Metabolism of verapamil in cultures of rat alveolar epithelial cells and pharmacokinetics after administration by intravenous and inhalation routes

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Pulmonary metabolism of verapamil

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Nonstandard Abbreviations: AECs, alveolar epithelial type II cells; CYP, cytochrome P450; DMEM, Dulbecco’s modified Eagle Medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography-mass spectometry; MSPD, matrix solid-phase dispersion; PBS, phosphate-buffered saline; RAM, restricted access material; rpm, revolutions per minute; RMV, respiratory minute volume.
Abstract:
Administration of therapeutic entities by inhalation opens new possibilities for drug entry into systemic circulation, but this requires passage through the alveolar epithelium. Little is known about the pulmonary metabolism of verapamil. Specifically, this cardiovascular drug suffers from extensive first pass metabolism. We therefore evaluated the metabolism of verapamil in cultured alveolar epithelium and compared findings with results after administration by inhalation and intravenous routes. Specifically, cell culture of alveolar epithelium was characterised by gene expression of surfactant proteins A, B, C and D, by immunohistochemistry of surfactant protein C, by staining for laminar bodies and by gene expression of CYP monooxygenases. During six days of culture expression of all cellular differentiation markers was obvious, albeit at different levels. With testosterone as substrate we found alveolar epithelial cells to produce several stereo- and site-specific hydroxylation products. This provided evidence for metabolic competence of cultured alveolar epithelial cells. With verapamil as substrate only limited production of metabolites was observed in cell culture assays and similar results were recorded after administration by inhalation and intravenous routes. Likewise, elimination of verapamil from lung tissue and plasma was similar by both routes of administration. In conclusion, administration of verapamil by inhalation abrogated extensive first pass metabolism frequently seen after oral application and this may well be extended to the development of drugs with similar pharmacokinetic defects.
Introduction

The lung is composed of approximately 40 different cell types with diverse functions within the respiratory tract (Warburton et al., 1998). Current data suggests that metabolism of drugs and other xenobiotics is thought to be associated with mainly six different cell types: type I and type II alveolar epithelial cells, alveolar macrophages, capillary endothelial cells, ciliated and non-ciliated (Clara cells) bronchiolar epithelial cells (Willey et al., 1997; Anttila et al., 1997, Baron and Voigt, 1990). Based on animal studies, the primary site of pulmonary cytochrome P450 (CYP) monooxygenase activity resides in Clara cells and type II alveolar epithelial cells (AECs) (Bend et al., 1985; Voigt et al., 1990). In particular, AECs serve two major functions, both of which are essential for the maintenance of normal lung physiology. Firstly, AECs synthesize and secrete pulmonary surfactant (Kasper and Singh, 1995), and secondly, they function as progenitors of newly formed pulmonary alveolar epithelium after lung injury and during normal lung cell turnover (Uhal, 1997). Additional functions include detoxification of airborne xenobiotics (Adamson et al., 1977) with the lung being the primary site for entry into systemic circulation. For elimination, certain xenobiotics undergo oxidation by phase I enzymes and additional reactions to foster excretion from the body (Guengerich and Shimada, 1991). Although most of these oxidations are beneficial, some may lead to reactive metabolites and cellular damage (Guengerich, 2000).

In addition to drug delivery designed to treat pulmonary disease, administration of therapeutic agents by the inhalation route has been reported for the immunsupressive agent cyclosporin (Keenan et al., 1992), interferons (Halme et al., 1994), α1-antitrypsin (McElvaney et al., 1991) amongst others.

We investigated the metabolism and pharmacokinetics of verapamil, a synthetic papaverin derivative, with L-type-calcium channel blocking activity and therapeutic use as an antianginal, antihypertensive and anti-rythmic agent (Woosley and Roden, 1983; Padrini et al., 1985; Piovan et al., 1995). Unfortunately, this drug suffers from extensive hepatic first
pass metabolism. Because of its extensive metabolism verapamil becomes rapidly inactivated and this necessitates frequent dosage of this drug. This has stimulated research into new release forms and alternative routes for drug application. The objective of the present study was therefore to evaluate the plasma clearance and metabolism of verapamil after drug administration by inhalation. Specifically, rats were given single doses of verapamil by inhalation and intravenous routes and the plasma and lung tissue concentrations of verapamil and its metabolites were measured at several time points using a validated and previously published HPLC-MS method (Walles et al., 2002). Further, we investigated the metabolism of verapamil in cultures of AECs, e.g. the site of drug entry into systemic circulation. Thus, the work in this manuscript explored the use of drug delivery by inhalation to avoid extensive first pass metabolism.
Materials and Methods

