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Research Paper

In-vitro respiratory drug absorption models possess nominal functional P-glycoprotein activity

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Abstract

Objectives The P-glycoprotein (P-gp) efflux pump is known to be present within several major physiological barriers including the brain, kidney, intestine and placenta. However, the function of P-gp in the airways of the lung is unclear. The purpose of this study was to use the highly specific P-gp inhibitor GF120918A to investigate the activity of the P-gp transporter in the airways to determine whether P-gp could influence inhaled drug disposition.

Methods P-gp activity was measured as a change in digoxin transport in the presence of GF120918A in normal human bronchial epithelial (NHBE) cells, Calu-3 cell layers and the ex-vivo rat lung.

Key findings The efflux ratios (ERs) in NHBE and Calu-3 cells were between 0.5 and 2, in contrast to 10.7 in the Caco-2 cell control. These low levels of GF120918A-sensitive polarised digoxin transport were measured in the absorptive direction in NHBE cells (ER = 0.5) and in the secretory direction in Calu-3 cells (ER = 2), but only after 21 days in culture for both cell systems and only in Calu-3 cells at passage >50. The airspace to perfusate transfer kinetics of digoxin in the ex-vivo rat lung were unchanged in the presence of GF120918A.

Conclusions These results demonstrated that although low levels of highly culture-dependent P-gp activity could be measured in cell-lines, these should not be interpreted to mean that P-gp is a major determinant of drug disposition in the airways of the lung.

Keywords airways; drug disposition; normal human bronchial epithelium; P-gp; transport

Introduction

The airways of the lung provide an excellent opportunity for drug delivery as they possess an extensive air–epithelium interface which allows almost instantaneous drug uptake and onset of action.[1] However, administering a clinically relevant dose of therapeutic agent reproducibly to a target site within the airways remains a technical challenge due to the innate ability of the lung to filter and remove particulate matter. Over the last decade considerable effort has been expended in generating and administering drug rich aerosols with ever-increasing efficiency. This has resulted in the development of inhaled systems that deliver over 50% of their dose to the distal regions of the lungs.[2] However, improving drug deposition efficiency may not always translate into a more effective inhaled medicine, as the clinical effects are not only dependent upon effective aerosol generation and deposition, but also effective regional or systemic disposition.

Once a therapeutic agent is dissolved in the lining fluid of the airways it is subject to multiple processes that determine retention within the mucosa or absorption to the systemic circulation. The clearance mechanisms that operate at the epithelial surface include mucociliary clearance, inactivation by macrophages, presystemic metabolism, absorption and physicochemical degradation.[3] As it is impossible to deconvolute the post-deposition processes in the lung using in-vivo models, a wide range of in-vitro systems has been developed. The three most commonly employed in-vitro disposition models are: immortalised respiratory epithelial cell lines; primary cultures of epithelial cells; and the isolated perfused lung.[3] Each of these systems is capable of measuring absorptive drug

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transport and discriminating between passive diffusional flux and active transport mechanisms.

Transporter proteins in the gastrointestinal tract, placenta, airways and blood–brain barrier can actively transport a wide range of chemicals and therefore have the potential to influence drug disposition. The *mdr*1 P-glycoprotein (P-gp), a 170 kDa membrane bound protein that acts as an ATP-dependent efflux pump, is one of the most studied epithelial drug transporters in the airways of the lung.[4–10] However, previous attempts to characterise P-gp expression and function in the respiratory epithelium *in vitro* have generated contradictory results and therefore the importance of P-gp in inhaled drug delivery is debatable (Table 1).[11–16] In Calu-3 cells for example, Florea *et al.*[11] demonstrated the location of P-gp to be predominantly serosal, using confocal laser scanning microscopy, and found the absorptive transport of flunisolide to be enhanced, whereas Hamilton *et al.*[13] detected the majority of P-gp expression on the mucosal surface of the cell membrane, using immunofluorescent staining, and found rhodamine 123 to be subject to secretory efflux.[11,13,17]

