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Evidence of P-glycoprotein mediated apical to basolateral transport of flunisolide in human broncho-tracheal epithelial cells (Calu-3)

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1 Transepithelial transport of flunisolide was studied in reconstituted cell monolayers of Calu-3, LLC-PK1 and the MDR1-P-glycoprotein transfected LLC-MDR1 cells.

2 Flunisolide transport was polarized in the apical (ap) to basolateral (bl) direction in Calu-3 cells and was demonstrated to be ATP-dependent. In LLC-MDR1 cells, flunisolide was transported in the bl to ap direction and showed no polarization in LLC-PK1 cells.

3 Non-specific inhibition of cellular metabolism at low temperature (4°C) or by 2-deoxy-D-glucose (2-d-glu) and sodium azide (NaN_3) abolished the polarized transport. Polarized flunisolide transport was also inhibited by the specific Pgp inhibitors verapamil, SDZ PSC 833 and LY335979.

4 Under all experimental conditions and in the presence of all used inhibitors, no decrease in the TransEpithelial Electrical Resistance (TEER) values was detected. From all inhibitors used, only the general metabolism inhibitors 2-deoxy-D-glucose and NaN_3, decreased the survival of Calu-3 cells.

5 Western blotting analysis and confocal laser scanning microscopy demonstrated the presence of MDR1-Pgp at mainly the basolateral side of the plasma membrane in Calu-3 cells and at the apical side in LLC-MDR1 cells. Mass spectroscopy studies demonstrated that flunisolide is transported unmetabolized across Calu-3 cells.

6 In conclusion, these results show that the active ap to bl transport of flunisolide across Calu-3 cells is facilitated by MDR1-Pgp located in the basolateral plasma membrane.
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Keywords: Flunisolide; Calu-3 (human submucosal gland adenocarcinoma); P-glycoprotein (MDR1-Pgp); ABC transporter; active transport; confocal microscopy; Western blotting

Abbreviations: ABC transporter, ATP binding cassette transporter; air, air-interface; ap, apical; bl, basolateral; clogP, calculated partition coefficient; 2-d-glu, 2-deoxy-D-glucose; DTT, 2,3-dihydroxybutane-1,4-dithiol; FCS, foetal calf serum; MDR1, MDR1-P-glycoprotein; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NaN_3, sodium azide; P, permeability; PMSF, phenylmethanesulfonyl fluoride; sub, submerged; TEER, transepithelial electrical resistance

Introduction

Asthma and chronic obstructive pulmonary diseases (COPD) are the major respiratory airways disorders that affect over 100 million people world-wide, with the prevalence increasing amongst children (Jeffery, 1998). The respiratory condition of the patients is characterized by airway inflammation, obstruction and hyper-responsiveness to stimuli such as environmental allergens, viral respiratory tract infections, irritant drugs, food additives, exercise and cold air. Treatment with inhaled corticosteroids effectively suppresses eosinophilic inflammation, ameliorating the symptoms in asthma (Meagher *et al*., 1996). In COPD patients it has been shown that treatment with fluticasone propionate, over a time period of at least 6 months, may be of clinical benefit (Paggiaro *et al*., 1998). Fluticasone propionate and flunisolide are members of the latest generation of highly potent, inhalable glucocorticoids, derived from hydrocortisone (Figure 1). They were merely empirically designed to have high airway selectivity and efficient hepatic metabolism in order to prevent side-effects (Brattsand *et al*., 1997; Chaplin *et al*., 1980; Teitelbaum *et al*., 1981). Due to lack of suitable *in vitro* models, the absorption and transport mechanism of synthetic corticosteroids across epithelia of the pulmonary system remain unknown.

In the present study, we have chosen the human, mucus-producing, submucosal gland carcinoma cell line (Calu-3) as a tool to identify the mechanism of flunisolide transepithelial transport in the airways. Calu-3 cells were reconstituted as functional epithelial monolayers on permeable filter supports and grown at an air-interface or under submerged culture conditions (Cavet *et al*., 1997; Foster *et al*., 2000). The transport of flunisolide showed a polarized transport in the

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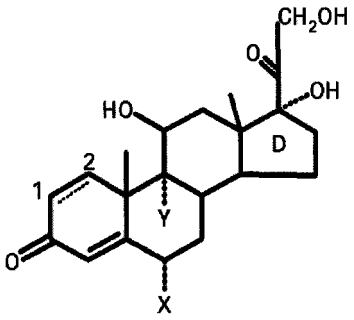


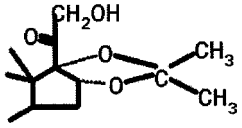
Glucocorticoid	X	Y	D	clogP
Hydrocortisone	H	H	D	1.16
Flunisolide	F	H		1.13

Figure 1 Structure of flunisolide in current use for inhalation obtained by chemical modifications of the hydrocortisone backbone. Lipophilic substitution is introduced at the 16α, 17α positions as acetonide to confer selectivity for the glucocorticoid receptor for inhalation therapy. Notice that the C1-C2 bond is unsaturated in hydrocortisone and saturated in flunisolide. Estimation of the clogP (logP is the partition coefficient in *n*-octanol/water system) was performed by using the computational software Pallas 1.2 based on the fragmental model (Rekker, 1997).