**Cell culture media and reagents.** Dulbecco’s modified Eagle Medium (DMEM) and phosphate-buffered saline (PBS) were purchased from Biochrom (Berlin, Germany). The DMEM used to culture primary AECs was supplemented with L-glutamine 4.4 mM (PAA Laboratories GmbH, Cölbe, Germany), prednisolone 0.67 µg/ml (Merck, Darmstadt, Germany), glucagon 0.0156 µg/ml (Novo, Mainz, Germany), insulin 6.3 µg/ml (Hoechst AG, Strasbourg, France), penicillin 200 units (U)/ml, streptomycin 200 µg/ml (Biochrom, Berlin, Germany) and 5% rat serum (PAA Laboratories GmbH, Cölbe, Germany). Rat tail collagen was prepared as described by Elsdale and Bard (1972) and was used to coat 12-well culture plates (Biochrom, Berlin, Germany).

**Cell isolation and culture.** AECs were isolated as previously described by Richards et al. (1987) with slight modifications. Male Sprague Dawley rats weighing approximately 120 g were anaesthetized with ketamine (100 mg kg⁻¹)/Rompun (10 mg kg⁻¹) by intraperitoneal injection. After tracheotomy, the trachea was cannulated for artificial ventilation of the lung and blood was removed from the lung by perfusion with ice cold PBS via the pulmonary artery. The perfused lung plus trachea were then explanted and placed in a 50 ml plastic tube containing 30 ml ice cold PBS. The lungs were lavaged three times with 6 ml PBS and subsequently 6 ml 0.25 % trypsin-EDTA solution were instilled into the lung via the trachea. The lung was incubated with trypsin for a total of 45 min at 37°C and every 15 min, 4 ml 0.25 % trypsin-EDTA solution was added via the trachea. Trachea and bronchi were removed and the lung tissue was chopped into 1-2 mm pieces. 10 ml DNase (250 µg/ml) (Invitrogen GmbH, Karlsruhe, Germany) was added to the suspension, incubated for 10 min at 37°C in a shaking waterbath (60 rpm) and then filtered through a nylon mesh with 100 and 60 µm pore size. The resulting cell suspension was layered on a discontinuous Percoll gradient (heavy density 1.089 and low density 1.040) which was centrifuged at 250 g at 4°C for 30 min. The cell fraction at the interface between heavy and low density gradient was removed and mixed.
with PBS containing 50 µg/ml DNase. The cell suspension was again centrifuged at 140 g at 4°C for 6 min. The resulting cell pellet was resuspended in culture medium and subsequently cultured for one hour on plastic to remove rapidly adhering cells including lung macrophages. Subsequently, the cell suspensions were cultivated on rat tail collagen-coated 12-well plates. Approximately 4 x 10^6 cells were seeded per well in 1 ml culture medium and cultured for up to 7 days.

**Histochemistry.** Cytospin preparations of freshly isolated cells and on day 3 and 6 in culture were prepared at an approximate density of 1 x 10^6 cells per slide and frozen at –80°C. These cells were characterized by staining for alkaline phosphatase as a marker for epithelial cells by the method of Edelson et al. (1988) and by tannic acid polychrome stain for lamellar bodies, as described by Mason et al. (1985). The expression of surfactant protein C was analysed by immunoperoxidase staining using the DAKO LSAB+ Kit (DAKO, Hamburg, Germany) in conjunction with a polyclonal rabbit anti human surfactant protein C antibody (Byk Gulden, Konstanz).

