Assessment of a New Cell Culture Perfusion Apparatus for *In Vitro* Chronic Toxicity Testing Part 2: Toxicological Evaluation

Paul Jennings[1], Christian Koppelstaetter[2], Walter Pfaller[2], Jean-Paul Morin[3], Thomas Hartung[4] and Michael P. Ryan[1]

[1]Pharmacology Dept, The Conway Institute of Biomolecular and Biomedical Research, IRL-Dublin, [2]Department of Physiology, University of Innsbruck, A-Innsbruck, [3]INSERM, Faculté de Médecine Pharmacie, Université de Rouen, F-Rouen, [4]Biomedical Pharmacology, University of Konstanz, D-Germany

Summary

The goal of replacement, refinement and reduction of animal testing is critically dependent on the development and assessment of novel in vitro methodologies and the further development of existing methodologies. Here, we evaluated the use of a modified perfusion cell culture apparatus for application to chronic in vitro nephrotoxicity testing using DMSO, SDS, paracetamol and cyclosporine A as test compounds.

Renal epithelial monolayers were cultured on microporous growth supports and exposed to test compounds under static or perfusion conditions. Alamar Blue reduction, gamma-glutamyl transpeptidase activity (GGT), lactate dehydrogenase activity (LDH) and remnant protein were used to assay cell toxicity. There was no significant difference in IC_{50} values between static and perfusion cultures up to 72 hours exposure. However, the perfusion system allowed continuous real-time monitoring of plasma membrane damage, which gives important information of time, duration and scale of toxicity.

The complexity of the system restrains its use to low-throughput analysis. However, the real and theoretical advantages of this and similar systems merit further investigations.

Zusammenfassung: Evaluierung eines neuen Zellkultur-Perfusionssystems für chronische *in vitro* Toxizitätstests. Teil 2: Toxikologische Evaluation

Das Ziel des 3R Konzeptes (replacement, refinement, reduction of animal testing) ist, im Bereich der Chemikalientestung Ersatzmethoden für Tierversuche zu entwickeln und bestehende Methoden zu verbessern. In dieser Arbeit wurde ein von uns modifiziertes Zellkultur-Perfusionssystem als Testmodell für Langzeit in vitro Toxizitätstests unter Verwendung von DMSO, SDS, Paracetamol und Cyclosporin A als Testsubstanzen evaluiert. An Nierenepithelzellen wurden sowohl mit der konventionellen „statischen" Zellkultur als auch im Perfusionssystem Untersuchungen mit verschiedenen Substanzen durchgeführt.

Die IC_{50} Werte zeigten keine signifikanten Unterschiede zwischen der „statischen"- und der Perfusionskultur nach 72 Stunden Toxinexposition. Zusätzlich zu den physiologischeren Bedingungen, welche die Perfusionskultur garantiert, konnten auch Echtzeit-Beobachtungen der Plasmamembranschädigung an Hand der Aktivität spezifischer Enzyme (Gamma-Glutamyl Transferase bzw. Laktatdehydrogenase) durchgeführt werden, was vor allem über die Kinetik der Toxizität Auskunft gibt, jedoch im „statischen" Zellkulturmodell nicht möglich ist.

Es bleibt zum jetzigen Zeitpunkt unklar, ob die Perfusions- verglichen mit der konventionellen Zellkultur ein Mehr an Information bietet. Die offensichtlichen Vorteile des neuen Systems verdienen jedoch eine weitere Verwendung in der Erforschung chronischer Toxizität.

Keywords: perfusion, cell culture, toxicity, chronic, DMSO, paracetamol, Cyclosporine A

1 Introduction

In the first paper we discussed the technical aspects of using a perfusion culture system with renal cell cultures (Koppelstaetter et. al., 2004). In this paper we assess the use of the modified system for toxicological studies. There were two aspects to this study. Firstly, to compare toxicological sensitivities of renal cell cultures under perfusion culture and static culture conditions. 72 h was chosen as the longest possible time to conduct these experiments in order to limit the effect of malnutrition on the static cultures (under normal cell culture conditions cells are fed every 2 to 3 days). The second part of this study was conducted to assess the possibility of conducting longer-term exposures and to attempt monitoring of cell vitality continuously.

