Development of an *In Vitro* Model for Vascular Injury with Human Endothelial Cells

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Summary
The aim of the present work was to establish an in vitro screening assay for drug candidates using human endothelial cells as a model for vascular injury after intravenous application. Different endpoints for viability and functionality of endothelial cells were investigated in human umbilical vein endothelial cells (HUVEC) and in immortalised human endothelial cells (IVEC). Cellular viability was determined by measuring ATP content and by the AlamarBlue assay. For comparison, the toxicity of the selected compounds was also tested in a murine fibroblast cell line (3T3 cells). Selected endpoints for endothelial cell-specific function were vascular permeability, determined by measurement of the transendothelial resistance and the diffusion of tracer molecules (FITC-dextran), and the release of prostaglandin und thromboxane as indicators for prothrombotic or vasoconstrictory action. Five compounds (cyclosporin A, mitomycin C, menadione, amrinone and rolipram) were selected due to their known effects on the vasculature. The cytotoxicity of all compounds was similar in endothelial and 3T3 cells. ATP content and AlamarBlue metabolism did not differ significantly except for amrinone. A dose-dependent decrease of transendothelial resistance and an increase in FITC-dextran permeability could be measured in HUVEC cells for the tested compounds, but the sensitivity was not higher than that of the cytotoxicity assays. Increased prostaglandin or thromboxane release was detected for all compounds at cytotoxic concentrations and for rolipram also at non-toxic concentrations. In conclusion, for a first ranking of drug candidates, cytotoxicity assays on any of the three cell types used are appropriate. For a more detailed characterisation of individual compounds, functional assays on HUVEC cells are proposed.

Zusammenfassung: Vorschlag für ein *in vitro* Modell zur Bestimmung der vaskulären Toxizität an humanen Endothelzellen
Ziel der vorliegenden Arbeiten war es, einen in vitro Test an humanen Endothelzellen als Modell für vaskuläre Toxizität nach intravenöser Gabe für das Screening von Arzneimittelkandidaten zu etablieren. Verschiedene Endpunkte zur Bestimmung der Viabilität und Funktion von Endothelzellen wurden an humanen Endothelzellen aus der Umbilikalvene (HUVEC-Zellen) und an immortalisierten humanen Endothelzellen (IVEC-Zellen) untersucht. Die Zellviabilität wurde mittels des ATP-Gehalts und des AlamarBlue Tests ermittelt. Zum Vergleich wurde die Toxizität an einer murinen Fibroblastenzelllinie (3T3-Zellen) getestet. Zum Nachweis der Funktionalität der Endothelzellen wurde die vaskuläre Permeabilität mittels Messung des transendothelialen Widerstands und der Diffusion von Tracermolekülen (FITC-Dextran), sowie die Prostaglandin- und Thromboxanfreisetzung als Indikator für eine prothrombotische und vasokonstriktorische Wirkung von Substanzen untersucht. Fünf Testsubstanzen (Cyklosporin A, Mitomycin C, Menadion, Amrinone und Rolipram) wurden aufgrund ihrer bekannten Wirkung auf Gefäße ausgesucht. Die Zytotoxizität der Modellsubstanzen war für Endothelzellen und 3T3-Zellen ähnlich. Die beiden Endpunkte ATP-Gehalt und AlamarBlue-Umsetzung unterschieden sich mit Ausnahme der Testsubstanz Amrinone nicht. Eine dosisabhängige Abnahme des transendothelialen Widerstandes und eine Zunahme der Permeabilität konnte in HUVEC-Zellen gemessen werden. Diese beiden Endpunkte wiesen jedoch keine höhere Empfindlichkeit als die Zytotoxizität auf. Ein Anstieg der Prostaglandin- oder Thromboxanfreisetzung zeigte sich bei allen Substanzen bei zytotoxischen Konzentrationen, für Rolipram auch bei niedrigeren Dosen. Für ein erstes Ranking von möglichen Arzneimittelkandidaten bietet sich daher ein Zytotoxizitätstest an einer der drei Zellarten an. Für eine genauere Charakterisierung einzelner Substanzen werden funktionelle Messungen an HUVEC-Zellen vorgeschlagen.

Keywords: in vitro, endothelial cells, HUVEC, cytotoxicity, transendothelial permeability

1 Introduction

A large number of drugs must be administered by the intravenous route because fast onset of action is desired or due to limited bioavailability by other routes of administration. A disadvantage of intravenous dosing is the potential to create high local concentrations of the compound in the blood vessel. Hence local intolerance reactions can occur. This is reflected in the guidelines asking for local tolerance testing in laboratory animals prior to human use (EMEA, 2001). Of course compounds can also exert vascular injury distant to the injection site. Toxicity of a xenobiotic to the vas-

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culature can result e.g. in vascular leakage, hypertension, inflammation or the induction of thrombosis (Boor et al., 1995; Hoorn et al., 1995; Idirs et al., 2003; Remuzzi and Bertani, 1989).

