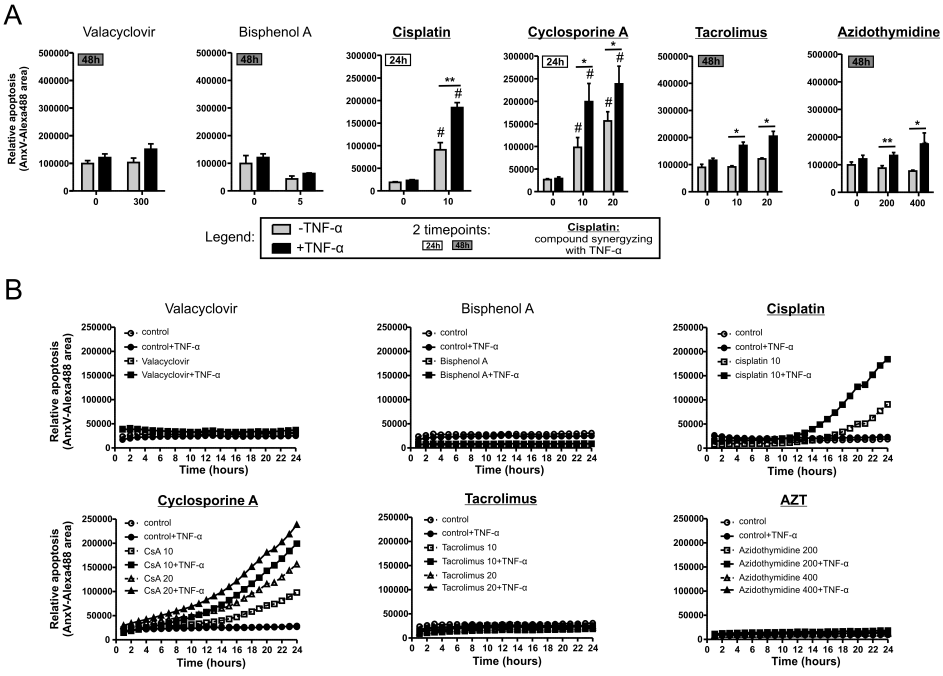

Figure 4. The synergistic apoptotic response of renal cells exposed to the four compounds in combination with TNF-α was confirmed with live apoptosis. IM-PTECs were exposed to two different concentrations of the 4 synergistic nephrotoxicants and the two non-nephrotoxicants in combination or not of TNF-α (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 ± S.E.M. * P ≤ 0.05, ** P ≤ 0.01 and # P ≤ 0 .05 compared to vehicle-treated cells.