Received 19 April 2004; received in final form and accepted for publication 17 May 2004

2 Materials and methods

The perfusion system was obtained from Minucells and Minutissue Vertriebs GmbH, Germany and was modified according to the accompanying technical paper (Koppelstaetter et. al., 2004). Alamar Blue was obtained from Accumed International Ltd. The lactate dehydrogenase cytotoxicity assay was obtained from Boehringer Mannheim (Roche). Human fibronectin and rat tail collagen were obtained from Becton and Dickinson. Foetal calf serum, L-alanyl-L-glutamine (glutamax), penicillin, streptomycin and glutamine were obtained from GibcoBRL. All other medium additives were of tissue grade quality and were obtained from Sigma. All tissue culture plastic ware (Falcon® Labware) was obtained from Becton Dickinson GmbH. Cyclosporin A was a generous gift from Novartis Pharmaceuticals Ltd. All other reagents and chemicals were obtained from Sigma at the highest grade available.

2.1 Cell culture

LLC-PK_1 cells (ATCC CRL 1392) of serial passage 180 to 192 were grown to confluence in a 37°C, H_2O saturated, 5% CO_2 incubator. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 5 mM glucose, 127 mM NaCl, 2 mM L-glutamine, 2 mM pyruvate, 30 µM phenol red, 100 U/ml penicillin and 100 µg/ml streptomycin. Culture medium was replaced every second to third day. HK-2 cells were treated as described in Koppelstaetter et al., 2004.

Confluent monolayers were removed by 0.5% (w/v) trypsin, 0.2% (w/v) EDTA and subcultured at a split ratio of 1:6 for propagation or at a seeding density of approximately 4 x 10^4 cells/cm^2 on growth supports for experiments.

2.2 Comparison between toxicity under static and perfusion culture conditions

Confluent HK-2 cell monolayers grown on fibronectin-coated polycarbonate filters in filter carriers were exposed to dimethyl sulfoxide (DMSO) at 0, 32, 100, 317, 1005 and 3176 mM concentrations or paracetamol (APAP) at 0, 0.17, 0.66, 2.65, 10.6 and 42.3 mM for 24 and 72 h under static and perfusion conditions. For static culture conditions 1 filter carrier per well of a 6 well plate was submerged in 3 ml medium. In the perfusion culture, 6 filter carriers were inserted into each modified perfusion chamber with a medium perfusion rate of 1 ml/h. (Perfusion culture conditions and perfusion equipment handling are described in more detail in Koppelstaetter et al., 2004.) At the end of 24 and 72 h cell monolayers were analysed for Alamar Blue reduction and cell homogenates were analysed for GGT activity, LDH activity and protein content. All perfusion and static experiments were carried out in a paired fashion, in 3 independent experiments with 3 to 6 replicates.

2.3 IC_{50} generation

IC_{50} values were calculated from percentage control data using a sigmoidal dose-response (variable slope) calculation with GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA, using the following equation:

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{((\text{LogIC50} - X)*\text{HillSlope})}}$$

where X is the logarithm of concentration and Y is the response. "Bottom" was set as 0 (as 0 represents no response, which is true if there is no viable cells remaining).

2.4 Real time cell toxicity monitoring under perfusion conditions

LLC-PK_1 cells were grown to confluence on collagen-coated aluminium oxide filters in filter carriers and transferred to perfusion culture conditions. After one day of perfusion, medium reservoir bottles were exchanged for medium bottles containing DMEM-Ham's F12 1% (v/v) FCS and toxin (SDS or Cyclosporin A, CsA). The time coinciding with toxin-containing medium entering the perfusion chamber was set as T0. Samples were collected aseptically from the effluent perfusion lines for 1 hour intervals over a period of 80 to 120 h. Samples were stored at 4°C until the end of the experiment when all samples were measured for LDH and/or GGT activity.

2.5 Assays

All the assays carried out are described in the accompanying technical paper Koppelstaetter et al., 2004.