The aim of the present work was to establish an *in vitro* assay to determine the local tolerance of test compounds after intravenous application as well as vascular injury in general. This assay is thought primarily for the screening of drug candidates to reduce the number of compounds tested in the necessary ensuing *in vivo* experiments. One of the problems in establishing an *in vitro* assay for vascular injury is the complexity of the interactions of the different constituents of the vasculature and the blood that are involved in the occurrence of vascular toxicity. Blood vessels comprise three layers: the intima made up of a monolayer of endothelial cells, the media consisting mainly of smooth muscle cells and the adventitia made of fibrous tissue (Boor et al., 1995). The endothelium as the innermost layer has not only a barrier function between the blood and the organs, it also plays a major role in controlling the transfer of nutrients, hormones and white blood cells and is involved in the control of the blood pressure, the blood fluidity and coagulation reactions.

Besides the cells of the blood, the endothelial cell is the cell type that is exposed to the highest concentration of the drug substance and is consequently especially susceptible to a potential toxic action of a drug applied intravenously. Therefore, in a first approach, the assay system should investigate the effects of test compounds on endothelial cells *in vitro*.

In order to reflect the complexity of endothelial damage, two kinds of endpoints were investigated: cell viability and endothelial-specific function after treatment with a test compound. Cell viability was studied in three cell types. Human umbilical vein endothelial cells (HUVEC) were used as an *in vitro* model of human endothelial cells. However, HUVEC cells are primary cells with a finite life span, which might display batch to batch differences, even though cells pooled from different donors are used in these experiments. Therefore, an immortalised venous endothelial cell line (IVEC) was also investigated. This cell line was obtained by transfection of human umbilical vein endothelial cells with large T and small t antigen DNA of the SV40 virus (Vicart et al., 1993). The murine fibroblast cell line Balb/3T3 was examined for comparison with the endothelial cells. The viability of endothelial and fibroblast cells was measured by two methods, both based on the detection of the metabolic activity of the cells. In the ATP assay, the ATP content of the cells is determined via a luminescent signal produced by an ATP-dependent luciferase reaction. The AlamarBlue Assay uses an oxidation-reduction indicator that is reduced to a fluorescent form in the reducing environment of growing cells.

The permeability of the endothelial monolayer and mediator release were selected as endpoints for the functionality of endothelial cells. Since one of the possible adverse effects on blood vessels is increased vascular leakage, the permeability of the endothelial monolayer to ions and macromolecules was determined by measuring the transendothelial resistance and the diffusion of the tracer molecule FITC-dextran (Jiang et al., 1999).

Furthermore, two important mediators synthesised by endothelial cells were investigated: prostacyclin (prostaglandin I_2, PGI_2), a vasodilator and potent inhibitor of platelet aggregation, and thromboxane A_2 (TXA_2), which induces vasoconstriction and platelet aggregation. Since both prostacyclin and TXA_2 have a short half-life of 30 sec or a few minutes respectively, their stable hydrolysed metabolites 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and thromboxane B_2 (TXB_2) were measured (Moncada and Vane, 1978).

The test compounds used were selected due to their known toxic effects on the vasculature. Intravenous administration of the DNA cross-linking anticancer drug mitomycin C is known to induce veno-occlusive disease in lung or liver. The inhibition of proliferation and direct cytotoxicity towards endothelial cells are discussed as underlying mechanisms (Lazarus et al., 1982; Hoorn et al., 1995). The immunosuppressive agent cyclosporin A induces vasoconstriction and renal damage characterised amongst others by thrombotic microangiopathy (Mason et al., 1990; Remuzzi and Bertani, 1989). Menadione (Vitamin K3), a cofactor of prothrombin synthesis given intravenously for hypothrombinemia, osteoporosis and as an anticancer drug, has been shown to induce glutathione depletion, vasoconstriction and endothelial cell toxicity (Lee et al., 1999; McAmis et al., 2003). Finally, two phosphodiesterase (PDE) inhibitors were selected for this study, the PDE 3 inhibitor amrinone and the PDE 4 inhibitor rolipram. PDE 3 and 4 inhibitors are known to result in vasculitis in animal models (Larson et al., 1996; Joseph et al., 1996) but the relevance of these findings for humans is uncertain.

2 Materials and methods

2.1 Chemicals

Cyclosporin A was obtained from Biomol (Hamburg, Germany), mitomycin C from Roche Diagnostics (Mannheim, Germany), amrinone and menadione from Sigma (Steinheim, Germany) and rolipram from Alexis (Lausen, Switzerland).

2.2 Cell culture

HUVEC cells (CellSystems, St. Katherinen, Germany) were cultivated on gelatinised (0.5%) cell culture dishes in EGM medium (EBM medium supplemented with the EGM singlequots fetal bovine serum, hEGF, hydrocortisone and bovine brain extract) (CellSystems). IVEC cells (Vicart et al., 1993) were cultured in MEM medium (Gibco) with 10% fetal calf serum (FCS, Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin, also on gelatine-coated (0.5%) cell culture dishes. Balb/3T3 cells (clone A31, ATCC, Rockville, USA) were grown in high glucose (4.5 g/l) DMEM medium (Gibco), 10% FCS, 4 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. When cells had grown confluent they were passaged by trypsinisation. HUVEC cells were used for experiments up to passage 8, IVEC cells were used between passage 25 and 35 and 3T3 cells up to passage 25.