**Testosterone hydroxylation assay.** Testosterone served as a substrate to probe for activity of CYP monooxygenases in cultured AECs (Arlotto et al., 1991). Therefore, AECs of day 3 in culture were exposed to culture medium containing 100 µM testosterone (Sigma) for 2, 4, 8, 12, and 24 h. Testosterone and its metabolites 2-α-hydroxytestosterone, 6-α-hydroxytestosterone, 6-β-hydroxytestosterone, 16-α-hydroxytestosterone and androstendione (Sigma) were analysed by high-performance liquid chromatography (HPLC) according to Arlotto et al. (1991) with slight modifications. 11-α-hydroxyprogesterone (Sigma) was used as an internal standard for the quantitative determination of testosterone and its metabolites using the following procedures:

1 µg 11-α-hydroxyprogesterone was added to 1 ml of cell culture supernatant. Following addition of 100 µl isopropanol, the samples were extracted with 5 ml ethylacetate by gentle shaking for 20 min. Extracts were evaporated to dryness, and the residues were reconstituted
in 100 µl of the mobile phase (water/methanol/acetonitrile, 60/25/15, v/v/v), and 80 µl of the sample was injected onto the HPLC system (HPLC series 1100, Hewlett Packard, Hamburg, Germany). The mobile phase was delivered at a flow rate of 1 ml/min using the HP 1100 Quaternary Pump. Chromatographic separation of metabolites was achieved on a C18 Nucleosil column with the dimensions 250 x 4 mm and a particle size of 5 µm (Macherey-Nagel, Düren, Germany) at a temperature of 30°C. The mobile phase consisted of water (solvent A), methanol (solvent B) and acetonitrile (solvent C), and analysis was initiated with an isocratic elution of 60% A, 25% B and 15% C for 12 minutes, followed by 45% A, 40% B and 15% C for 3 minutes and finally 45% A, 45% B and 10% C thereafter. The total run time was 30 minutes per sample. Testosterone and metabolites were detected by UV-absorption at 238 nm by comparison of retention times of individual reference standards.

**CYP induction in cultured AECs.** At day 3 in culture AECs were treated for 48 h with 10 µM Aroclor 1254, a well known inducer of CYP monooxygenases (Borlak and Thum, 2001; Borlak et al., 1996).

**Verapamil metabolism.** At day 3 in culture AECs were treated with 300 nM Verapamil at day 3 in culture for 12 h. This concentration (300 nM Verapamil) is based on therapeutic plasma values (Hamann et al., 1984). Culture supernatant was removed and frozen at –80°C to await analysis.

**Preparation of lung microsomes.** Lungs were cut into small pieces and homogenised with an ultraturrax (Ika, Staufen, Germany) in KCl-solution (0.15 M, pH 7.4) and thereafter centrifuged for 30 min at 11,000 g and 4°C. The resulting supernatant was decanted and once again centrifuged at 110,000 g and 4°C for 60 min. The resultant pellet was washed and resuspended in KCl-solution (0.15 M, pH 7.4) and centrifuged for 40 min at 110,000 g and 4°C. Then, the microsomal fraction was transferred into TRIS-sucrose buffer (0.25 M sucrose, 20 mM TRIS, 5 mM EDTA, pH 7.4). The protein content was determined according to Smith.
et al. (1985). Microsomal solutions were frozen in liquid nitrogen and stored at -80°C until further use.

**Lung microsomal metabolism assays.** A 1 ml reaction mixture containing 500 µg microsomal protein in 0.1 M Tris buffer pH 7.4 + 1 mM NADPH (Sigma, Taufkirchen, Germany) was incubated with 300 nM verapamil for up to 60 min at 37°C. After incubation the samples were frozen in liquid nitrogen and stored at –80°C until analysis.

**Gene expression profiling.** RNA was isolated from cultured AECs from day 3 to 7 using the Nucleo Spin RNAII Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s recommendation. For reverse transcription 1 µg RNA was denaturated for 5 min at 65°C and reverse transcription was initiated by Omniscript Reverse Transcriptase (Qiagen, Hilden, Germany). This reaction was carried out for 60 min at 37°C and stopped by heating to 95°C for 5 min. The resulting cDNA was frozen at –20°C until further use.