apical (luminal) to basolateral (serosal) direction, indicating the presence of an active efflux system. The presence of P-glycoprotein (Pgp) efflux pumps in mammalian lung tissue have been previously demonstrated (Arvelo *et al*., 1995; Nooter *et al*., 1996; Oberli *et al*., 1994), and might play a key role in the active transport mechanism of flunisolide. As members of the ATP-Binding Cassette (ABC) superfamily, P-glycoproteins are ATP-dependent drug efflux pumps initially identified as proteins that confer multidrug resistance (MDR) (Gottesman *et al*., 1993). The gene products of MDR1 are Pgp (140–180 kDa) plasma membrane glycoproteins that actively decrease the intracellular concentrations of a wide variety of structurally diverse chemotherapeutic agents with preference for relatively hydrophobic, amphiphilic drugs, whereas the closely related MDR2-Pgp are not associated with drug resistance (Ruetz *et al*., 1994). Based on the observation that Pgp is expressed in the apical membrane of mucosal cells in the intestine, in the brush border of the renal proximal tubules, in the biliary membrane of hepatocytes and in capillary endothelial cells of brain and testes (Cordon Cardo *et al*., 1989; Schinkel *et al*., 1994; de Boer *et al*., 1998; Sugawara *et al*., 1988; Thiebaut *et al*., 1987), a role for Pgp in the protection of the organism from xenobiotics has been proposed (Schinkel *et al*., 1995).

The aim of this study was to investigate the role of MDR1-Pgp in the transport mechanism of the synthetic glucocorticoid flunisolide across Calu-3 cells and in the polarized epithelial pig kidney cell lines LLC-PK1 (Gstraunthaler *et al*., 1985) and MDR1-Pgp transfected LLC-MDR1 (Schinkel *et al*., 1995). The present results showed that flunisolide is transported unmetabolized across Calu-3 cell monolayers and that the polarized transport is ATP dependent and susceptible to inhibition by Pgp blockers. Furthermore, Western blotting and confocal microscopy studies were used to demonstrate the presence and localization of Pgp in Calu-3 cells.

Methods

Chemicals

Flunisolide® (6α-fluor-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione-16,17-acetonide) was a gift from Boehringer Ingelheim (Ingelheim am Rhein, Germany). The Pgp inhibitors LY335979 (Eli Lilly Comp., Indianapolis, IN, U.S.A.), SDZ PSC 833 (Novartis, Basel, Switzerland) and the monoclonal antibody C219 (Dako Corp., Santa Barbara, CA, U.S.A.) directed against Pgp, were kindly supplied by the Division of Pharmacology, LACDR, Leiden University. Hank's Balanced Salt Solution (HBSS, pH 7.4) and Dulbecco's Modified Eagle Medium (DMEM, 4.5 g l^{-1} glucose, pH 7.4) were from Gibco–BRL (Basel, Switzerland). N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES), sodiumazide (NaN_3), 2-deoxy-D-glucose (2-d-glu), verapamil, and all other chemicals of analytical grade were obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands).

Cell culture

LLC-PK1 and LLC-MDR1 cells were kindly provided by the Division of Pharmacology, LACDR, Leiden University and were cultured as previously described (van der Sandt *et al*., 2000) on Transwells® (Corning, Schiphol-Rijk, The Netherlands). Calu-3 cells (#HTB-55) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.) at passage number (PN) 19. The experiments were performed in 18 days old, differentiated and polarized Calu-3 cells of PN 20 to PN 62. Calu-3 cells were seeded at a seeding density of 1×10^5 cells cm^{-2} on collagen-coated Transwells® and grown either at an air interface (i.e. apical culture medium removed after 1 day in culture) or in submerged state (i.e. culture medium present at both apical and basolateral side) at 37°C in a 90% humidified incubator and 5% CO_2 (Meaney *et al*., 1999). The tightness of the cell monolayers was assessed by measuring transepithelial electrical resistance (TEER) using a Millicell®-ERD apparatus equipped with chop-stick electrodes (Millipore Corp., Bedford, MA, U.S.A.).

Transport studies

Prior to the actual transport studies, the cell culture medium was removed and the cells were allowed to equilibrate in HBSS buffered with HEPES (30 mM, pH 7.4). After 2 h, 2 ml of a flunisolide solution (115 μM) in HBSS/HEPES (30 mM HEPES, pH 7.4) was applied in the donor compartment and samples of 200 μl were withdrawn from the acceptor chamber at $t = 10$, 20, 30, 40, 50, 60, 80, 100, 120, 150 and 180 min. The TEER was measured before and after the experiment. According to the experimental set-up, the experiments were performed at 37°C or at 4°C, respectively. The samples were analysed by isocratic h.p.l.c. analysis (see below). The active transport inhibition studies were performed at 37°C by

incubation with ATP synthesis inhibitors NaN_3 (3 mM) and 2-deoxy-D-glucose (50 mM), the general ATP-Binding-Cassette (ABC) inhibitor verapamil (20 μM), or the specific Pgp inhibitors, SDZ PSC 833 (1 μM) and LY335979 (1 μM).

H.P.L.C. analysis and mass spectrometry

Samples from transport studies were analysed using an isocratic h.p.l.c. analysis method on a Spectra Physics (Eindhoven, The Netherlands) P200 h.p.l.c.-system. A reversed phase ChromSpher (Varian Chrompack Benelux, Bergen op Zoom, The Netherlands) C18 column (10×3.0 mm, particle size 5 μm) was used as stationary phase and the mobile phase consisted of an aqueous 1% (v v^{-1}) acetic acid solution and acetonitrile (73 : 27 v v^{-1}). At a flow-rate of 1.0 ml min^{-1}, using a 100 μl injection loop and UV-detection at 240 nm, the retention time (t_{ret}) was 5.5 min and the detection limit was 50 ng ml^{-1}.