2.3 Cytotoxicity assays

HUVEC, IVEC or 3T3 cells were seeded into 96 well plates at a density of 1 x 10^4 cells/well in 200 µl of the respective culture medium. Opaque-walled 96 well plates were used for the ATP assay. The cells were allowed to adhere for one day. Then medium was removed and replaced with medium containing the test compound in medium with 1% DMSO (6 wells per concentration, control wells with 1% DMSO) and cells were incubated for 24 h.

For the AlamarBlue™ Assay (Serotec, Düsseldorf, Germany) the medium was removed and 100 µl of 5% AlamarBlue reagent in medium were added. After incubation for 1 h at 37°C, fluorescence was measured at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The ATP content was determined with the CellTiter-Glo™ Luminescent Cell Viability Assay (Promega, Madison, US) according to the manufacturer's procedure.

2.4 Transendothelial resistance

HUVEC and IVEC cells were seeded onto the gelatine-coated microporous membrane of transwell plates (pore diameter 0.4 µm, insert growth area 1 cm^2) (Costar, Bodenheim, Germany) at a density of 2 x 10^5 cells/well and cultured with 0.5 ml of medium in the upper compartment and 1.5 ml in the lower compartment. Cells were grown to confluence with a change of medium every two to three days. The electrical resistance between the upper (apical) and lower (basolateral) compartment was measured using a resistance meter (Millicell-ERS, Millipore, Bedford, US) approximately every third day. The resistance of the cell monolayer was calculated by substracting the mean of wells without cells from the measured resistance in the sample wells: $R_{cell\ monolayer} = R_{sample} - R_{mean\ blanks}$. When cells reached confluence and a constant resistance, they were treated with test compounds (in medium with 1% DMSO, in the upper compartment only; 3 wells per concentration) and electrical resistance was measured 1, 5, 10 and 24 h after addition of the compound.

2.5 Permeability assay

Endothelial cells were grown on transwells and treated with test compounds as described above. After 24 h of incubation, medium was renewed in both compartments under addition of 0.1 mg/ml FITC-dextran (Av MW 21200, Sigma) to the upper compartment. After 1, 4 and 8 h 200 µl of medium were taken from the lower compartment and replaced by the same volume of fresh medium. Fluorescence was measured in the samples at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. IC_{50} values were determined as the concentration showing a permeability P of 50% ($P_{blanks} - P_{controls}$).

2.6 Prostacyclin and thromboxane A2 release

HUVEC cells were incubated with the test compounds as described for the cytotoxicity assays. After 24 h the cell culture medium was collected and stored at -20°C until further use. Prostacyclin and thromboxane A_2 release into the medium were monitored by measurement of their stable derivates 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and thromboxane B_2 (TXB_2) by ELISA using the respective Assay Designs Correlate-CLIA™ kits (Biotrend, Köln, Germany) according to the manufacturer's instructions.

3 Results

3.1 Cytotoxicity

The cytotoxicity of the five test compounds in HUVEC, IVEC and 3T3 cells after 24 h incubation was determined by the AlamarBlue assay and the ATP assay. The concentration response curves were similar for both endpoints (shown for mitomycin C in Fig. 1; IC_{50} values see Tab. 1). Only for amrinone the AlamarBlue assay was more sensitive than the ATP assay in all three cell types. Complete cell death could not be reached with amrinone or cyclosporin A due to solubility reasons. The IC_{50} values of the tested compounds were in the same range for all three cell types (Fig. 2 and Tab. 1).

3.2 Transendothelial resistance

HUVEC cells exhibited a steady resistance of 25.0 ± 7.6 $\Omega \cdot cm^2$ (n=90) after approximately 10 days in culture. In contrast IVEC cells showed less than half that resistance with 11.3 ± 7.4 $\Omega \cdot cm^2$ (n=72) even after 12 days in culture. Since the blank values were in the range of 116.6 ± 7.2 $\Omega \cdot cm^2$ (n=30), significant changes between blanks and cells or treated and untreated cells could not be measured with a manageable number of wells for IVEC cells.

Treatment of HUVEC cells with test compounds resulted in a concentration- and time-dependent decrease of transendothelial resistance. The earliest decrease in TER was seen after 5 to 24 hours, depending on compound and concentration. Since the most marked decrease was detected after 24 h, the 24 h-values were used for further calculations. Figure 2 shows the dose-dependent decrease of resistance for the example mitomycin C. IC_{50} values are

Tab. 1: Cytotoxicity of amrinone, cyclosporin A, rolipram, menadione and mitomycin C on HUVEC, IVEC and 3T3 cells.
The table shows the IC_{50} values after 24 h incubation measured by AlamarBlue and ATP assay (mean of ≥ 2 experiments).