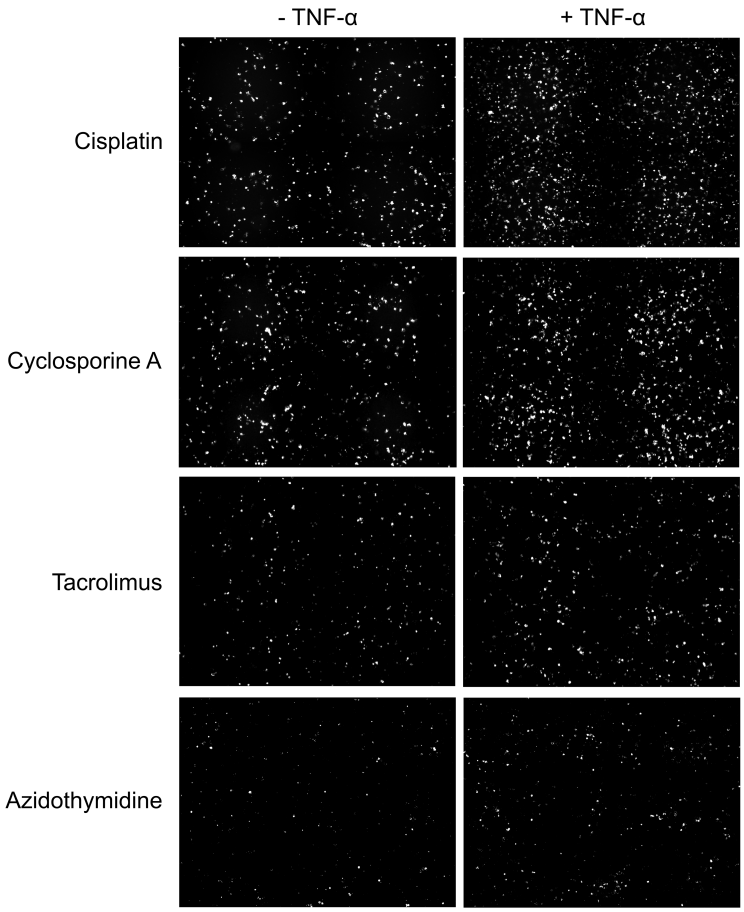

Figure 5. Annexin-V staining of apoptotic cells after exposure to the drugs synergizing with TNF-α. Annexin-V staining of the apoptotic cells was visualized by a BD Pathway 855 imager for IM-PTECS exposed to compounds in combination of not with TNF-α. Increased annexin-V stained cells was demonstrated 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 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-κB 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-κB 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-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 (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-κB pathway (46). It could be that in our IM-PTECs methotrexate and TNF-α protected the cells via enhanced activation of the NF-κB pathway. It is unknown what the role of JNK is in the activation of the NF-κB 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-κB pathway in renal cells or other cell lines and organs, including acetaminophen (54), gentamycin (55, 56) and neomycin (57, 58). Therefore, by incorporating NF-κB 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-κB as a common mechanism for compounds that induce synergistic apoptosis, in the future, other read-outs such as a NF-κB translocation and activation should be incorporated.

Acknowledgements

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References

1. Li W, Choy DF, Lam MS, Morgan T, Sullivan ME, Post JM. Use of cultured cells of kidney origin to assess specific cytotoxic effects of nephrotoxins. Toxicology *in vitro* : an international journal published in association with BIBRA 2003;17:107-113.

2. Li W, Lam M, Choy D, Birkeland A, Sullivan ME, Post JM. Human primary renal cells as a model for toxicity assessment of chemo-therapeutic drugs. Toxicology *in vitro* : an international journal published in association with BIBRA 2006;20:669-676.

3. Weber F, Freudinger R, Schwerdt G, Gekle M. A rapid screening method to test apoptotic synergisms of ochratoxin A with other nephrotoxic substances. Toxicology *in vitro* : an international journal published in association with BIBRA 2005;19:135-143.

4. Wu Y, Connors D, Barber L, Jayachandra S, Hanumegowda UM, Adams SP. Multiplexed assay panel of cytotoxicity in HK-2 cells for detection of renal proximal tubule injury potential of compounds. Toxicology *in vitro* : an international journal published in association with BIBRA 2009;23:1170-1178.

5. Zhang L, Mu X, Fu J, Zhou Z. *In vitro* cytotoxicity assay with selected chemicals using human cells to predict target-organ toxicity of liver and kidney. Toxicology *in vitro* : an international journal published in association with BIBRA 2007;21:734-740.

6. Bush KT, Keller SH, Nigam SK. Genesis and reversal of the ischemic phenotype in epithelial cells. The Journal of clinical investigation 2000;106:621-626.

7. Kays SE, Schnellmann RG. Regeneration of renal proximal tubule cells in primary culture following toxicant injury: response to growth factors. Toxicol Appl Pharmacol 1995;132:273-280.

8. Cordes N. Integrin-mediated cell-matrix interactions for prosurvival and antiapoptotic signalling after genotoxic injury. Cancer letters 2006;242:11-19.

9. Yalavarthy R, Edelstein CL. Therapeutic and predictive targets of AKI. Clin Nephrol 2008;70:453-463.

10. Araujo LP, Truzzi RR, Mendes GE, Luz MA, Burdmann EA, Oliani SM. Annexin A1 protein attenuates cyclosporine-induced renal hemodynamics changes and macrophage infiltration in rats. Inflamm Res 2012;61:189-196.