3 Results

3.1 Comparison between toxicity under static and perfusion conditions

The toxicity profile of APAP and DMSO was compared between perfusion and static conditions at 24 h and 72 h. Toxicity was measured by end-point determination of Alamar Blue, remnant protein, remnant LDH and remnant GGT. IC_{50} values are shown in Table 1 and Table 2. Two examples of IC_{50} curves are shown in Figure 1.

At 24 h APAP resulted in dose-dependent toxicity as evidenced by the four parameters used (Alamar Blue reduction, remnant protein, remnant LDH activity and remnant GGT activity). Morphological examination of the cell monolayers by phase contrast microscopy showed extensive cell rounding and loss of monolayer integrity at 43.2 mM only (not shown). APAP toxicity was more pronounced at 72 h. Morphological examination of the cell monolayers by phase contrast microscopy showed extensive cell rounding and loss of monolayer integrity at both 10.6 mM and 42.3 mM (not shown). Monolayers were intact at lower concentrations.

DMSO demonstrated dose dependent toxicity at 24 h. Remnant protein, remnant LDH activity and end-point Alamar Blue reduction gave similar results. Morphological evaluation of cell monolayers by phase contrast microscopy revealed extensive destruction of the HK-2 monolayer at 1005 mM DMSO and complete destruction at 3176 mM. At 72 h toxicity was apparent at 317 mM DMSO. 1005 and 3176 mM DMSO resulted in complete cell lethality for all parameters measured. Morphological evaluation of the cell monolayers by phase contrast microscopy showed that no cells were left attached at 1005 mM and 3176 mM. DMSO was significantly more toxic at

72 h than 24 h for 1005 mM as assessed by end-point Alamar Blue (0.8% control ± 0.2 for 72 h and 24.4 ± 3.4 for 24 h with P = 0.002 using pooled static and perfusion data). However, there was no increase in toxicity at lower concentrations at 72 h and IC_{50} values remained similar at both time points. Thus, there appears to be threshold tolerance to DMSO, and once this is exceeded the cells do not recover.

From the IC_{50} data it is clear that there was little difference in sensitivity to toxin exposure when conducted either under perfusion culture conditions or under static culture conditions. APAP was more toxic at 72 h than at 24 h under both conditions.

3.2 Inter-laboratory comparison

The end-point Alamar Blue (resazurin) data from 3 independent laboratories was compared. The average results are plotted in Figure 2. Error bars represent the standard error between laboratories.

3.3 LLC-PK$_1$ cells exposed to perfused SDS

LLC-PK$_1$ cells perfused with SDS-containing medium resulted in elevated LDH release with 173 µM SDS but not with 34.6 µM or 6.9 µM (Fig. 3), i.e. an all-or-nothing effect. Similar results were demonstrated in static plastic-adhered cell cultures (not shown). Elevated LDH activity was detected in the outflow beginning at 6 h when perfused with 173 µM SDS. LDH release increased over the next 8 h, reached a plateau and then decreased exponentially until 80 h. The increase in effluent LDH fitted (r^2=0.989) to a Boltzmann sigmoidal curve plotted on the graph (equation is Y = Bottom + (Top-Bottom)/(1 + exp((V50-X)/Slope)). The V50 value (time at which LDH release was half maximal) was 10.68 h. Since the chamber volume is 5 ml with filter carriers, it should take at least 5 h before the chamber concentration of SDS reaches the inflow concentration of 173 µM. Thus, correcting for the lag of concentration accumulation, V50 = 5.68 h. After a maximum toxicity (plateau) at 26 h, the LDH release fell again until 80 h.

3.4 LLC-PK1 cells exposed to perfused CsA

When LLC-PK1 cells were perfused with CsA, a dose- and time-dependent elevation of effluent LDH and GGT activity was detected (Fig. 4). The onset of the release of GGT was slightly ahead of the onset of LDH release. This would be expected, considering that GGT is located at the brush border. LDH release was prolonged as compared to GGT release.

Tab. 1 and 2: IC_{50} values
HK-2 cells were treated with APAP and DMSO for 24 h and 72 h under perfusion and static culture conditions. Log IC_{50} values (50% inhibitory concentration) were generated using a sigmoidal dose response curve (variable slope) using the following equation Y = Bottom + (TopBottom) / (1+10^ ((LogEC_{50}-X) *HillSlope)). No statistical significance was found between static and perfusion culture conditions (analysed using an unpaired t-test). SEM values represent standard error of IC_{50} generation and not experimental variation. (Analysis does not permit the calculation of SEM for non-log values). "nd" denotes "not determined" and "nc" denotes "no convergence".