	Cyclosporin A		Mitomycin C		Menadione		Amrinone		Rolipram	
IC_{50} (µg/ml)	AlamarBlue	ATP	AlamarBlue	ATP	AlamarBlue	ATP	AlamarBlue	ATP	AlamarBlue	ATP
HUVEC	22	22	14	13	2.1	2.8	83	>550	250	170
IVEC	13	11	13	15	1.6	1.6	73	103	400	315
3T3	20	16	19	n.d.	1.0	1.0	130	475	250	120

n.d.: not determined

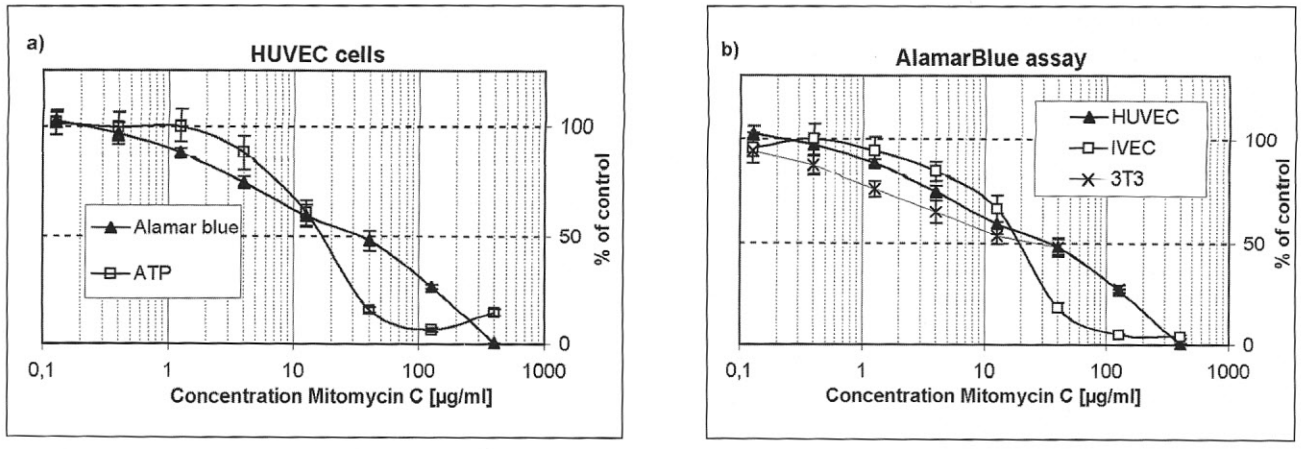

Fig. 1: Exemplary concentration-response curves for a) the endpoints AlamarBlue reduction and ATP content in HUVEC cells and b) the endpoint AlamarBlue reduction in HUVEC, IVEC and 3T3 cells after treatment with mitomycin C.

summarised in Table 2. In the case of rolipram, no significant decrease was seen up to the highest concentration tested (750 µg/ml).

3.3 Permeability assay

The amount of FITC-dextran permeating the microporous membrane increased almost linearly with time within the observed 8 hours in blank wells or wells with cells (data not shown). Since the 8 h values gave the best discrimination between doses, these were used for further calculations. The FITC-dextran permeability in a confluent layer of HUVEC cells was approximately 13% of that in blank wells. Treatment with test compounds resulted in a dose-dependent increase of permeability (shown for mitomycin C in Fig. 3; IC_{50} values are summarised in Tab. 2). Similar to the measurement of TER, no effect was detected for rolipram up to the highest concentration tested. In contrast, confluent IVEC cells revealed a much higher permeability for FITC-dextran (approx. 50% compared to blank wells) than the HUVEC cells. Although a time- and dose-dependent increase of permeability could also be detected in IVEC cells after treatment with compounds, the separation of different treatment conditions was less clear than for HUVEC cells (data not shown).

3.4 Prostacyclin and thromboxane A_2 release

Treatment of HUVEC cells with all tested compounds resulted in a dose-dependent increase of 6-keto-$PGF_{1\alpha}$ and/or TXB_2 release. This increase usually runs in parallel with a decrease of cell viability, but was increased also at non-toxic concentrations as in the case of rolipram. For mitomycin C, a strong increase in 6-keto-$PGF_{1\alpha}$ release was noted, but TXB_2 release remained constant despite cytotoxicity (Fig. 4, Tab. 2).

4 Discussion

Both the two endothelial cell types, HUVEC and IVEC cells, as well as the 3T3 fibroblast cells are suitable models to test the cytotoxicity of compounds. For the few compounds tested almost identical IC_{50} values were obtained in all three cell types and no clear endothelial cell-specific toxicity could be observed. Also the endpoint AlamarBlue reduction generally had a similar sensitivity to the ATP assay or was slightly more sensitive

Tab. 2: IC_{50} values for cytotoxicity (AlamarBlue assay), transendothelial resistance and FITC-dextran permeability as well as concentrations leading to doubling of the release of 6-keto-$PGF_{1\alpha}$ and TXB_2 in HUVEC cells after 24 h incubation with test compounds.

IC_{50} (µg/ml)	Cytotoxicity IC_{50}	TER IC_{50}	Permeability IC_{50}	6-k$PGF_{1\alpha}$ 200%	TXB_2 200%
cyclosporin A	22	35	200	20	>250
mitomycin C	14	20	140	>400	20
menadione	2	4	5	20	>20
amrinone	83	500	>650	170	170
rolipram	250	>750	>750	60	400

">": IC_{50} value could not be reached up to highest concentration tested (limiting factor: mainly solubility of compound)

in the case of amrinone. This is in concordance with data from the MEIC (Multicenter Evaluation of In vitro Cytotoxicity programme) study which showed a high correlation of results from *in vitro* cytotoxicity assays with different cell types and different viability endpoints (Clemedson and Ekwall, 1999).