In order to assess the chemical stability of flunisolide after transport across Calu-3 cells, a random selection of samples from the transport studies were analysed by direct infusion mass spectrometry (MS) using a Finnigan MAT900 (San José, CA, U.S.A.) mass spectrometer. The method is based on an electro-spray interface followed by soft negative ionisation of the analytes. The electro-sprayed droplets made brief contact with high temperature (200°C) leading to instantaneous evaporation of the sheet fluid, 0.1% (v v^{-1}) acetic acid and acetonitrile (90/10), resulting in the attachment of an acetate anion to the analytes and rendering the complex negatively charged. The m/z ratios were determined by the mass spectrometer.

Cell survival

Cell survival of confluent, 18 days old, Calu-3 cells was assessed by an MTT colorimetric assay in 96-well plates (Ax *et al*., 2000; Holt *et al*., 1987). Prior to the 1 h MTT (5 mg ml^{-1} in HBSS/HEPES) treatment, the Calu-3 cells were exposed for 2 h to flunisolide, ATP synthesis or Pgp inhibitors at the same end concentrations used for the transport experiments. After lysis of the cells in NaOH/SDS, 0.01/1.0% (w v^{-1}), the absorbance was measured at 590 nm in a Bio-Rad 96-well plate reader (Alphen a/d Rijn, The Netherlands). Values of eight measurements were normalized to 100% for the control group (exposure to transport medium HBSS/HEPES).

Western blotting for Pgp

Calu-3, LLC-PK1 and LLC-MDR1 cells were investigated for the presence of the 170 kDa Pgp by Western blot analysis (Schinkel *et al*., 1991). The cells were grown in 75 cm^2 culture flasks lyzed using TSE (10 mM Tris-HCl, 250 mM sucrose 1 mM, EGTA; pH 7.4) or lysisbuffer (50 mM Tris-HCl, 150 mM NaCl, 1% nonidet p-40; pH 8.0) both supplemented with inhibitors (1 μM DTT, 0.005 μg ml^{-1} leupeptin, 0.01 μg ml^{-1} aprotinin, 10 μM orthovanadate, 50 μM NaF and 5 μM PMSF). Protein concentrations were determined using Bradford reagent (Bio-Rad, Alphen a/d Rijn, The Netherlands). Proteins (25 μg) were resolved by 7.5% SDS-acryl/bisacrylamide gel electrophoresis and transferred to Immobilon™-P membranes (Millipore, Badford, M.A., U.S.A.). The blots were blocked overnight in TBS-T (5 M NaCl, 200 mM Tris, 1% Tween 20; pH 7.4) with 5% protifar at 4°C, washed, incubated for 1 h at room temperature with the monoclonal antibody C219 (mouse-anti-Pgp) in TBS-T (2% protifar), and incubated at room temperature for 1 h with a Horse radish Peroxidase labelled goat-anti-mouse second antibody (Jackson Immuno Research Laboratories Inc., West Grove, PA, U.S.A.) in TBS-T (2% protifar) under constant shaking. Antibody binding was visualized with the ECL™-kit Western blotting detection reagents (Amersham Pharmacia Biotech, Rosendaal, The Netherlands).

Confocal Laser Scanning Microscopy (CLSM)

Calu-3 and LLC-MDR-1 cells were grown on collagen coated glass coverslips, fixed with 3.7% formaldehyde in PBS for 10 min, blocked with 0.1% Triton (v v^{-1})/0.5% BSA (w v^{-1}) for 60 min at 4°C, incubated with the murine monoclonal antibody C219 for 60 min, washed and incubated with a M-α-FAK second antibody labelled with Alexa488 (Zymed, CA, U.S.A.). After a 5 min postfixation in 3.7% (v v^{-1}) formaldehyde, the nuclei were stained for 15 min with Hoechst 33258 (2 μg ml^{-1}) in PBS. Staining for Pgp was visualized by a Bio-Rad Confocal Laser Scanning Microscope (Bio-Rad, Alphen a/d Rijn, The Netherlands). The confocal pictures were obtained by scanning the Calu-3 cell monolayer in the x,y plane with a z-step of 20 nm from the apical to the basolateral side and the x,z plane was reconstituted by the Comos (BioRad) software.

Data analysis and statistics

The transport of flunisolide across the epithelial cells is expressed as the percentage transported (% $cm^{-2}\,h^{-1}$) or permeability (cm sec^{-1}). Calculation of the permeability (P) has the advantage of being independent of experimental design, surface area, time of experiment and drug concentration. P is calculated using equation 1:

$$P = k \cdot V_R / A \cdot 60 \quad (1)$$

The volume in the receiver chamber V_R (ml) was kept at 2 ml for all experiments and the surface area A was determined by the filter size (4.71 cm^2). The transport rate constant k (min^{-1}) was determined by linear regression from the linear part of the Cumulative-Fraction-Absorbed (FA_{cum}) versus time. FA_{cum} was calculated by a summation of the amount of flunisolide transported across the cell monolayer in time (m_i) divided by the total amount applied in the donor chamber as shown in equation 2:

$$FA_{cum} = \sum m_i / m_{tot} \quad (2)$$

Estimation of the hydrophobicity of a compound is given by the logP, where P is the partition coefficient in an *n*-octanol/water system. We have estimated the clogP value of several glucocorticoids used for inhalation by using the Pallas 1.2 computational software (AH Systems Group, MD, U.S.A.) based on the fragmental model (Rekker, 1977).

Data are presented as means $\pm$ s.d. Comparison tests were performed by using an unpaired Student's *t*-test (2-tailed). *P* values <0.05 were considered significant.

Results

The chemical modifications of the hydrocortisone backbone used for the development of synthetic corticosteroids with potent topical anti-inflammatory activities are presented in Figure 1. The estimated hydrophobicity values of the compounds (clogP) is calculated by predicting the sum of fragmental values according to the fragment model (Rekker, 1977). Flunisolide is an uncharged molecule, due to absence of basic or acidic functional groups, and shows a low hydrophobicity value. Flunisolide is halogenated on the six position and displays amphiphatic characteristics due to the presence of a lipophilic, aromatic ring at one side and the more hydrophilic, electronegative acetonide moieties at the opposite side of the molecule. These data suggest that flunisolide meets the criteria necessary for being a substrate for Pgp, as reviewed by Meijer *et al*. (1997).