For PCR amplification of cDNA, a 25 µl reaction mixture was prepared containing 10 x polymerase reaction buffer, 3 mM MgCl₂, 0.4 mM dNTPs (Invitrogen, Karlsruhe, Germany), 400 nM concentration of the 3´ and 5´ specific primer (synthesized by GIBCO, Hilden, Germany) (Table 1), 1 U Hot Star Taq™ polymerase (Qiagen, Hilden, Germany) and 1 µl of cDNA. PCR reactions were carried out in a thermocycler (T3, Biometra, Göttingen, Germany) using the following melting, annealing, and extension cycling conditions: denaturation for 45 sec at 94°C, annealing for 60 sec at 55°C, and extension for 60 sec at 72 °C for GAPDH (30 cycles), CYP 2C11, CYP 4A1 (32 cycles) and CYP 3A1, CYP 2E1, CYP 2J3, CYP 1B1 (36 cycles). PCR conditions for CYP 1A1 (34 cycles), CYP 1A2 (38 cycles), CYP 2B1/2 (36 cycles) and CYP 3A2 (38 cycles) were: denaturation for 45 sec at 94°C, annealing for 60 sec at 57°C, and extension for 60 sec at 72 °C. Each PCR started with a 15 min denaturation step at 95°C and ended with a 10 min elongation step. Primer sequences are given in table 1. PCR reactions were done within the linear range of amplification, after having established the chosen cycles to be in the mid exponential phase for every primer set.
used. Amplification products were separated on a 1.8% agarose gel and were visualized by ethidium bromide under UV transillumination.

Quantification of PCR-products was done with the program NIH Image V.1.62 and the software of the Kodak Image Station version ID 3.5. Data are expressed as the ratio of gene of interest (nominator) to the housekeeping gene, GAPDH.

Statistical analysis. Each analysis was based on n = 4 independent isolations. Means and standard deviations were calculated with the Microsoft Excel software package (version 2000). The Student’s *t*-Test (unpaired) was used to detect statistical differences (p ≤ 0.05).

Inhalation studies. For 14 days and prior to exposure, animals were acclimated to external procedures to avoid any unphysiological breathing pattern. Verapamil was administered as dry aerosol by spray drying 0.8 % (v/w) aqueous solution. Cascade impactor measurements of the aerosol size distribution revealed a mass median aerodynamic diameter of 1.4 µm and a geometric standard deviation of 1.5. A total of 21 male Sprague Dawley rats weighing approximately 250 – 300g were exposed to 50 mg/m³ verapamil by nose-only inhalation for 10, 20, 30, 40 (each time period 3 animals) and 60 minutes (9 animals). After the 10, 20, 30 and 40 minute time point 3 animals were killed immediately. In the case of the 60 minute time point, 3 animals were killed immediately after the end of inhalation, whereas a further 3 animals were killed 60 minutes after the end of inhalation and further 3 animals were killed 180 minutes after the end of inhalation to enable direct comparison with the intravenous treatment group. The total inhaled dose after 60 minutes of exposure was calculated from the respiratory minute volume (RMV) and the aerosol concentration to be 1.56 mg/kg. Based on an empirical correlation established by Stahl (Stahl, 1967) a value of 0.52 l/min was chosen for the RMV in this calculation. From the inhaled dose only the fraction deposited in the lung can become bioavailable. This fraction is estimated to be 30 %, so that the relevant body dose of verapamil after 60 minute inhalation is approximately 0.5 mg/kg. In the case of intravenous application 21 animals were given an intravenous dose of exactly 0.5 mg/kg.
verapamil via the tail vein. After intravenous injection 3 animals were killed after 10, 20, 30, 40, 60, 120 and 240 min. The blood was collected and centrifuged at 4000 rpm for 15 min at 4°C. Resultant plasma was frozen at –20°C. Further, lungs were carefully explanted and frozen at –80°C. Blank plasma and tissue samples from untreated rats were obtained as well.

**Bioanalytical assay for verapamil and its metabolites.** Verapamil and its major metabolites were extracted by a column-switching technique with a restricted access material (RAM) and by a matrix solid-phase dispersion method (MSPD) to analyze lung tissue samples as reported previously (Walles et al., 2002). Identification of the metabolites was achieved by liquid chromatography-mass spectrometry (LC-MS) as described elsewhere (Walles et al., 2002). The abundances of the metabolites were calculated using the peak ratio of the metabolites relative to that of verapamil. Only metabolites observed with a single-to-noise ratio (s/n) > 3 were quantified.
Results

Isolation of alveolar epithelial cells. A yield of $21.7 \pm 5.9 \times 10^6$ cells per rat lung was obtained. Immediately after isolation, the trypan blue exclusion test revealed almost all cells (> 95%) to be viable. Following 24-48 hours in culture, AECs generally formed confluent monolayers (Figure 1a).