The heterogeneity of the previously reported data on P-gp activity in the airways is probably a consequence of the different cell culture conditions, the use of different substrates and most importantly the use of nonspecific P-gp inhibitors. Digoxin is a metabolically stable P-gp substrate that has been used to investigate P-gp activity in the lung.[18] Digoxin is not entirely selective for P-gp as it is also a substrate for organic anion transporting polypeptide transporters, the presence of which in the lung is unclear.[19] Poor transporter selectivity of P-gp substrates is common and complicates the study of P-gp activity, making the use of selective inhibitors essential. The use of GF120918A (the hydrochloride salt of GF120918, 9,10-dihydro-5-methoxy-9-oxo-N-[4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]phenyl]-4-acridine-carboxamide), a third generation selective P-gp inhibitor, will help to resolve this problem.[20] Whilst GF120918A has been used both *in vitro* and *in vivo* to investigate P-gp influence on the disposition of various test molecules, it has not been used previously to investigate P-gp activity in the airway epithelium. Unlike the more commonly used nonspecific inhibitors such as verapamil, GF120918A is highly specific for P-gp and much more potent. For example, the IC50 value (concentration required for 50% inhibition) in murine monocyte leukaemia cells P388/dx for verapamil was 2.91 ± 0.80 μM compared with 0.011 ± 0.004 μM for GF120918A.[21]

The primary objective of this study was to identify functional activity of P-gp in ex-vivo and in-vitro respiratory drug absorption models. The P-gp substrate digoxin and selective P-gp inhibitor GF120918A were used to measure and confirm P-gp activity, respectively. The aim of the study was to determine whether P-gp was active in the respiratory epithelium and could influence the disposition of inhaled xenobiotics. In addition, the influence of culture conditions in generating the conflicting data reported for P-gp activity in

Table 1 Literature reporting the activity of P-glycoprotein in cultured human respiratory epithelial cell layers

Cells	Substrate(s)	Inhibitor(s)	Direction of polarised transport	Efflux ratio	Time in culture (days)	Passage	Other method of P-gp detection	Localisation of P-gp
Calu-3[11]	Flunisolide	NaN_3, 2-deoxy-D-glucose, SDZ PSC 833, LY335979, verapamil, 4°C	A–B	~0.25	18	20–62	Western blot, immunohisto-chemistry	Basolateral
Calu-3[12]	Ciprofloxacin, digoxin, vinblastine	[a]	No polarised transport	Not detected	14–21	21–37	–	–
Calu-3[13]	Rhodamine 123, calcein, acetoxy-methylester	Ciclosporin, vinblastine, taxol, sodium azide	B–A	11.5	13–15	19–40	Western blot	Apical
Calu-3[14]	Ciclosporin, saquinavir, ritonavir	Quinidine	B–A	Ciclosporin :1.6[b]	10	–	Western blot	Apical
16HBE14o-[9]	Rhodamine 123	Verapamil	B–A	2.95	7	47–87	Immunohisto-chemistry	Apical
16HBE14o-[15]	Digoxin	Verapamil	No polarised transport	Not detected	6–20	33–38	–	–
NHBE[16]	Rhodamine 123	Verapamil	B–A	2.95	6-8	2–3	RT-PCR	Apical
Alveolar type I-like cells[10]	Rhodamine 123	Verapamil	B–A	3.09	8	–	Immunohisto-chemistry, RT-PCR, Western blot	Apical

Cell lines included were Calu-3 and 16HBE14o-, primary normal human bronchial epithelial (NHBE) and human alveolar type-I like cells. [a]Norfloxacin, enoxacin, pefloxacin, and ofloxacin used to investigate whether an active transport was involved in ciprofloxacin transport, not actually as P-glycoprotein (P-gp) inhibitors. [b]Bidirectional transport of saquinavir and ritonavir measured in treated cells. RT-PCR, reverse transcription polymerase chain reaction; –, data not available.

respiratory epithelial cells was investigated by comparing different respiratory epithelial cell models under different culture conditions. Caco-2 cell monolayers were used as a positive control for P-gp activity.[22]