Transport studies of flunisolide in Calu-3 cell monolayers at 37°C

The cumulative transport of flunisolide during transport experiments across Calu-3 cell monolayers, cultured at an air-interface (air) or under submerged (sub) conditions and performed at 37°C are presented in Figure 2a,b. Data clearly show that the flunisolide transport is polarized in the apical to basolateral direction. The ap→bl transport was linear ($R^2=0.94$) during 90 min before achieving a steady-state situation. The bl→ap transport was linear ($R^2=0.96$) over a period of 180 min. No significant differences in transport were observed between cells grown at an air-interface and under submerged conditions, indicating that the activity of the transporter is comparable for both culture conditions. The integrity of the cell monolayers remained intact as no decrease in TEER was detected (begin 440 ± 25 Ωcm^2, end 480 ± 30 Ωcm^2) at the end of the experiment.

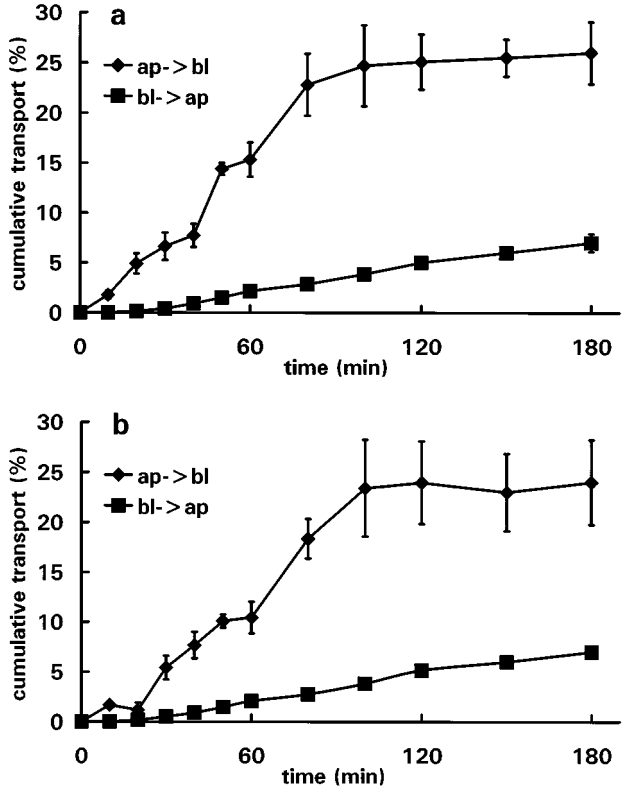


Figure 2 Cumulative transport of flunisolide (%) for air interface (a) and submerged (b) grown Calu-3 cell monolayers at 37°C. Flunisolide was tested at 115 μM final concentration. At t=0 flunisolide was applied in the donor compartment (apical or basolateral) and the percentage of flunisolide appearing in the acceptor compartment at t=10, 20, 30, 40, 50, 60, 80, 100, 120, 150 and 180 min was measured by h.p.l.c. and plotted. Data represent mean±s.d. from three experiments and triplicate determination.

Investigation of the active flunisolide transport across Calu-3 cell monolayers

In order to further investigate the active mechanism responsible for the apical to the basolateral transport of flunisolide, transport studies were performed under control conditions (37°C), at low temperature (4°C) as general inhibitor of the cellular metabolism, or by using NaN_3 (3 mM) and 2-deoxy-D-glucose (50 mM) as metabolic inhibitors of ATP synthesis. Figure 3a shows that at 4°C the permeability of flunisolide in the ap→bl direction was significantly decreased to the same magnitude as the bl→ap permeability. In contrast to the data obtained at low temperature, in the presence of NaN_3 and 2-deoxy-D-glucose the ap→bl permeability of flunisolide was similar to the control situation and bl→ap permeability was significantly increased to the same value as the control. Furthermore, the integrity of the cell monolayers was intact, as no decrease in TEER values were detected (data not shown). These studies clearly show that the polarized transport of flunisolide across air-interface and submerged grown Calu-3 cell monolayers involves an ATP dependent process.

Transport studies of flunisolide in LLC-PK1 and LLC-MDR1 cell monolayers

In order to investigate the substrate specificity of the relatively hydrophilic glucocorticoid flunisolide for Pgp, we have used the well established LLC-PK1 cells as Pgp negative control and LLC-MDR1 cells as Pgp positive control for performing transport studies. Table 1 gives an overview of the flunisolide permeability across Calu-3 (air and sub), LLC-PK1 and LLC-MDR1 cell monolayers. In LLC-MDR1 cells, flunisolide undergoes a polarized bl→ap transport ($P<0.05$) due to Pgp expression at the apical side of the plasma membrane, while flunisolide permeability in LLC-PK1 cells is similar for both transport routes. In contrast to LLC-MDR1 cells, the permeability of flunisolide in Calu-3 cells is polarised in the ap→bl direction. No decrease in TEER was detected at the end of the transport studies (data not shown). The permeability data in LLC-MDR1 indicate that flunisolide is a substrate for active efflux by Pgp.