Tab. 1: Paracetamol (APAP) IC_{50} data

	24 h exposure Log IC_{50} (mM)	mM	72 h exposure Log IC_{50} (mM)	mM
Alamar Blue assay				
Static culture	1.43 ± 0.62	27.0	1.01 ± 0.04	10.3
Perfusion culture	1.32 ± 0.06	20.8	1.03 ± 0.03	10.6
LDH activity per filter				
Static culture	1.10 ± 0.06	12.5	0.56 ± 0.13	3.6
Perfusion culture	0.92 ± 0.16	8.2	0.80 ± 0.10	6.2
GGT activity per filter				
Static culture	n.c.		0.99 ± 0.13	9.8
Perfusion culture	1.17 ± 1.731	14.7	0.91 ± 0.13	8.1
Protein per filter				
Static culture	1.24 ± 0.89	17.4	0.67 ± 0.04	4.4
Perfusion culture	1.14 ± 0.22	13.8	0.92 ± 4.7	8.3

Tab. 2: DMSO IC_{50} data

	24 h exposure Log IC_{50} (mM)	mM	**72 h exposure** Log IC_{50} (mM)	mM
Alamar Blue assay				
Static culture	2.65 ± 0.01	449	2.56 ± 0.04	367
Perfusion culture	2.38 ± 0.01	237	2.59 ± 0.03	392
LDH activity per filter				
Static culture	2.58 ± 0.01	383	2.33 ± 0.11	214
Perfusion culture	2.32 ± 0.12	207	2.53 ± 0.01	335
GGT activity per filter				
Static culture	n.d.		2.39 ± 0.01	243
Perfusion culture	n.d.		2.18 ± 0.08	151
Protein per filter				
Static culture	2.56 ± 0.08	361	2.50 ± 0.85	316
Perfusion culture	2.48 ± 0.45	299	2.53 ± 1.08	340

4 Discussion

4.1 Perfusion and static toxicity testing and IC_{50} generation

These experiments were carried out to determine whether there is an alteration in sensitivity of cell toxicity under perfusion in comparison to static culture conditions. Two different test substances were used, DMSO, a relatively non-toxic organic solvent, and paracetamol (APAP), a well-characterised hepatic and renal toxicant. APAP was more toxic at 72 h than at 24 h. This is a similar finding to one previously reported (Riddell et al., 1986) and demonstrates the importance of incubation time for *in vitro* toxicity assays. There was no significant difference between APAP toxicity in

the static and perfusion culture models at 24 h or 72 h for any of the end-points analysed (Tab. 1). APAP is reported to exhibit toxicity above 2 mM plasma concentration in humans (Forrest et al., 1982). Here, we report an IC_{50} of 10 mM using the Alamar Blue assay, with toxicity beginning above 2.5 mM (Fig. 1).

Unlike for APAP, there was no time-related difference in DMSO toxicity. 3162 mM and 1000 mM DMSO resulted in significant toxicity at 24 and 72 h. Since DMSO is a non-specific toxin there appears to be a threshold value between 100 mM and 316 mM, which is constant for different cultured cells. 1% (140 mM) DMSO has been used in the culture medium of primary hepatocytes to improve differentiated function (Isom et al., 1985; Zurlo and Arterburn, 1996). There was no significant difference between DMSO toxicity under static or perfusion culture conditions (Tab. 2). There is not sufficient data on DMSO toxicity in man for a direct comparison.