Cell characterization. Table 2 summarizes the results obtained from cytospin preparations of rat AECs. Over 60% of freshly purified AECs and over 70% of AECs (day 3 in culture) were positive for surfactant protein C, tannic acid and alkaline phosphatase staining (Figure 1b-d, Table 2). We therefore provide evidence for the expression of markers of alveolar epithelial type II cells. Notably, at day 6 in culture, a significant decrease in staining for alkaline phosphatase (59%), lamellar bodies (70%) and surfactant protein C (63%) was observed (Table 2). Further, transcript level of surfactant proteins A and B remained stable during culture, but the expression of genes coding for surfactant protein C and surfactant protein D decreased progressively. Figure 2 depicts a representative ethidium bromide stained gel of surfactant protein coding genes at day 3 to 7 in culture.

We also investigated expression of CYP monooxygenases in cultures of AECs. With the exception of CYP 1A2, CYP 2C11, CYP 3A1, CYP 4A1, which were below the level of detection, expression of transcripts of CYP isoforms 1A1, CYP 1B1, CYP 2B1/2, CYP 2E1, CYP 2J3 and CYP 3A2 was similar during cell culture. Further, CYP 1A1, CYP 1B1 and CYP 2E1 mRNA expression was increased 6-fold, 2-fold and 3-fold, respectively after treatment of AECs with Aroclor 1254, a known CYP monooxygenases inducer (Figure 3). The mRNA expression of genes coding for CYP 2B1/2, CYP 3A2 and CYP 2J3 was unchanged after treatment of the AECs with Aroclor 1254 (Figure 3). Further, treatment of AECs with verapamil had no influence on CYP gene expression (data not shown).

To confirm the metabolic competence of the cultured AECs, the cells were incubated with 100 µM testosterone for 24 h and the production of metabolites was analysed by HPLC. There
was a time-dependent increase in the formation of testosterone metabolites, as shown in Figure 4. Androstendione was the main metabolite produced, followed by smaller amounts of 6-β-hydroxytestosterone, 6-α-hydroxytestosterone, 16-α-hydroxytestosterone, and 2-α-hydroxytestosterone (see Figure 4). Approximately 5% of the testosterone was metabolically converted in a 24 h assay period.

**Inhalation studies.** The plasma and lung concentrations of verapamil after administration by intravenous and inhalation routes are depicted in Figure 5 and 6. After bolus intravenous administration a characteristic bi-exponential plasma clearance was observed. The characteristic start term clearance time constant for both the plasma and the lung tissue is 0.05 min⁻¹. Long term clearance is characterized by a time constant of 0.013 min⁻¹ for the plasma and 0.009 min⁻¹ for the lung tissue. In fitting the data for the inhalation route we considered deposition at a constant rate and clearance running in parallel during the exposure period and only clearance afterwards. In both cases we used the same clearance time constants as for the intravenous application. For the inhalation route the plasma data were fitted by assuming a transfer time period of 7 min between the moment of particle deposition and entrance into the circulation system. For obvious reasons this delay time is zero for the build-up of concentration of verapamil in the lung tissue. In Table 3 some relevant kinetic parameters for intravenous administration of verapamil and administration by inhalation are compared. The C_max value is higher for the intravenous administration since the entire dose is given as a short bolus as opposed to inhalation, where the same dose is administered over a time period of 60 minutes. AUC1 denotes the value obtained by integration of the time period covered experimentally, i.e. t = 250 min, whereas AUC2 represents the integral extended to t = ∞. (Integration was done mathematically using the mathematical expressions of the fitted curves.) There is less than 5% difference between the two AUC values for the plasma and less then 11% for the lung tissue, therefore indicating that the experimental time period covers most of the kinetics of verapamil. Further, the AUC after of intravenous and inhalation
compare very well, the value for inhalation being 10% higher. Since the nominal doses after administration by inhalation and intravenous routes were nearly equal, we conclude that the bioavailability of verapamil after inhalation is essentially the same as after intravenous administration.