Influence of Pgp inhibitors on the transport of flunisolide in Calu-3 cells

For studying the involvement of Pgp in the polarised transport of flunisolide across Calu-3 cells, we have used the Pgp inhibitors verapamil, PSC-SDZ 833 and LY335979 (Tsuruo *et al*., 1981; Bezombes *et al*., 1998; Boesch *et al*., 1991; Dantzig *et al*., 1999). Figure 3b shows the permeability

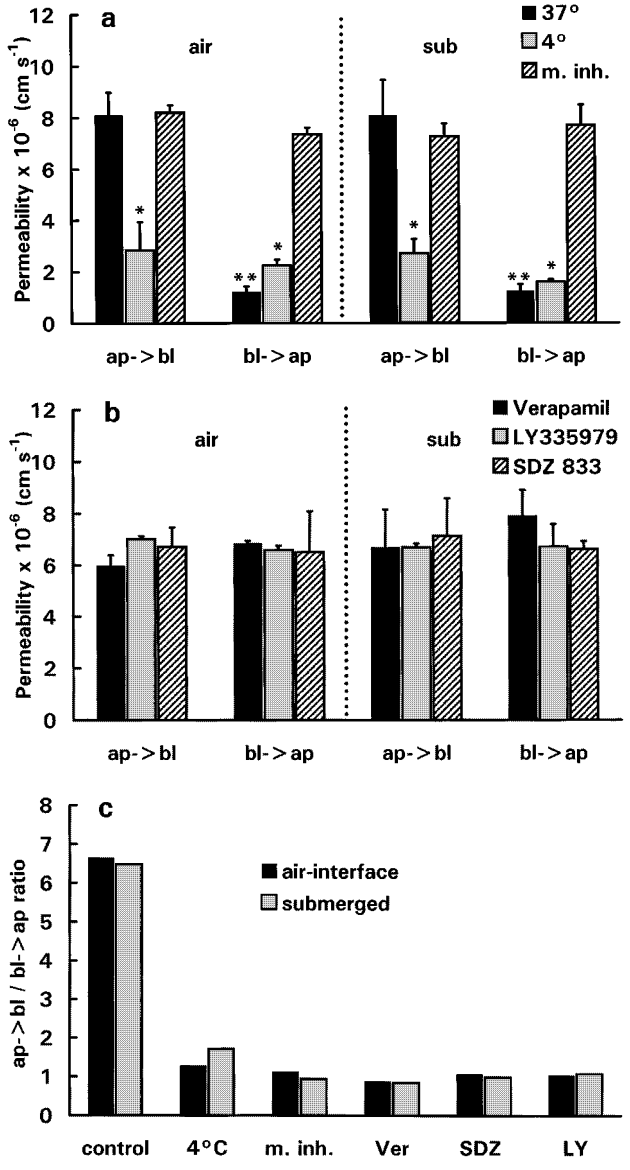


Figure 3 Permeability of flunisolide (115 μM) across Calu-3 cell monolayers for the ap bl and bl ap direction as calculated from transport experiments. (a) shows the permeability data in the control situation (37°C), the suppression of cell metabolism at 4°C and in the presence of the ATP production inhibitors sodium azide (3 mM) and 2-deoxy-D-glucose (50 mM). (b) shows the effect of Pgp inhibitors as verapamil (20 μM), LY335979 (1 μM) and SDZ PSC 833 (1 μM) on the permeability of flunisolide. In panel c, the ap→bl/bl→ap ratios are depicted for control, metabolism inhibition and Pgp inhibition of the flunisolide transport across air and sub grown Calu-3 monolayers. Data represent mean ± s.d. from three experiments and triplicate determination. Data were compared by using an unpaired two-tailed Student's *t*-test, differences were considered to be significant when $P<0.05$; * significantly lower than control, $P<0.05$; ** significantly lower than control, $P<0.01$.

of flunisolide across Calu-3 cells in the control situation (37°C) and in the presence of specific Pgp inhibitors. The polarized transport of flunisolide was completely abolished by the inhibitors, demonstrating that Pgp is involved in the ap→bl transport of flunisolide. The integrity of the cell monolayers was not disrupted, as no decrease in TEER was detected (data not shown).

Cell survival

The effect of flunisolide, inhibitors of ATP synthesis and Pgp inhibitors on the viability of Calu-3 cells was tested by using the MTT test. The cell survival data are presented in Figure 4. The metabolic inhibitors NaN_3 and 2-D-deoxy-glucose significantly reduced ($P<0.01$) the mitochondrial activity of Calu-3 cells to 23 ± 12% of the control value. Flunisolide and the Pgp inhibitors verapamil, SDZ PSC833 and LY335979 showed a slight but not significant reduction in cell viability.

Immunoblot analysis and CLSM visualization of Pgp

The levels of MDR1-Pgp in cell lysates of LLC-PK1, LLC-MDR1 and Calu-3 cells were analysed by immunoblotting. In Figure 5, the 170 kDa Pgp band was present in LLC-MDR1 and Calu-3 cells and was absent in LLC-PK1 cells. Pgp is present in Calu-3 cells after 7 days in culture and the Pgp levels were increased in fully differentiated 19 days old cells.

For the CLSM studies, LLC-PK1, LLC-MDR1 and Calu-3 cells were fixed and the Pgp levels were visualized by a sandwich of the C219 monoclonal antibody against Pgp and an Alexa labelled goat anti mouse antibody. The x,y planes in Figure 6a,c show that in Calu-3 and LLC-MDR1 cells Pgp is present as compact (green) dots in the plasma membranes but also in the cytoplasm (nuclei are stained red). The x,z planes in Figure 6b,d show that in Calu-3 cells Pgp is predominantly located at the basolateral membrane, in contrast to LLC-MDR1 cells where Pgp is predominantly located at the apical membrane.