HUVEC cells grown to confluence displayed a transendothelial resistance of ~ 25 $\Omega \cdot cm^2$. This is in the range of published data on resistance of non-brain endothelial systems including HUVEC cells of 1-30 $\Omega \cdot cm^2$ (Dye et al., 2001), but significantly below that of brain endothelial cells (several hundred $\Omega \cdot cm^2$, Rutten et al., 1987) or epithelial cell types (e.g. CaCo-2 cells ~ 200 $\Omega \cdot cm^2$, own observation). The relatively low resistance and high variability of the measurement, caused for example by the dependency of the resistance on temperature and the angle of electrodes, necessitate highly accurate handling to perform the assay. Compared to HUVEC cells, IVEC cells revealed a lower resistance of ~ 11 $\Omega \cdot cm^2$, so that combined with the relatively high variability, IVEC cells were not suitable to measure significant changes between treated and untreated cells. Also, the permeation of FITC-dextran was significantly higher in IVEC cells than in HUVEC cells. Transendothelial permeability to ions, macromolecules and cells through paracellular clefts is regulated by tight junctions and adherence junctions. These are similar to those in the corresponding epithelial cells, but typically less organised in endothelial cells, reflecting the generally higher permeability of endothelial monolayers (Dye et al, 2001). The immortalisation process in IVEC cells was most probably associated with a loss of organised cell-cell junctions, resulting in increased permeability. This is in concordance with other differences detected between HUVEC and IVEC cells, e.g. altered expression of the IL-1 receptor (Wautier et al., 1999).

Treatment of HUVEC cells with the compounds resulted in a time- and dose-dependent decrease in the transendothelial resistance. However, at least for the five compounds tested, this endpoint was not more sensitive than the endpoint cytotoxicity. In contrast,

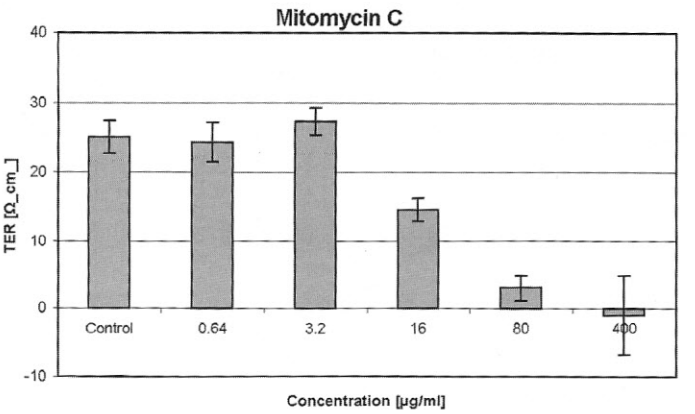

Fig. 2: Transendothelial resistance of HUVEC cells measured 24 h after addition of different doses of mitomycin C. Values are means of three wells for treated and 30 wells for control cells an blanks ± SD.

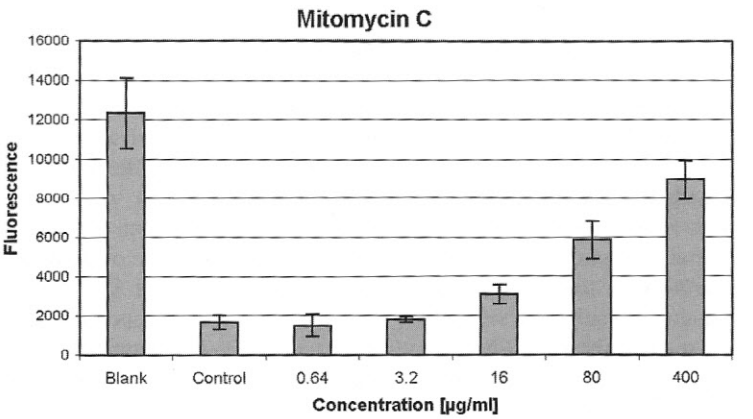

Fig. 3: Permeability for FITC-dextran. The diagrams show the 8 h values for the fluorescence of permeated FITC-dextran to the lower compartment after treatment of HUVEC cells for 24 h with different doses of mitomycin C. Values are means of three wells for treated and 30 wells for control cells and blanks ± SD.