11. Pabla N, Dong Z. Cisplatin nephrotoxicity: mechanisms and renoprotective strategies. Kidney international 2008;73:994-1007.

12. Quiros Y, Vicente-Vicente L, Morales AI, Lopez-Novoa JM, Lopez-Hernandez FJ. An integrative

overview on the mechanisms underlying the renal tubular cytotoxicity of gentamicin. Toxicol Sci 2011;119:245-256.

13. Naughton CA. Drug-induced nephrotoxicity. Am Fam Physician 2008;78:743-750.

14. Akcay A, Nguyen Q, Edelstein CL. Mediators of inflammation in acute kidney injury. Mediators Inflamm 2009;2009:137072.

15. Ramesh G, Brian Reeves W. Cisplatin increases TNF-alpha mRNA stability in kidney proximal tubule cells. Renal failure 2006;28:583-592.

16. Ramesh G, Kimball SR, Jefferson LS, Reeves WB. Endotoxin and cisplatin synergistically stimulate TNF-alpha production by renal epithelial cells. American journal of physiology. Renal physiology 2007;292:F812-819.

17. Liu M, Chien CC, Burne-Taney M, Molls RR, Racusen LC, Colvin RB, Rabb H. A pathophysiologic role for T lymphocytes in murine acute cisplatin nephrotoxicity. Journal of the American Society of Nephrology : JASN 2006;17:765-774.

18. Ghosh J, Das J, Manna P, Sil PC. Acetaminophen induced renal injury via oxidative stress and TNF-alpha production: therapeutic potential of arjunolic acid. Toxicology 2010;268:8-18.

19. Ramesh G, Reeves WB. TNF-alpha mediates chemokine and cytokine expression and renal injury in cisplatin nephrotoxicity. The Journal of clinical investigation 2002;110:835-842.

20. Ramesh G, Reeves WB. TNFR2-mediated apoptosis and necrosis in cisplatin-induced acute renal failure. American journal of physiology. Renal physiology 2003;285:F610-618.

21. Piao RL, Liu YY, Tian D, Ma ZH, Zhang M, Zhao C, Niu JQ. Adefovir dipivoxil modulates cytokine expression in Th1/Th2 cells in patients with chronic hepatitis B. Molecular medicine reports 2012;5:184-189.

22. Asvadi I, Hajipour B, Asvadi A, Asl NA, Roshangar L, Khodadadi A. Protective effect of pentoxyfilline in renal toxicity after methotrexate administration. European review for medical and pharmacological sciences 2011;15:1003-1009.

23. Helal GK, Aleisa AM, Helal OK, Al-Rejaie SS, Al-Yahya AA, Al-Majed AA, Al-Shabanah OA. Metallothionein induction reduces caspase-3 activity and TNFalpha levels with preservation of cognitive function and intact hippocampal neurons in carmustine-treated rats. Oxidative medicine and cellular longevity 2009;2:26-35.

24. Pogrebniak HW, Matthews W, Pass HI. Chemotherapy amplifies production of tumor necrosis factor. Surgery 1991;110:231-237.

25. Remick DG, Nguyen DT, Eskandari MK, Strieter RM, Kunkel SL. Cyclosporine A inhibits TNF production without decreasing TNF mRNA levels. Biochemical and biophysical research communications 1989;161:551-555.

26. Yard BA, Pancham RR, Paape ME, Daha MR, van Es LA, van der Woude FJ. CsA, FK506, corticosteroids and rapamycin inhibit TNF alpha production by cultured PTEC. Kidney international 1993;44:352-358.

27. Cicora F, Roberti J, Lausada N, Gonzalez P, Guerrieri D, Stringa P, Raimondi C. [Immunosuppression in kidney donors with rapamycin and tacrolimus. Proinflammatory cytokine expression]. Medicina 2012;72:3-9.