Mass spectrometry

Figure 7 shows a representative mass spectrum of flunisolide containing samples taken from the donor compartment at the end of transport studies. The molecular weight of Flunisolide is 435 Da and after the soft negative ionization it is complexed to an acetate anion with a M_w of 59 resulting in an m/z ratio of 494. All other peaks in the mass spectrum are memory peaks of the standards used for calibration. The mass spectrum was recorded over a wide range to assess the presence of flunisolide metabolites or glutathion (GSH) complexes. The theoretical m/z ratio of a flunisolide-GSH complex is 802, but no such peak was detected. The data demonstrate that flunisolide is transported unchanged across Calu-3 cell monolayers.

Discussion

Numerous clinical studies have shown the benefits of synthetic corticosteroids for the treatment of asthma or COPD (Rodrigo *et al*., 1998; Jarad *et al*., 1999; Jeffery, 1998; Keatings *et al*., 1996; Paggiaro *et al*., 1998). Despite the great efforts towards an optimisation of the chemical structure, bio-availability and bio-transformation of synthetic corticosteroids, little is known about the mechanism underlying their absorption in the pulmonary system. Nowadays, transport across epithelial barriers can easily and accurately be studied in cell culture systems cultivated on permeable membranes. Calu-3 cells have recently been suggested as an appropriate model for the nasal and airway epithelium (Foster *et al*.,

Table 1 Permeability (P) of flunisolide across Calu-3, LLC-PK1 and LLC-MDR1 cell monolayers

	$P_{ap\ bl}$ (10^{-6} cm s^{-1})	$P_{bl\ ap}$ (10^{-6} cm s^{-1})	
Calu-3 (sub)	8.05±1.4	1.25±0.1	$P<0.01$
Calu-3 (air)	8.11±0.9	1.22±0.2	$P<0.01$
LLC-PK1	9.69±0.5	9.22±1.5	ns
LLC-MDR1	7.03±0.4	10.4±1.0	$P<0.01$

Permeability (ap→bl and bl→ap) of flunisolide was calculated by linear regression of the linear part of the cumulative transport data of flunisolide (115 μM) at 37°C. Data represent mean±s.d. from three experiments and triplicate datermination. Data were compared by using a unpaired two-tailed Student's t-test, differences were considered to be significant when $P<0.05$; ns, not significant.

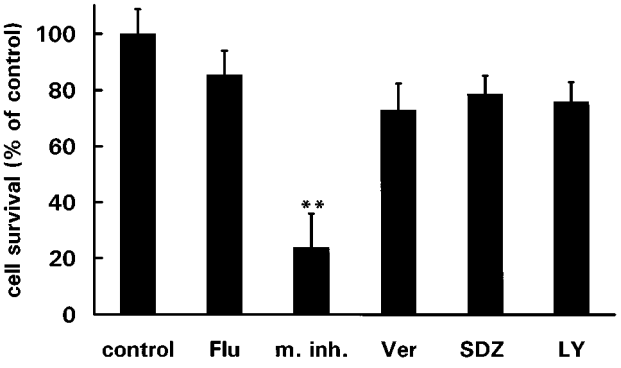


Figure 4 Survival of 18 days old, confluent Calu-3 cells. The cells were exposed to HBSS/HEPES (control), flunisolide (115 μM), ATP production inhibitors NaN_3 (3 mM) and 2-deoxy-D-glucose (50 mM), verapamil (20 μM), LY335979 (1 μM) and SDZ PSC 833 (1 μM), for 2 h followed by an 1 h incubation with MTT (5 mg ml^{-1}). After cell lysis, the optical absorption was assayed at 590 nm in a plate reader. Data represent mean±s.d. from three experiments and octaduplicate determination. Data were compared by using an unpaired two-tailed Student's t-test, differences were considered to be significant when $P<0.05$; ** significantly lower than control, $P<0.01$.

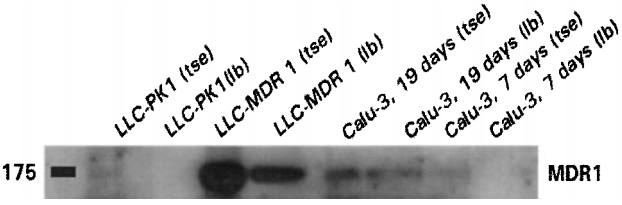


Figure 5 Western blot analysis of LLC-PK1, LLC-MDR1 and Calu-3 (7 and 19 days in culture) cell lysates. Membrane proteins (25 μg) were resolved on 7.5% SDS-acryl/bisacrylamide and transferred to nitrocellulose by electroblotting. Protein levels were analysed with the C219 monoclonal antibody raised against MDR1. Primary antibody was visualized by enhanced chemiluminscence. Size of the molecular weight marker is indicated in kDa; lb=lysis buffer, tse=10 mM Tris-HCl, 250 mM sucrose, 1 mM EGTA, pH 7.4.

2000; Witschi & Mrsny., 1999; Winton *et al*., 1998) as they form tight monolayers, expressing the tight junction associated protein ZO-1 and the adherin protein E-cadherin, present apical villi and produce mucous secretions.