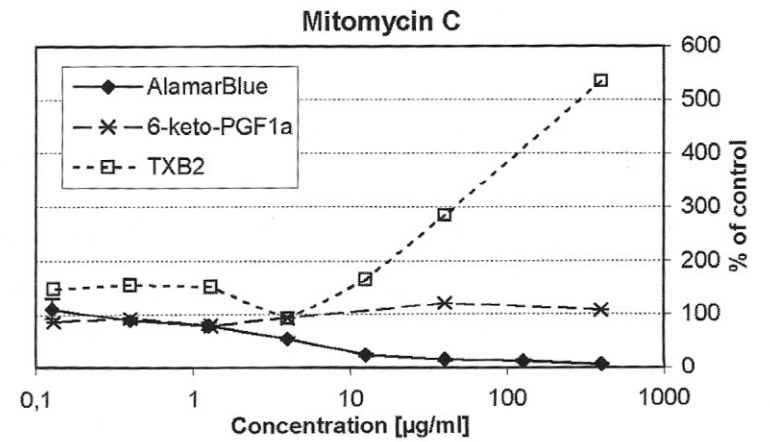

Fig. 4: Release of 6-keto-$PGF_{1\alpha}$ and TXB_2 into the medium by HUVEC cells treated with mitomycin C for 24 h compared to the cytotoxicity of the compounds measured by AlamarBlue assay.

the IC_{50} values for the transendothelial resistance (Tab. 2) were slightly higher for all test compounds than the IC_{50} values for cytotoxicity. This could at least in part be due to the fact that the test compounds were given only to the upper cell compartment of the transwells and might have had a certain dilution effect in the higher volume of medium in the lower compartment. For menadione the obtained values were in the range of published data, which describe increased permeability in rat brain barrier endothelial cells or cytotoxicity in pulmonary artery endothelial cells at comparable concentrations (15 or 100 µM, i.e. 2.6 or 17 µg/ml) (Lagrange et al., 1999; McAmis et al., 2003). No significant reduction of transendothelial resistance was noted for rolipram up to the highest dose tested. This could also be a specific effect, since it has been shown that rolipram reduces the increase in vascular permeability induced by platelet-activating factor in guinea-pig airways *in vivo* (Ortiz et al., 1992).

Similarly, a dose dependent increase of the permeability to FITC-dextran was seen for all compounds except for rolipram. The IC_{50} values (Tab. 2) were even slightly higher than those of the TER measurement. This might be explained by the fact that at moderately cytotoxic concentrations the monolayer is no longer able to retain ions but still able to retain high molecular weight molecules like FITC-dextran. Even at concentrations that caused complete cell death and abolition of TER, FITC-dextran was partially retained, possibly due to remnants of dead cells on the microporous membrane. Although the sensitivity of the TER and the permeability assay was not higher than that of the cytotoxicity assays for the tested compounds, the ranking of the IC_{50} values remained the same for all three endpoints, cytotoxicity (AlamarBue), TER and permeability: menadione < mitomycin C < cyclosporin A < amrinone < rolipram.

The higher IC_{50} values of the two PDE inhibitors rolipram and amrinone fit well with the assumption that PDE 3 and 4 inhibitors causing vasculitis exert their primary effects on vascular smooth muscle cells, causing relaxation due to increased cAMP levels (Joseph et al, 1996) in contrast to menadione, mitomycin C and cyclosporin A, for which direct toxicity on endothelial cells is discussed (Hoorn et al., 1995; Lee et al., 1999; Mason, 1990).

The release of the two vascular mediators prostacyclin and thromboxane after treatment of HUVEC cells with the test compounds was generally increased at cytotoxic concentrations, which might be partially explained by a decrease in membrane integrity resulting in the release of mediators. However specific effects can also be detected, since the two mediators are released (relative to control cells) in different ratios, depending on the compound. For mitomycin C the release of 6-keto-$PGF_{1\alpha}$ was not increased, even at concentrations causing cytotoxicity and increased release of TXB2. At least for one compound, i.e. rolipram, an activation of the endothelial cells could be detected, since an increase of 6-keto-$PGF_{1\alpha}$ release was seen at non-cytotoxic concentrations, thus showing a higher sensitivity of this endpoint. The release of TXB2 was increased only slightly. The difficulty of interpreting the measured mediator release and comparing it to the *in vivo* situation can be shown by the example of cyclosporin A. In this study, cyclosporin A increased prostaglandin release up from about 10 µg/ml more strongly than it increased thromboxane release. The literature data are conflicting for this compound, depending on the species and the model system. Whereas an increased prostacyclin release was shown e.g. in cultured rat microvascular endothelial cells at concentrations of 0.01 to 1 µg/ml (Lau et al., 1989), it was reduced in HUVEC cells at concentrations of 1 to 100 µg/ml (Voss et al., 1988). An increase of thromboxane release was detected in rat urine *in vivo* or in aortic ring *in vitro* after treatment with cyclosporin A (Oriji and Schanz, 2001; Remuzzi and Bertani, 1989), but release was depressed or only modestly increased in humans (Mason, 1990). The inhibition of induced mediator release as described for some compounds, e.g. the inhibition of stimulated prostaglandin release caused by menadione in porcine aortic endothelia cells (Barchowsky et al., 1989), was not investigated in this study.