**Identification of metabolites.** In multiple LC-MS experiments we found the N-dealkylated product of verapamil (Figure 7) as the main metabolite in plasma and lung tissue after intravenous application (representing 10% (plasma) and 2% (lung) of the peak ratio relative to verapamil). This metabolite was detected in lung tissue after administration by inhalation as well, but was not quantified because of a s/n ratio of < 3. Further, after intravenous application two additional metabolites (9 and 19, Figure 7) were identified in plasma and lung tissue (representing 1% and 0.4% of the peak ratio relative to verapamil). Additionally, the carbinolamin (Figure 7) was detected in lung tissue after intravenous drug administration, but once again was too small to be quantified (s/n ratio < 3). Further details regarding verapamil biotransformation are given elsewhere (Walles et al., 2003).

With cultures of AECs and with microsomal lung fractions metabolism of verapamil proceeded with the production of metabolite D617, carbinolamin and norverapamil (Figure 7). Additionally, with microsomal fractions of rat lung metabolite 24 was observed (Figure 7).
Discussion

This study aimed for a better understanding of pulmonary metabolism of verapamil. It is the first report on metabolism of verapamil in cultured AECs and with lung microsomes. We observed limited metabolism of verapamil in metabolically competent cultures of AECs and with lung microsomes. The extensive hepatic first pass metabolism of this drug can be avoided by administration of the compound via the inhalation route.

AECs have been reported to express CYP monoxygenases (Baron and Voigt, 1990) and are likely to play an important role in the pulmonary metabolism of drugs. To investigate the metabolic competence of cultured AECs lung tissue was explanted and cells were isolated as described previously by Richards et al. (1987). AECs and other lung cells overlap in cell density (wt/vol) (Bingle et al., 1990) and cell diameter (Crapo et al., 1982; Haies et al., 1981), which hampers efficient separation from other pulmonary cells. Characterization of AEC in culture should involve several cellular markers (Dobbs, 1990). We investigated AEC cultures by phase contrast microscopy and by studying lamellar bodies in the cytosol of cultured AECs and by investigating surfactant protein C protein expression. We show that isolated and cultivated AECs retained certain differentiation markers during culture. The lamellar bodies contained surfactant, a morphological hallmark of AECs. Laminar bodies are the main cellular storage compartment of pulmonary surfactant, and AECs produce surfactant protein A, B, C and D (Kasper and Singh, 1995). In our study gene expression of all four surfactant proteins was confirmed during 7 days of culture albeit at varying intensities. Noteworthy, cells other than AECs also express surfactant proteins. For example, surfactant protein A is expressed in alveolar macrophages and Clara-cells, as determined by immunocytochemistry (Walker et al., 1986; Williams et al., 1988). Nonetheless, surfactant protein C expression is restricted to AECs (Kasper and Singh, 1995) and therefore, surfactant protein C is a valuable marker for the characterization of this particular cell type. Indeed, surfactant protein C
expression remained constant, when freshly isolated and day 6 cultured cells are compared (Table 2).

Additionally, alkaline phosphatase was proposed as marker for AECs. Although cells other than AECs express alkaline phosphatase activity as well, alveolar macrophages lack this phosphatase activity (Cohn and Weiner, 1963). A histochemical stain for alkaline phosphatase can therefore be used to distinguish AECs from alveolar macrophages (Edelson et al., 1988). In our study, 74% of freshly isolated AECs were positive for alkaline phosphatase staining.

Though AECs retained some of their differentiation marker, alkaline phosphatase activity, tannic acid polychrome staining and surfactant protein C expression declined with time, e.g. between day 3 and 6 in culture (Table 2). We therefore chose 3 day old cultures for studying the metabolism of testosterone and verapamil.

The metabolic competence of the cultured AECs was studied by measuring the mRNA expression levels of CYP monooxygenases and production of stereo- and site-specific hydroxylation products of testosterone and reasonable correlations were found. We further show treatment of cultures of AEC’s with Aroclor 1254 to induce expression of certain CYP monooxygenases, i.e. CYP 1A1, CYP 1B1 and CYP 2E1, and therefore provide evidence for cultured AECs to respond to CYP monooxygenase inducing agents.