It is worth considering that absolute exposure to both toxins was different for perfusion and static experiments. In the static set-up, each filter was provided a volume of 3 ml and in perfusion each filter was exposed to 4 ml over 24 h and 12 ml over 72 h (6 filters per chamber with a flow rate of 1 ml/h). Thus the exposure ratio for 24 h was 1:1.33 and for 72 h was 1:4, static to perfusion respectively, but there was no noticeable increase of toxicity under perfusion conditions for DMSO or APAP. APAP is reabsorbed in the renal tubules by simple diffusion (Duggin and Mudge, 1975). Thus the intracellular concentration of APAP is in equilibrium with the extracellular concentration (Iida et al., 1989). The provision of 3 ml of medium containing APAP may be enough to keep intracellular levels of APAP constant over the 72 h interval and thus concentrations under static and perfusion conditions remain comparable. It is unlikely that DMSO will be concentrated within cells and thus a critical concentration rather than absolute amount would be expected for toxicity.

The inter-laboratory comparison of Alamar Blue end-point data from 3 independent laboratories executing the experiments in the same way showed very similar results (Fig. 2), and demonstrates the success of standardisation of methodologies.

4.2 Real time measurement of toxicity

The assessment of toxicity under perfusion conditions allows a unique avenue of toxicological evaluation, which is not possible with conventional methods of cell

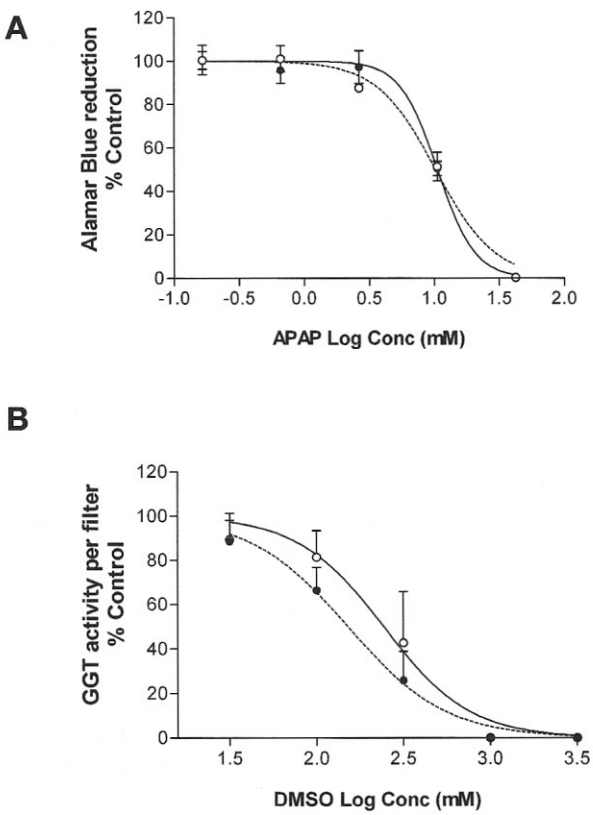

Fig. 1: Two examples of the generated IC_{50} curves.
(A) End-point Alamar Blue evaluation of HK-2 monolayers exposed to a concentration range of paracetamol (APAP) under static culture (o) and perfusion culture (=) conditions. (B) Remnant GGT activity per filter of HK-2 monolayers exposed to a concentration range of DMSO for 72 h static culture (o) and perfusion culture (=) conditions.