A direct comparison of the *in vitro* results with the *in vivo* situation is complicated since local concentrations after i.v. administration are generally unknown (and the performance of adequate *in vivo* experiments would go beyond the scope of this work). Comparison to the systemic plasma levels is clearly less meaningful. Nevertheless, at least for mitomycin C and menadione, the obtained IC_{50} values are not far from the plasma levels reached at doses resulting in vascular dysfunction *in vivo*: After intravenous infusion of 60 mg/m² mitomycin C to patients over 60 min or of 30 mg/m² over 15 min, peak plasma levels of ~ 2 µg/ml were reached. Although no information on local effects are available, repeated administration at this dose is known to result in systemic vascular effects, e.g. veno-occlusive disease in the liver (Karanes et al., 1986). Considering the higher local concentrations at the injection site, the plasma levels *in vivo* are very close to the *in vitro* cytotoxic concentration of 14 µg/ml in our experiments. For menadione, clinical plasma levels of 0.5-7.4 µM have been observed (Akman et al., 1988; Lee et al., 1999). Although primary toxicity in humans *in vivo* was hemolysis (Margolin et al., 1995), menadione at a concentration of 5 µM resulted in contraction in an *ex vivo* model of isolated rat aortic rings, and in increased blood pressure in rats *in vivo* from an intravenous dose of 1 mg/kg (Lee et al., 1999). In our results, cytotoxicity was seen at only slightly higher concentrations of ~ 2 µg/ml (~ 12 µM).

In conclusion, several endpoints were investigated in endothelial cells. A cytotoxicity assay using any of the three cell types would be suitable for a first ranking of compounds. For a more comprehensive assessment of substance-related effects on endothelial cells, the functional assays such as permeability and mediator release are recommended. Transendothelial resistance and permeability can be measured in HUVEC cells whereas IVEC cells were not suitable due to the lower resistance and higher leakage. Although the sensitivity of TER and the permeability assay was not higher than for the endpoint cytotoxicity

for the few compounds tested, the measurement of the maintenance of the barrier function of endothelial cells represents an interesting functional endpoint that also allows time-dependent measurement, since both methods are non-destructive.

Clearly there are some limitations of the assays presented here. All parameters were determined after up to 24 h of incubation with the test substance. Although in the case of intravenous administration local exposure to the high concentrations is generally short, at maximum in the range of minutes, repeated dosing or continuous infusion of drugs is also performed. Furthermore, some toxicological effects are known to take longer times to develop.

The 24 h incubation time, a typical exposure time used for *in vitro* methods (Clemedson and Ekwall, 1999), was also applied here, since it presents a good compromise between the usually short exposure *in vivo* and the possibly longer time required to develop toxic effects, and as it is suitable for a fast and practicable *in vitro* screening assay for the intended application. However, longer evaluation times might be of interest and can be performed especially using the TER assay within the experiment as described above.

Moreover, further endothelial cell functions could represent interesting endpoints. Endothelial cells release several other factors which exert antithrombogenic or thrombogenic influences, depending on their state of activation or injury, e.g. the antithrombotic tissue-type plasminogen activator (t-PA) or the prothrombotic factors plasminogen activator inhibitor (PAI) and von Willebrand factor. Inflammatory parameters, such as the expression of adhesion molecules like ICAM-1 and VCAM-1 that play a key role in the adhesion of leukocytes and therefore the control of inflammation (Tervaert and Kallenberg, 1997), would be additional options.

Due to the limitation to endothelial cells of large vein origin, vessel type-specific effects could not be detected. For a comprehensive assessment of effects on the vasculature, the effects of test compounds on vascular smooth muscle cells and interactions with the different constituents of blood and vasculature would have to be investigated as well. However, this cannot be achieved in a simple *in vitro* cell culture assay.

However, in our opinion, the assays presented here are well suited for the screening of drug candidates or different formulations of one compound for possible effects on endothelial cells. With reference to the 3R principle the assay system presented here is not intended as a replacement of the *in vivo* assay. It shall rather be seen as a screening assay used to reduce the number of compounds tested in *in vivo* experiments to the most promising candidates.

References

Akman, S., Carr, B., Leong, L. et al. (1988). Phase I Trial of Menadiol Sodium Diphosphate (Synkavite) (M) in Advanced Cancer. *Proc. Am. Soc. Clin. Oncol. 7*, 24 Meet., 76.

Barchowsky, A., Tabrizi, K., Kent, R. S. and Whorton, A. R. (1989). Inhibition of prostaglandin synthesis after metabolism of menadione by cultured porcine endothelial cells. *J. Clin. Invest. 83*, 1153-1159.

Boor, P. J., Gottlieb, A. I., Joseph, E. C. et al. (1993). Chemical-induced vasculature injury. Summary of the symposium presented at the 32nd annual meeting of the Society of Toxicology, New Orleans, Louisiana, March 1993. *Toxicol. Appl. Pharmacol. 132*, 177-195.

Clemedson, C. and Ekwall, B. (1999). Overview of the final MEIC results: I. The in vitro-in vivo evaluation. *Toxicol. In Vitro 13*, 657-663.

Dye, J. F., Leach, L., Clark, P. and Firth, J. A. (2001). Cyclic AMP and acidic fibroblast growth factor have opposing effects on tight and adherens junctions in microvascular endothelial cells in vitro. *Microvasc. Res. 62*, 94-113.

EMEA (2001). Note for guidance on non-clinical local tolerance testing of medicinal products (CPMP/SWP/2145/00) of The European Agency for the Evaluation of Medicinal Products.