28. Nguyen DT, Eskandai MK, DeForge LE, Raiford CL, Strieter RM, Kunkel SL, Remick DG. Cyclosporin a modulation of tumor necrosis factor gene expression and effects *in vitro* and *in vivo*. Journal of immunology 1990;144:3822-3828.

29. Peces R, Urra JM. Effect of calcium-channel blocker on tumour necrosis factor alpha (TNF alpha) production in cyclosporin-treated renal transplant recipients. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association 1995;10:871-873.

30. Weimer R, Melk A, Daniel V, Friemann S, Padberg W, Opelz G. Switch from cyclosporine A

to tacrolimus in renal transplant recipients: impact on Th1, Th2, and monokine responses. Human immunology 2000;61:884-897.

31. Araujo LP, Truzzi RR, Mendes GE, Luz MA, Burdmann EA, Oliani SM. Interaction of the anti-inflammatory annexin A1 protein and tacrolimus immunosuppressant in the renal function of rats. American journal of nephrology 2010;31:527-533.

32. Lauzurica R, Pastor MC, Bayes B, Malumbres S, Homs M, Llopis MA, Bonet J, et al. Subclinical inflammation in renal transplant recipients: impact of cyclosporine microemulsion versus tacrolimus. Transplantation proceedings 2007;39:2170-2172.

33. Pichler RH, Franceschini N, Young BA, Hugo C, Andoh TF, Burdmann EA, Shankland SJ, et al. Pathogenesis of cyclosporine nephropathy: roles of angiotensin II and osteopontin. Journal of the American Society of Nephrology : JASN 1995;6:1186-1196.

34. Zager RA, Johnson AC, Geballe A. Gentamicin suppresses endotoxin-driven TNF-alpha production in human and mouse proximal tubule cells. American journal of physiology. Renal physiology 2007;293:F1373-1380.

35. Geleilete TJ, Melo GC, Costa RS, Volpini RA, Soares TJ, Coimbra TM. Role of myofibroblasts, macrophages, transforming growth factor-beta endothelin, angiotensin-II, and fibronectin in the progression of tubulointerstitial nephritis induced by gentamicin. Journal of nephrology 2002;15:633-642.

36. De Simone C, Tzantzoglou S, Santini G, Vullo V, di Orio F, Leuter C, Jirillo E, et al. Clinical and immunologic effects of combination therapy with intravenous immunoglobulins and AZT in HIV-infected patients. Immunopharmacology and immunotoxicology 1991;13:447-458.

37. Morlat P, Chene G, Ragnaud JM, Mercie P, Grobost F, Bernard N, Lacoste D, et al. Evolution of tumor necrosis factor-alpha serum concentrations in HIV infected individuals treated with zidovudine. Pathologie-biologie 1996;44:716-719.

38. van Erk MJ, Wopereis S, Rubingh C, van Vliet T, Verheij E, Cnubben NH, Pedersen TL, et al. Insight in modulation of inflammation in response to diclofenac intervention: a human intervention study. BMC medical genomics 2010;3:5.

39. Puigvert JC, de Bont H, van de Water B, Danen EH. High-throughput live cell imaging of apoptosis. Curr Protoc Cell Biol 2010;Chapter 18:Unit 18 10 11-13.

40. Stokman G, Qin Y, Genieser HG, Schwede F, de Heer E, Bos JL, Bajema IM, et al. Epac-Rap signalling reduces cellular stress and ischemia-induced kidney failure. Journal of the American Society of Nephrology : JASN 2011;22:859-872.

41. Benedetti G, Fredriksson L, Herpers B, Meerman J, van de Water B, de Graauw M. TNF-α-mediated NF-κB Survival Signalling Impairment by Cisplatin Enhances JNK Activation Allowing Synergistic Apoptosis of Renal Proximal Tubular Cells. Biochemical pharmacology 2012.

42. Du S, Hiramatsu N, Hayakawa K, Kasai A, Okamura M, Huang T, Yao J, et al. Suppression of NF-kappaB by cyclosporin a and tacrolimus (FK506) via induction of the C/EBP family: implication for unfolded protein response. Journal of immunology 2009;182:7201-7211.