In this study, flunisolide was used as model corticosteroid for performing transport studies across Calu-3 cell mono-

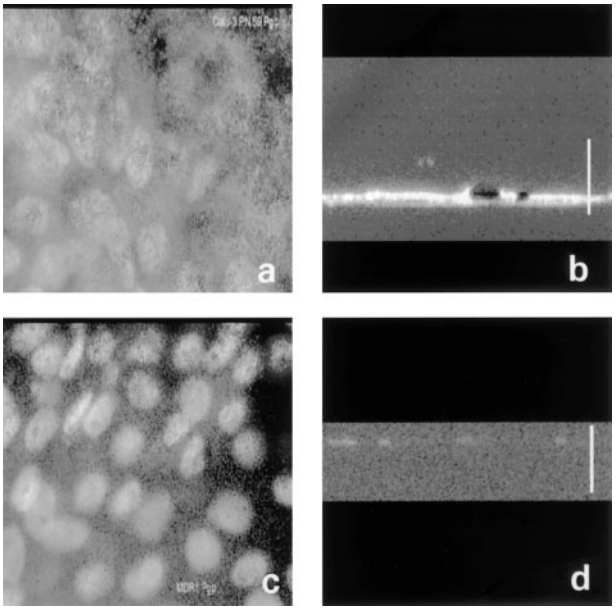


Figure 6 Representative confocal photomicrographs showing immunostaining for MDR1 (green) and nuclei (red) in Calu-3 (a) and LLC-MDR1 (c). The cells were grown on coverslips for 19 days (Calu-3) and 3 days (LLC-MDR1), and treated as described under Methods. The C219 primary antibody was visualized by a M-α-FAK second antibody labelled with Alexa488. (b) and (d) represent CLSM vertical xz cross sections across Calu-3 and LLC-MDR1, respectively, going from the apical to the basolateral direction. Notice the label distribution at the basolateral side of the plasma membrane in Calu-3 cells and at the apical side in LLC-MDR1 cells. Calibration bar=20 μm.

layers cultured in air and sub conditions, which proved to have similar transport characteristics. Under control conditions at 37°C, the transepithelial transport of flunisolide across Calu-3 cell monolayers showed to be polarized in the ap→bl direction. At low temperature (4°C), the permeability of flunisolide in the ap→bl direction decreased significantly ($P<0.01$) to the same level as in the bl→ap direction, which slightly increased. The main disadvantage of the non-specific metabolic inhibition by lowering the temperature is that the rigidity of the cell membranes is increased due to reduced fluidity of the plasma membrane phospholipids (Bates *et al*., 1985) and the diffusion rate of compounds in solution is decreased, hence the diffusion rate is temperature dependent. These physical conditions will eventually result in an overall lower permeability of flunisolide across Calu-3 cell monolayers and may bias the interpretation of the results. The use of metabolic inhibitors of the ATP synthesis is a more elegant method to investigate ATP-dependent transport phenomena. Efficient inhibition of the intracellular ATP synthesis is achieved when using 2-deoxy-D-glucose as inhibitor of the Krebs cycle, and sodium azide that uncouples the oxidative phosphorylation route. The polarized transport of flunisolide was again abolished in this case, due to an increase of the bl→ap permeability to the same level as the ap→bl permeability. These data clearly demonstrate that the polarized transport of flunisolide across Calu-3 cell monolayers involves an ATP-driven export mechanism.

The chemical structural features of flunisolide as amphiphatic molecule and halogenation, combined with the initial transport and inhibition data, lead to the hypothesis that

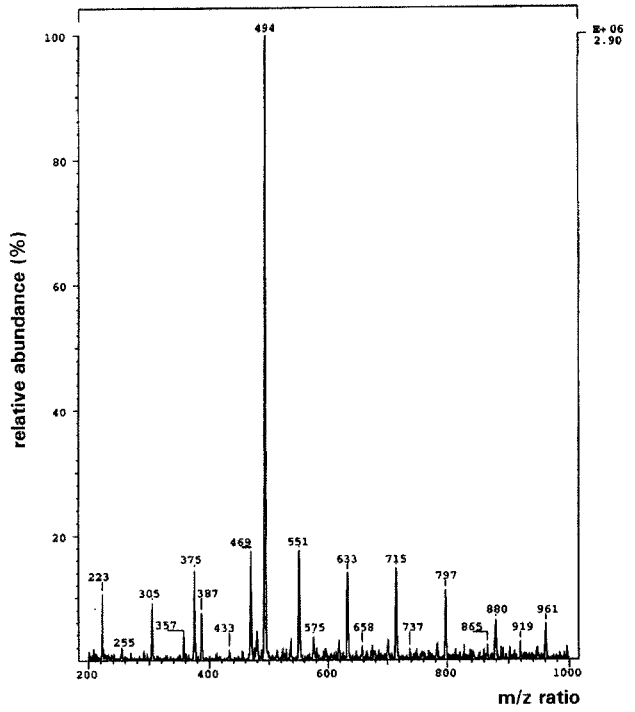


Figure 7 Typical direct flow infusion mass spectrum of flunisolide containing samples from transport studies. Peak at m/z ratio of 494 is ascribed to the flunisolide/acetate complex. All other peaks are reference compounds used for calibration. Notice the absence of a peak at m/z ratio 802, the theoretical molecular weight of a flunisolide/gluthation/acetate complex.

MDR1-Pgp might be involved in the polarized ap→bl transport of flunisolide in Calu-3 cells. Luker *et al.* (1999) demonstrated the involvement of Pgp in the intracellular trafficking of cholesterol from the membrane pool towards the endoplasmatic reticulum (ER), enhancing cholesterol esterification, and being susceptible to inhibition by steroids like progesteron and dehydroepiandrosterone. Steroid hormones have previously been shown to interact with MDR-1Pgp and inhibit their activity (Qian *et al.*, 1990; Yang *et al.*, 1989). After investigating 12 steroid hormones, Debry *et al.* (1997) concluded that the ability of a steroid to inhibit Pgp activity is strongly correlated to the general hydrophobicity of the steroid. The great chemical resemblance between flunisolide and cholesterol or steroids, supports the hypothesis that Pgp is involved in the polarised transport of flunisolide.