We link the metabolic competence of cultured AECs to metabolism of verapamil and compare findings with results from microsomal incubation assays. With lung microsomal fractions and with AECs a total of 4 metabolites was observed with norverapamil being the major metabolite. In strong contrast the hepatic metabolism of verapamil resulted in 25 phase I and 14 phase II metabolites as recently reported by us (Walles et al., 2003). In human liver abundant expression of CYP enzymes resulted in extensive metabolism of verapamil and it’s metabolism is primarily catalysed by CYP 3A4, CYP 3A5, CYP 2C8, CYP 2C18, CYP 2D6, and CYP 2E1 (Tracy et al., 1999). Although the expression of an array of different CYP isoforms was detectable in cultured AECs of rat lung, only the rat orthologue to CYP 3A5 is
likely be a major contributor to pulmonary oxidation of verapamil. Hithertho, we show a good correlation between the limited expression of CYP isoforms relevant to verapamil metabolism and its limited metabolism in the alveolar epithelium.

We also investigated the metabolism of verapamil in vivo after administration by intravenous and inhalation routes. The level of verapamil in lung tissue, as well as in plasma, increased with inhalation time. This suggests fast transfer of verapamil via intracellular or paracellular transport from epithelium into lung tissue and blood. An important finding of our study was the similar elimination of verapamil from lung tissue and plasma after administration by intravenous and inhalation routes. The limited metabolism of verapamil in cultured AECs and in lung tissue after administration by inhalation is in good agreement and we demonstrate that therapeutic plasma levels can be achieved. With lung tissue we observed norverapamil as major metabolite, but the amounts were too small for proper quantification. Amongst many other metabolites this metabolite is known to be a major breakdown product of verapamil. We found rat hepatocytes to produce large amounts of norverapamil (40% peak ratio in the MS-mode relative to verapamil) as well (Walles et al., 2003). Obviously, inhalation of verapamil abrogated extensive hepatic metabolism and we observed a kinetic of plasma elimination similar to intravenous administration. Notably, norverapamil was not detected in rat plasma after administration by inhalation, though three additional metabolites (carbinolamin, and metabolites 9, 19, see Figure 5) were identified after intravenous application.

Our study clearly demonstrates the advantage of a drug application by inhalation for verapamil to achieve therapeutic plasma levels without suffering the consequences of extensive hepatic first pass metabolism. The need for frequent drug usage may therefore be overcome. In conclusion, drugs with pharmacokinetic defects, such as verapamil, may benefit from drug administration by inhalation to enter into systemic drug circulation.
Acknowledgments

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References


Legends for Figures

Figure 1. Histo- and immunohistochemical characterization of primary cultures of rat AECs at day 3 in culture: a) Phase contrast micrograph (original magnification x 100); b) tannic acid polychrome stain for lamellar bodies (see dark granules in the photograph); c) surfactant protein C expression (see brown coloring in photograph); d) alkaline phosphatase staining (see red coloring in photograph).

Figure 2. Ethidium bromide stained PCR amplification products of mRNA encoding for surfactant protein A, B, C and D in primary cultures of rat AECs at day 3 (lane 2), 4 (lane 3), 5 (lane 4), 6 (lane 5) and 7 (lane 6) in culture.

Figure 3. Ethidium bromide stained PCR amplification products of mRNA encoding for CYP monoxygenases in primary cultures of rat AECs: Lane 1: ladder, lane 2: control, lane 3: after treatment with 10 µM Aroclor 1254 for 48 h at day 3 in culture.

Figure 4. Production of testosterone metabolites in AECs on day 3 in culture. Approximately 4 x 10^6 cells were used for incubation with 100 µM testosterone for 24 h. Results are given as means and standard deviations of at least four independent experiments.

Figure 5. Plasma concentration of verapamil after intravenous injection and after inhalation.

