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 release of prostaglandin and 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.

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-
cultures 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 of the vasculature and the blood that are involved in the reduction of 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-dextrans (Jiang et al., 1999).

Furthermore, two important mediators synthesised by endothelial cells were investigated: prostacyclin (prostaglandin I\(_3\), PGI\(_3\)), 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 venoocclusive 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 hypothyrombinaemia, 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 10⁴ 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 (Srotec, 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 cm²) (Costar, Bodenheim, Germany) at a density of 2 x 10⁴ 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 subtracting the mean of wells without cells from the measured resistance in the sample wells: \( R_{\text{cell monolayer}} = R_{\text{sample}} - R_{\text{mean blanks}} \). 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. IC₅₀ values were determined as the concentration showing a permeability P of 50% (\( P_{\text{blanks}} - P_{\text{controls}} \)).

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 A₂ release into the medium were monitored by measurement of their stable derivates 6-keto-prostaglandin F₁α (6-keto-PGF₁α) and thromboxane B₂ (TXB₂) 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; IC₅₀ 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 IC₅₀ 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 Ω·cm² (n=90) after approximately 10 days in culture. In contrast IVEC cells showed less than half that resistance with 11.3 ± 7.4 Ω·cm² (n=72) even after 12 days in culture. Since the blank values were in the range of 116.6 ± 7.2 Ω·cm² (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. IC₅₀ values are

Tab. 1: Cytotoxicity of amrinone, cyclosporin A, rolipram, menadione and mitomycin C on HUVEC, IVEC and 3T3 cells.
The table shows the IC₅₀ values after 24 h incubation measured by AlamarBlue and ATP assay (mean of ± 2 experiments).

<table>
<thead>
<tr>
<th></th>
<th>Cyclosporin A</th>
<th>Mitomycin C</th>
<th>Menadione</th>
<th>Amrinone</th>
<th>Rolipram</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀ (μg/ml)</td>
<td>AlamarBlue</td>
<td>ATP</td>
<td>AlamarBlue</td>
<td>ATP</td>
</tr>
<tr>
<td>HUVEC</td>
<td>22</td>
<td>22</td>
<td>14</td>
<td>13</td>
<td>2.1</td>
</tr>
<tr>
<td>IVEC</td>
<td>13</td>
<td>11</td>
<td>13</td>
<td>15</td>
<td>1.6</td>
</tr>
<tr>
<td>3T3</td>
<td>20</td>
<td>16</td>
<td>19</td>
<td>n.d.</td>
<td>1.0</td>
</tr>
</tbody>
</table>

n.d.: not determined
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; IC₅₀ 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 A₂ release
Treatment of HUVEC cells with all tested compounds resulted in a dose-dependent increase of 6-keto-PGF₁α and/or TXB₂ release. This increase usually runs in parallel with a decrease of cell viability, but was increased also at nontoxic concentrations as in the case of rolipram. For mitomycin C, a strong increase in 6-keto-PGF₁α release was noted, but TXB₂ 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 IC₅₀ 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

<table>
<thead>
<tr>
<th>IC₅₀ (µg/ml)</th>
<th>Cytotoxicity IC₅₀</th>
<th>TER IC₅₀</th>
<th>Permeability IC₅₀</th>
<th>6-kPGF₁α 200%</th>
<th>TXB₂ 200%</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyclosporin A</td>
<td>22</td>
<td>35</td>
<td>200</td>
<td>20</td>
<td>&gt;250</td>
</tr>
<tr>
<td>mitomycin C</td>
<td>14</td>
<td>20</td>
<td>140</td>
<td>&gt;400</td>
<td>20</td>
</tr>
<tr>
<td>menadione</td>
<td>2</td>
<td>4</td>
<td>5</td>
<td>20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>amrinone</td>
<td>83</td>
<td>500</td>
<td>&gt;650</td>
<td>170</td>
<td>170</td>
</tr>
<tr>
<td>rolipram</td>
<td>250</td>
<td>&gt;750</td>
<td>&gt;750</td>
<td>60</td>
<td>400</td>
</tr>
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</table>

“>”/: IC₅₀ 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 Ω cm⁻². This is in the range of published data on resistance of non-brain endothelial systems including HUVEC cells of 1-30 Ω cm⁻² (Dye et al., 2001), but significantly below that of brain endothelial cells (several hundred Ω cm⁻², Rutten et al., 1987) or epithelial cell types (e.g. CaCo-2 cells ~ 200 Ω 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 Ω 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,

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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 ± 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-PGF1α and TXB2 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\textsubscript{50} values for the transendothelial resistance (Tab. 2) were slightly higher for all test compounds than the IC\textsubscript{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\textsubscript{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\textsubscript{50} values remained the same for all three endpoints, cytotoxicity (AlamarBue), TER and permeability: menadione < mitomycin C < cyclosporin A < amrinone < rolipram.

The higher IC\textsubscript{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\textsubscript{1α} was not increased, even at concentrations causing cytotoxicity and increased release of TXB\textsubscript{2}. At least for one compound, i.e. rolipram, an activation of the endothelial cells could be detected, since an increase of 6-keto-PGF\textsubscript{1α} release was seen at non-cytotoxic concentrations, thus showing a higher sensitivity of this endpoint. The release of TXB\textsubscript{2} 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\textsubscript{50} values are not far from the plasma levels reached at doses resulting in vascular dysfunction in vivo: After intravenous infusion of 60 mg/m\textsuperscript{2} mitomycin C to patients over 60 min or of 30 mg/m\textsuperscript{2} 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 of 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**


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