Hoorn, C. M., Wagner, J. G., Petry, T. W. and Roth, R. A. (1995). Toxicity of mitomycin C toward cultured pulmonary artery endothelium. *Toxicol. Appl. Pharmacol. 130*, 87-94.

Idris, I., Gray, S., Donnelly, R. (2003). Rosiglitazone and pulmonary oedema: an acute dose-dependent effect on human endothelial cell permeability. *Diabetologia 46*, 288-290.

Jiang, W. G., Martin, T. A., Matsumoto, K. et al. (1999). Hepatocyte growth factor/scatter factor decreases the expression of occludin and transendothelial resistance (TER) and increases paracellular permeability in human vascular endothelial cells. *J. Cell. Physiol. 181*, 319-329.

Joseph, E. C., Rees, J. A. and Dayan, A. D. (1996). Mesenteric arteriopathy in the rat induced by phosphodiesterase III inhibitors: an investigation of morphological, ultrastructural, and hemodynamic changes. *Toxicol. Pathol. 24*, 436-450.

Karanes, C., Ratanatharathorn, V., Schilcher, R. B. et al. (1986). High-Dose Mitomycin-C with Autologous Bone Marrow Transplantation in Patients with Refractory Malignancies. Influence of Dose Schedule on Pharmacokinetics and Nonhematopoietic Toxicities. *Am. J. Clin. Oncol. Cancer Clin. Trials 9*, 444-448.

Lagrange, P., Romero, I. A., Minn, A. and Revest, P. A. (1999). Transendothelial permeability changes induced by free radicals in an in vitro model of the blood-brain barrier. *Free Radic. Biol. Med. 27*, 667-672.

Larson, J. L., Pino, M. V., Geiger, L. E. and Simeone, C. R. (1996). The toxicity of repeated exposures to rolipram, a type IV phosphodiesterase inhibitor, in rats. *Pharmacol. Toxicol. 78*, 44-49.

Lau, D. C., Wong, K. L. and Hwang, W. S. (1989). Cyclosporine toxicity on cultured rat microvascular endothelial cells. *Kidney Int. 35*, 604-613.

Lazarus, H. M., Gottfried, M. R., Herzig, R. H. et al. (1982). Veno-occlusive disease of the liver after high-dose mitomycin C therapy and autologous bone marrow transplantation. *Cancer 49*, 1789-1795.

Lee, J. Y., Lee, M. Y., Chung, S. M. and Chung, J. H. (1999). Menadione-induced vascular endothelial dysfunction

and its possible significance. *Toxicol. Appl. Pharmacol. 161*, 140-145.

Mason, J. (1990). Pharmacology of cyclosporine (sandimmune). VII. Pathophysiology and toxicology of cyclosporine in humans and animals. *Pharmacol. Rev. 41*, 423-434.

McAmis, W. C., Schaeffer, R. C. Jr., Baynes, J. W. and Wolf, M. B. (2003). Menadione causes endothelial barrier failure by a direct effect on intracellular thiols, independent of reactive oxidant production. *Biochim. Biophys. Acta 1641*, 43-53.

Moncada, S. and Vane, J. R. (1978). Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A2, and prostacyclin. *Pharmacol. Rev. 30*, 293-331.

Oriji, G. K. and Schanz, N. (2001). Nitric oxide in CsA-induced hypertension: role of beta-adrenoceptor antagonist and thromboxane A2. *Prostaglandins Leukot. Essent. Fatty Acids 65*, 259-263.

Ortiz, J. L., Cortijo, J., Valles, J. M. and Morcillo, E. J. (1992). Rolipram inhibits PAF-induced airway microvascular leakage in guinea-pig: a comparison with milrinone and theophylline. *Fundam. Clin. Pharmacol. 6*, 247-249.

Remuzzi, G. and Bertani, T. (1989). Renal vascular and thrombotic effects of cyclosporine. *Am. J. Kidney Dis. 13*, 261-272.

Rutten, M. J., Hoover, R. L. and Karnovsky, M. J. (1987). Electrical resistance and macromolecular permeability of brain endothelial monolayer cultures. *Brain Res. 425*, 301-310.

Tervaert, J. W. and Kallenberg, C. G. (1997). Cell adhesion molecules in vasculitis. *Curr. Opin. Rheumatol. 9*, 16-25.

Schilcher, R. B., Young, J. D., Ratanatharathorn, V. et al. (1984). Clinical pharmacokinetics of high-dose mitomycin C. *Cancer Chemother. Pharmacol. 13*, 186-90.

Vicart, P., Testut, P., Schwartz, B. et al. (1993). Cell adhesion markers are expressed by a stable human endothelial cell line transformed by the SV40 large T antigen under vimentin promoter control. *J. Cell Physiol. 157*, 41-51.

Voss, B. L., Hamilton, K. K., Samara, E. N. and McKee, P. A. (1988). Cyclosporine suppression of endothelial prostacyclin generation. A possible mechanism for nephrotoxicity. *Transplantation 45*, 793-796.

Wautier, M. P., Chappey, O., Vicart, P. et al. (1999). Immortalized human endothelial cells have a decreased response to IL-1 secondary to an IL-1 receptor defective expression restored by corticosteroids. *Cell Biol. Toxicol. 15*, 153-161.

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