43. Pallet N, Thervet E, Anglicheau D. c-Jun-N-Terminal Kinase Signalling Is Involved in Cyclosporine-Induced Epithelial Phenotypic Changes. Journal of transplantation 2012;2012:348604.

44. Jeon SH, Park HM, Kim SJ, Lee MY, Kim GB, Rahman MM, Woo JN, et al. Taurine reduces FK506-induced generation of ROS and activation of JNK and Bax in Madin Darby canine kidney cells. Human & experimental toxicology 2010;29:627-633.

45. Kurokawa M, Ghosh SK, Ramos JC, Mian AM, Toomey NL, Cabral L, Whitby D, et al. Azidothymidine inhibits NF-kappaB and induces Epstein-Barr virus gene expression in Burkitt lymphoma. Blood 2005;106:235-240.

46. Lo SZ, Steer JH, Joyce DA. Tumor necrosis factor-alpha promotes survival in methotrexate-exposed macrophages by an NF-kappaB-dependent pathway. Arthritis research & therapy 2011;13:R24.

47. Schwertz DW, Kreisberg JI, Venkatachalam MA. Gentamicin-induced alterations in pig kidney epithelial (LLC-PK1) cells in culture. The Journal of pharmacology and experimental therapeutics 1986;236:254-262.

48. Hu JJ, Lee MJ, Vapiwala M, Reuhl K, Thomas PE, Yang CS. Sex-related differences in mouse renal metabolism and toxicity of acetaminophen. Toxicology and applied pharmacology 1993;122:16-26.

49. Williams PD, Bennett DB, Gleason CR, Hottendorf GH. Correlation between renal membrane binding and nephrotoxicity of aminoglycosides. Antimicrobial agents and chemotherapy 1987;31:570-574.

50. Tune BM. Nephrotoxicity of beta-lactam antibiotics: mechanisms and strategies for prevention. Pediatric nephrology 1997;11:768-772.

51. Uwai Y, Ida H, Tsuji Y, Katsura T, Inui K. Renal transport of adefovir, cidofovir, and tenofovir by SLC22A family members (hOAT1, hOAT3, and hOCT2). Pharmaceutical research 2007;24:811-815.

52. Ho ES, Lin DC, Mendel DB, Cihlar T. Cytotoxicity of antiviral nucleotides adefovir and cidofovir is induced by the expression of human renal organic anion transporter 1. Journal of the American Society of Nephrology : JASN 2000;11:383-393.

53. Martins L, Ventura A, Branco A, Carvalho MJ, Henriques AC, Dias L, Sarmento AM, et al. Cyclosporine versus tacrolimus in kidney transplantation: are there differences in nephrotoxicity? Transplantation proceedings 2004;36:877-879.

54. Boulares AH, Giardina C, Inan MS, Khairallah EA, Cohen SD. Acetaminophen inhibits NF-kappaB activation by interfering with the oxidant signal in murine Hepa 1-6 cells. Toxicological sciences : an official journal of the Society of Toxicology 2000;55:370-375.

55. Juan SH, Chen CH, Hsu YH, Hou CC, Chen TH, Lin H, Chu YL, et al. Tetramethylpyrazine protects rat renal tubular cell apoptosis induced by gentamicin. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association 2007;22:732-739.

56. Volpini RA, Costa RS, da Silva CG, Coimbra TM. Inhibition of nuclear factor-kappaB activation attenuates tubulointerstitial nephritis induced by gentamicin. Nephron. Physiology 2004;98:p97-106.

57. Garcia-Trapero J, Carceller F, Dujovny M, Cuevas P. Perivascular delivery of neomycin inhibits the activation of NF-kappaB and MAPK pathways, and prevents neointimal hyperplasia and stenosis after arterial injury. Neurological research 2004;26:816-824.

58. Cuevas P, Diaz-Gonzalez D, Dujovny M. Glioma cell-associated sustained activation of the transcription factor, nuclear factor-kappa B, was inhibited by neomycin. Neurological research 2003;25:271-274.