Figure 6. Tissue concentration of verapamil after intravenous injection and after inhalation in rat lung.
Figure 7. Identified metabolites of verapamil in supernatants of cultures of rat AECs, rat lung microsomal fractions and in plasma and lung tissue of rats. For metabolite nomenclature see Walles et al. (2003).
**Tables**

**TABLE 1**

*Primers used for PCR analysis and product size. SP = surfactant protein.*

|----------|--------------------------------------|-------------------|-----------------------|
| CYP 1A1  | fwd: 5’-gtggtctggataaccacagctg  
rev: 5’-ccaaaggtgtgattacaggg | 331               | NM 012540.1          |
| CYP 1A2  | fwd: 5’-gtcaacctcaaggaatgcttg  
rev: 5’-ttggacaaatccttcctgagg | 236               | NM 012541.1          |
| CYP 1B1  | fwd: 5’-ctctcccccccataccacacta  
rev: 5’-aagaagacatgacccctagcgg | 350               | NM 012940.1          |
| CYP 2B1/2| fwd: 5’-gatgtcttcctgctggctcttg  
rev: 5’-actgtgctcctggagagctg | 549               | M 37134.1            |
| CYP 2C11 | fwd: 5’-ctctgctgtaaaccagctg  
rev: 5’-gatgaacagagatacttac | 248               | NM 019184.1          |
| CYP 2E1  | fwd: 5’-ctctgctgtaatctatccttg  
rev: 5’-gcagcctcagtaaattgggtgg | 473               | NM 031543.1          |
| CYP 2J3  | fwd: 5’-ggatgcccttaaataaaaga  
rev: 5’-gcctcagctctcctgaa | 364               | U 39943              |
| CYP 3A1  | fwd: 5’-atcgcctgatgtgagatccttg  
rev: 5’-gaagagacatgctgctcag | 579               | NM 013105.1          |
| CYP 3A2  | fwd: 5’-cttgccaactctcctagctgg  
rev: 5’-cagttaaccagacctagcgg | 116               | M 13646              |
| CYP 4A1  | fwd: 5’-ggggcaaaagaatacagc  
rev: 5’-agaggagctgtgctgctgctg | 344               | NM 016999.1          |
| GAPDH    | fwd: 5’-ggccagctatcctatgagt  
rev: 5’-ctagtgtgctgtgcagctg | 353               | BC009081.1           |
| SP-A     | fwd: 5’-gctgctgctgctgctgctg  
rev: 5’-agagagactgactgactg | 349               | NM 017329.1          |
| SP-B     | fwd: 5’-gctgctgctgctgctgctg  
rev: 5’-agagagactgactgactg | 349               | X 14778.1            |
| SP-C     | fwd: 5’-gggcaaaagaatacagcagc  
rev: 5’-gaaactcagaaacgctg | 284               | NM 017342.1          |
| SP-D     | fwd: 5’-agcccttccgctgagatc  
rev: 5’-ttgagacttgctggtctg | 343               | NM 012878.1          |
TABLE 2

Expression of differentiation markers in primary cultures of rat AECs. Results (positive cells as percentage of all cells) are given as means and standard deviations of at least 4 independent experiments. (Separate animals were used to obtain the cells of each individual experiment).

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 6</th>
</tr>
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<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>74% ± 14%</td>
<td>70% ± 8%</td>
<td>59% ± 23%</td>
</tr>
<tr>
<td>Tannic acid polychrome</td>
<td>not detected</td>
<td>87% ± 8%</td>
<td>70% ± 9%</td>
</tr>
<tr>
<td>Surfactant protein C</td>
<td>66% ± 22%</td>
<td>84% ± 12%</td>
<td>63% ± 20%</td>
</tr>
</tbody>
</table>
TABLE 3

Kinetic data based on time series of plasma concentrations of verapamil after administration by intravenous and inhalation routes.

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose [mg/kg]</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; [ng/g]</td>
<td>187</td>
<td>85</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0→240&lt;/sub&gt; [ng min/g]</td>
<td>6477</td>
<td>6999</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0→∞&lt;/sub&gt; [ng min/g]</td>
<td>6629</td>
<td>7268</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2α&lt;/sub&gt; [min]</td>
<td>13.8</td>
<td>13.8</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2β&lt;/sub&gt; [min]</td>
<td>55.5</td>
<td>77.0</td>
</tr>
</tbody>
</table>

* calculated value subject to uncertainty due to uncertainties related to respiratory minute volume and particle deposition efficiency.
Figure 3
Figure 7

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Verapamil</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metabolite 9</td>
<td>Metabolite 19</td>
<td>Metabolite 24</td>
<td>D-617</td>
</tr>
<tr>
<td>Carbinolamin</td>
<td>Norverapamil</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>