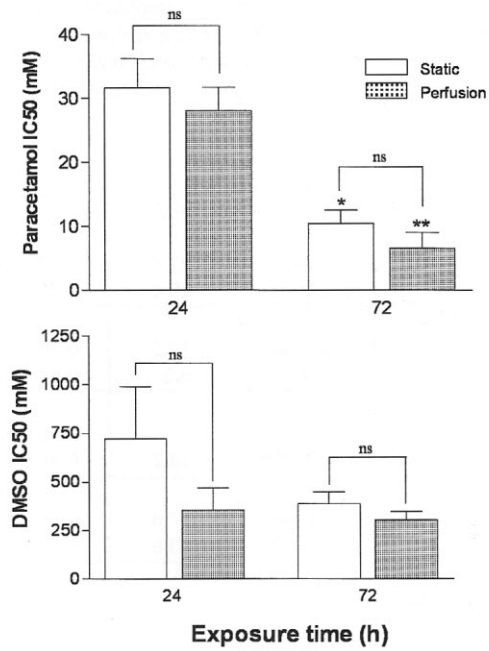

Fig. 2: Inter-laboratory comparison from 3 independent laboratories showing IC_{50} measurements of paracetamol (A) and DMSO (B) in the human kidney cell line HK-2 under both perfusion and static conditions.
Each value represents the IC_{50} calculated from the mean Alamar Blue data from 3 different laboratories (each experiment was carried out 3 times with 3 to 6 replicates). Error bars represent the standard error between laboratories. IC_{50}s were calculated with a sigmoidal dose response curve (variable slope) using the following equation Y = Bottom + (TopBottom) / (1+10^ ((LogEC_{50}-X) *HillSlope)). Data was analysed for statistical significance using one- way ANOVA and Tukey's post test. * and ** represent statistical significance versus 24 h exposure with $p < 0.05$ and 0.01 respectively.

culture and cell toxicity, i.e. real time determination of cell damage. In *in vivo* toxicological evaluation, urinary or blood-borne organ-specific markers are utilised to determine onset, quantity and duration of toxic damage. Under *in vivo* conditions the specificity of the marker for the organ or tissue of interest is an obvious prerequisite. Urinary markers such as gamma glutamyl transpeptidase (GGT) (Donadio et al., 1998), alpha glutathione S-transferase (Kilty et al., 1998) and fructose 1,6 bisphosphatase (Pfaller et al., 1994) have been used to sensitively determine real time damage of proximal tubular cells in humans. Perfusion culture allows the possibility of similar determinations with the advantage that the specificity of the marker measured is not critical since there are no higher organ systems.

SDS at 173 µM (50 µg/ml/h), but not at lower concentrations, resulted in almost immediate damage to the cells, evidenced by the detection of elevated LDH activity in the perfusion out-flow from as early as 6 h (Fig. 3). The ascent of effluent LDH activity fitted well to a sigmoidal plot, reaching a plateau at 26 h. Effluent LDH activity fell sharply over the next few hours, then levelled out and gradually reached baseline at 80 h. Morphological examination of the cell monolayers after 80 h (3 days) demonstrated complete monolayer destruction at 173 µM SDS exposure where cells exposed to 34.6 µM and 6.9 µM SDS and control monolayers were intact (not shown).

CsA at 83 µM (100 µg/ml/h) and 8.3 µM (10 µg/ml/h) resulted in elevated effluent LDH activity beginning at 26 h (Fig. 4). CsA also induced increases in effluent GGT activity (Fig. 4). Effluent GGT was present at above basal concentration at earlier time points than effluent LDH and returned to basal levels sooner. Since GGT is an integral brush border membrane protein (Pfaller et al., 1984), it would be expected to be released following damage to membrane integrity prior to cytosolic proteins, such as LDH. It has been previously shown that treatment of rats with CsA (50 mg/kg) induced elevated urinary GGT activity (Metz-Kurschel et al., 1990; Selvam and Adhirai, 1997). Interestingly, even though not all the cells had been damaged at higher doses of CsA (as evidenced by phase contrast evaluation of the cell monolayers in Fig. 5), the elevated effluent LDH and GGT activity returned to basal levels at 4 and 3 days respectively. This may be a result of CsA-induced toxico-tolerance. Mechanisms of such tolerance may involve increasing the extrusion of CsA via P-glycoprotein or the induction of stress proteins. CsA increases the expression of P-glycoprotein in rat proximal tubular cells in a time and dose dependent manner (Hauser et al., 1998). CsA also induces the expression of HSP 70 in LLC-PK_1 cells, which increases tolerance to subsequent exposure to CsA (Yuan et al., 1996).