In order to test this hypothesis we have performed transport studies with flunisolide across LLC-PK1 and LLC-MDR1 cell monolayers. The LLC-PK1 (Gstraunthaler *et al.*, 1985) cell line is a pig kidney epithelial cell line that is widely used for investigating the expression, cellular localization and kinetic characteristics of various efflux pumps. By transfecting cDNA coding for Pgp into the LLC-PK1 cells, the LLC-MDR1 cell line is created that expresses Pgp at the apical membrane of the cells (Schinkel *et al.*, 1995). Permeability data showed a polarized bl→ap transport of flunisolide in LLC-MDR1 cells that was absent in Pgp naïve LLC-PK1 cells. These results clearly demonstrate that flunisolide is a substrate for Pgp efflux.

The physiological role of Pgp in various tissues is still unclear. There is strong evidence that Pgp is closely involved in detoxification mechanisms of oncogenic cells, in the trafficking of cholesterol from the plasma membrane to the endoplasmatic reticulum and flippase activity for phospholipids (Gottesman *et al.*, 1993; Schinkel *et al.*, 1995; 1997; Bezombes *et al.*, 1998; Luker *et al.*, 1999). It has also been shown that Pgp displays a preference for relatively hydrophobic and amphiphilic drugs (Meijer *et al.*, 1997). The presence of Pgp in the lung has been shown by Sugawara *et al.* (1988), but its role in drug transport across the lung epithelium remains uncertain. We have used the Pgp inhibitors verapamil, SDZ-PSC 833 and LY335979 as pharmacological tools for studying the influence of Pgp on the transport of flunisolide across Calu-3 cells. Figure 3c shows the ap→bl/bl→ap permeability ratios where a ratio of 1 indicates no polarised transport. Despite of the lowering of the metabolic rate at 4°C and the inhibition of ATP synthesis by sodium azide and 2-deoxy-D-glucose, the ratios of flunisolide transport range between 1 and 2 units, showing that ATP depletion does not fully inhibit the Pgp activity. Although the TEER did not decrease, the cell survival measured by the MTT test shows a strong loss in viability that as a consequence is jeopardizing the integrity of the cell monolayers leading to increased permeability. On the other hand, the specific Pgp inhibitors verapamil, SDZ-PSC 833 and LY335979 showed ratios of exactly 1 unit suggesting that Pgp is involved in the transport of flunisolide across Calu-3 cell monolayers. The cell viability data from Figure 4 shows that the transport of flunisolide is not caused by toxic effects of the compounds on the Calu-3 cells, indicating that the observed transport is not due to a reduced integrity of the monolayers. Evidence for the integrity of the monolayers is also demonstrated by the observation that no decrease in TEER was detected at the end of the experiments (data not shown). Furthermore, Western blot analysis and *in-situ* hybridization studies using the C219 monoclonal antibody against Pgp have demonstrated the presence of Pgp in the Calu-3 cells and strong evidence was found for the basola teral localization of Pgp.

In accordance with our findings, immunocytochemical studies using the C219 monoclonal antibody, have demonstrated the distribution of Pgp in the basolateral membranes of serous acinar cells in human major and minor salivary glands (Uematsu *et al.*, 2001). It appears that basolateral Pgp in these cells is involved in the efflux of xenobiotics stemming from the interstitial fluid. We suggest that a similar mechanism is present in the Calu-3 cell line, which was derived from human submucosal gland cells.

Our findings are in contrast to the general acceptance that Pgp is mainly expressed at the apical side of epithelial cells that form a penetration barrier to exclude xenobiotics from entering the main circulation (Gottesman *et al.*, 1993; Schinkel *et al.*, 1995). In a recent study, Hamilton *et al.* (2001) have shown that the efflux of Rhodamine 123 in Calu-3 cells is polarized in the basolateral to apical direction, suggesting the presence of Pgp in the apical membrane of Calu-3 cells. However, previous studies (van der Sandt *et al.*, 2000, Masereeuw *et al.*, 1997) have demonstrated that transport data of Rhodamine 123 in cell lines expressing both Pgp and the organic cation carrier system (OCT) should be carefully interpreted. The presence of OCT in Calu-3 cells has not been demonstrated yet and its possible involvement in the transport of Rhodamine 123 across Calu-3 cells is therefore not fully understood.

In the past decade, more efflux pumps have been described in the literature. Next to Pgp, the Multi-drug Resistance Proteins (MRP) have been investigated and characterized. MRPs are transporters of multivalent organic anions, preferentially glutathione *S*-conjugates (Roelofsen *et al*., 1999). Flunisolide is metabolized to its 6β-OH metabolite by mouse liver microsomes, but no metabolizing activity is observed with mouse lung, intestine or kidney microsomes (Teitelbaum *et al*., 1981) indicating an unmodified transport across the lung tissue. The involvement of MRPs in the clearance of flunisolide is unlikely because, as Figure 7 shows, flunisolide is transported unmetabolized across the Calu-3 cell monolayers.

The pharmacokinetic profile of flunisolide in humans shows a fast absorption phase (Mollmann *et al*., 1997) and a short dwell-time in the pulmonary tissue (Derendorf *et al*., 1998) which has been related to high pulmonary solubility of flunisolide. The human submucosal gland adenocarcinoma cell line Calu-3 is a suitable cell line for the investigation of transport processes of corticoids in the upper airways of the respiratory system. The presence of MDR1-P-glycoprotein in Calu-3 cells was determined by Western blot analysis and *in-situ* hybridization. Flunisolide was found to be a substrate for Pgp and the transport across Calu-3 was polarized in the apical to the basolateral direction. We have demonstrated the presence of Pgp or a Pgp-related transporter at the basolateral side of Calu-3 cell monolayers, which is sensitive to inhibition by the specific Pgp inhibitors SDZ PSC 833 and LY335979. In conclusion, our studies provide the new insight that the active ab→bl transport of flunisolide is responsible for the transport phenomena that has a profound impact on the clinical use of corticosteroids in asthma therapy.

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