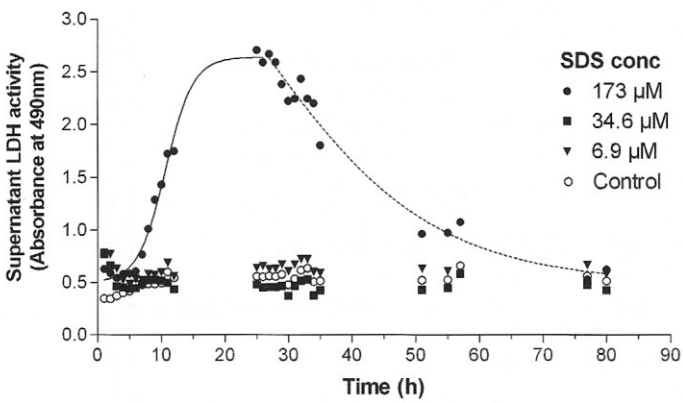

Fig. 3: The effect of continuous exposure of SDS on lactate dehydrogenase (LDH) release from LLC-PK1 cells.
LLC-PK1 cells were grown to confluence on collagen-coated aluminium oxide filters and transferred to perfusion chambers. SDS-containing medium was perfused at 1 ml/h for 80 h. Each perfusion chamber contained 6 filter carriers. LDH activity was assayed in effluent medium and expressed as arbitrary absorbance units as a function of time. Time 0 coincides with SDS entry into the perfusion chamber. Continuous line was calculated using a Boltzmann sigmoidal fit [equation: Y=Bottom+(Top-Bottom)/((1+exp.(V50-X)/Slope))] plotted from 0 to 26 h (r^2 = 0.989). Discontinuous line was also calculated using a Boltzmann sigmoidal fit plotted from 26 to 80 h (r^2 = 0.974).

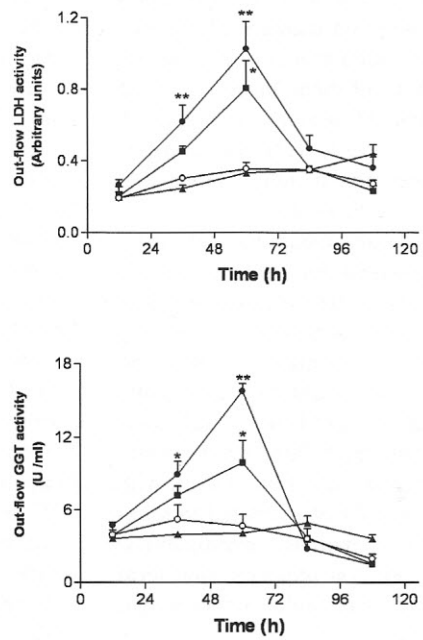

Fig. 4: The effect of continuous exposure to cyclosporine A (CsA) on lactate dehydrogenase (LDH) and gamma-glutamyl transferase (GGT) release from LLC-PK1 cells.
LLC-PK1 cells were grown to confluence on collagen-coated aluminium oxide filters and were transferred to perfusion chambers where they were continually exposed to 83µM (●), 8.3µM (■), 0.83µM (▲) and 0µM CsA (○) at 1 ml/h for a duration of 120 h. The effluent medium was assayed for LDH and GGT activity. Time 0 coincides with CsA entry into the perfusion chamber. Values were compared to time 0 using one-way analysis of variance with Dunnett's post-test, where * represents $p < 0.05$ and ** represents $p < 0.01$.

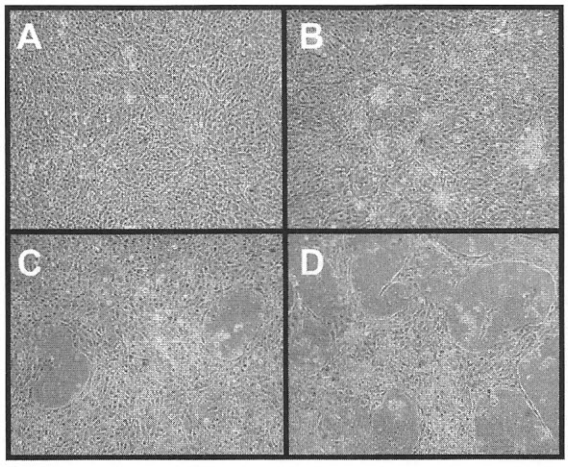

Fig. 5: Phase contrast morphology of LLC-PK1 continuously exposed to CsA for 120 h.
See legend of Figure 4 for details. A) Control, B) 0.83 µM CsA, C) 8.3 µM CsA and D) 83 µM CsA.

Measurement of real time toxicity *in vitro* is thus possible with the perfusion system. LDH and GGT are released in sufficient amounts from the perfusion effluent to be good markers of cell viability online. Such monitoring is advantageous when information on the onset and duration of toxicity is required. Moreover, *in vitro* toxicity data generated in this way may be more comparable to respective *in vivo* data.

The perfusion apparatus optimised here has potential for *in vitro* chronic nephrotoxicity testing. Perfusion offers the only *in vitro* alternative to carrying out long-term continuous or repeated low-dose chronic nephrotoxicity testing. However, this system is not readily suitable to medium/high throughput screening in its current state. Future work with this system should be restricted to long-term (weeks to months) investigations, which are based on previous short-term studies conducted under conventional culture conditions.

References

Donadio, C., Tramonti, G., Lucchesi, A. et al. (1998). Gamma-glutamyltransferase is a reliable marker for tubular effects of contrast media. *Ren. Fail. 20*, 319-324.

Duggin, G. G. and Mudge, G. H. (1975). Renal tubular transport of paracetamol and its conjugates in the dog. *Br. J. Pharmacol. 54*, 359-366.

Forrest, J. A., Clements, J. A. Prescott, L. F. (1982). Clinical pharmacokinetics of paracetamol. *Clin. Pharmacokinet. 7*, 93-107.

Hauser, I. A., Koziolek, M., Hopfer, U. and Thevenod, F. (1998). Therapeutic concentrations of cyclosporine A, but not FK506, increase P-glycoprotein expression in endothelial and renal tubule cells. *Kidney Int. 54*, 1139-1149.

Iida, S., Mizuma, T., Sakuma, N. et al. (1989). Transport of acetaminophen conjugates in isolated rat hepatocytes. *Drug Metab. Dispos. 17*, 341-344.

Isom, H. C., Secott, T., Georgoff, I. et al. (1985). Maintenance of differentiated rat hepatocytes in primary culture. *Proc. Natl. Acad. Sci. U S A 82*, 3252-3256.

Koppelstaetter, C., Jennings, P., Pfaller, W. et al. (2004) Assessment of a new cell culture perfusion apparatus for in vitro chronic toxicity testing. Part 1: Technical description. *ALTEX 21*, 51-60.

Kilty, C., Doyle, S., Hassett, B. and Manning, F. (1998). Glutathione S-transferases as biomarkers of organ damage: applications of rodent and canine GST enzyme immunoassays. *Chem. Biol. Interact. 111-112*, 123-135.

Metz-Kurschel, U., Wagner, K. and Mueller, M. K. (1990). The synthetic prostaglandin E1 analogue rioprostil reduces the nephrotoxic potential of cyclosporine A. *Transplant. Proc. 22*, 1713-1716.

Pfaller, W., Gstraunthaler, G., Kotanko, P. et al. (1984). Immunocytochemical localization of gamma-glutamyl-transferase on isolated renal cortical tubular fragments. *Histochemistry 80*, 289-293.

Pfaller, W., Thorwartl, U., Nevinny-Stickel, M. et al. (1994). Clinical value of fructose 1,6 bisphosphatase in monitoring renal proximal tubular injury. *Kidney Int. Suppl. 47*, S68-75.

Riddell, R. J., Panacer, D. S., Wilde, S. M. et al. (1986). The importance of exposure period and cell type in in vitro cytotoxicity tests. *ATLA 14*, 86-92. (Abstract)

Selvam, R. and Adhirai, M. (1997). Vitamin E pretreatment prevents cyclosporin A-induced crystal deposition in hyperoxaluric rats. *Nephron. 75*, 77-81.

Yuan, C. M., Bohen, E. M., Musio, F. and Carome, M. A. (1996). Sublethal heat shock and cyclosporine exposure produce tolerance against subsequent cyclosporine toxicity. *Am. J. Physiol. 271*, F571-578.

Zurlo, J. and Arterburn, L. M. (1996). Characterization of a primary hepatocyte culture system for toxicological studies. *In vitro Cell. Dev. Biol. Anim. 32*, 211-220.

Correspondence to

Dr. Paul Jennings
Institute of Physiology
University of Innsbruck
Labor Pfaller
Fritz-Pregl Strasse 3
A-6020 Innsbruck
Austria
e-mail: paul.jennings@uibk.ac.at