Materials and Methods

Materials

Normal human bronchial epithelial (NHBE) (Clonetics, first passage) cells were obtained from Cambrex Bio Science, Inc. (Walkersville, Maryland, US; now Lonza, Basel, Switzerland). Calu-3 and Caco-2 cells were purchased from the American Type Cell Culture Collection (ATCC, Rockville, US). [^{14}C]Mannitol (2.26 GBq/mmol, 3.39 mM) was purchased from Amersham (Amersham, UK) and [^{3}H]digoxin (869.5 GBq/mmol, 42.5 μM) from Perkin Elmer (Beaconsfield, UK). GF120918A was a gift from GlaxoSmithKline (Stevenage, UK). Bronchial epithelial cell basal medium (BEBM), hydrocortisone (0.5 mg/ml), insulin (5 mg/ml), transferrin (10 mg/ml), adrenaline (0.5 mg/ml), triiodothyronine (6.5 mg/ml), gentamicin (50 mg/ml), amphotericin (50 mg/ml), retinoic acid (0.1 ng/ml), epidermal growth factor (0.5 ng/ml human recombinant), bovine pituitary extract (BPE) and 0.1% trypsin-EDTA solution were obtained from Cambrex Bio Science, Inc. Dulbecco's modified Eagle's medium/Nutrient Mixture F12 HAM (DME/F12), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), glutamine (200 mM), penicillin 100 UI/ml–streptomycin 100 μg/ml solution, MEM non-essential amino acids solution (100 ×), 0.22% EDTA/0.25% trypsin solution, Hank's balanced buffer solution (HBSS), NaCl, KCl, $CaCl_2$, $MgSO_4$, $NaHCO_3$, KH_2PO_4, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), D-glucose, bovine serum albumin (BSA), heparin, phosphate buffered saline (PBS), dimethylsulfoxide (DMSO), ammonium acetate and acetone were obtained from Sigma-Aldrich (Poole, UK). Twelve-well polyester Transwell cell culture supports (0.4 μm pore size, 1.13 cm^2) were purchased from Corning Costar (Corning, High Wycombe, UK). Ready Protein$^+$ scintillation fluid was obtained from Beckman Coulter (High Wycombe, UK).

Normal human bronchial epithelial cell culture

Frozen passage-1 NHBE cell stocks were thawed according to the suppliers instructions and cultured in a 162 cm^2 flask using NHBE cell culture medium, e.g. BEBM supplemented with hydrocortisone (0.5 mg/ml), insulin (5 mg/ml), transferrin (10 mg/ml), adrenaline (0.5 mg/ml), triiodothyronine (6.5 mg/ml), gentamicin (50 mg/ml), amphotericin (50 mg/ml), retinoic acid (0.1 ng/ml), epidermal growth factor (0.5 ng/ml human recombinant), and BPE (35 mg/ml) at 37°C in a humidified atmosphere of 95% air/5% CO_2. The medium was changed every two days. When the culture reached approximately 70–80% confluency (day seven), cells were sub-cultured with 0.1% trypsin–0.1% EDTA solution and were seeded for permeability experiments on 1.13 cm^2 polyester Transwell inserts at a density of 2.5×10^5 cells/cm^2.[16] Cell layers were cultured on the Transwell inserts using two different media: NHBE medium as described above; or BEBM : DME/F12 (50 : 50) with all supplements as in NHBE cell culture medium except BPE.[16] After 24 h, the medium in the basolateral compartment was changed and the medium on the apical side of the cell layer was removed to directly expose the cells to ambient air–liquid culture condition. Medium in the basolateral compartment (500 μl) was changed every second day for seven days and then every day. Cell layers were used for drug transport studies only when the transepithelial electrical resistance (TER) value exceeded 400 Ω cm^2 at day 14 or 21 in culture.

Calu-3 cell culture

Calu-3 cell stocks were cultured in 162 cm^2 flasks in DME/F12 growth media supplemented with 10% (v/v) FBS, 1% (v/v) glutamine, 200 mM, 1% (v/v) penicillin 100 UI/ml–streptomycin 100 μg/ml solution and 1% (v/v) non-essential amino acid solution. The cells were maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2 and subcultured weekly using 0.22% (v/v) EDTA/0.25% (v/v) trypsin solution. For transport experiments, the cells (passage 33 or 53) were seeded at a density of 1.0×10^5 cells/cm^2 on 1.13 cm^2 polyester Transwell inserts. After the cells had attached to the Transwells overnight, the medium was removed from the apical cell surface and the cells were grown at an air–liquid interface using culture conditions described previously.[23] The culture medium in the basolateral chamber (500 μl) was changed every two to three days and cells were used for permeation studies after 14 or 21 days in culture.

Caco-2 cell culture

Caco-2 (passage 56) cells were seeded at 2.5×10^5 cells/cm^2 on 1.13 cm^2 polyester Transwell inserts in DMEM medium supplemented with 10% (v/v) FBS, 1% (v/v) glutamine 200 mM, 1% (v/v) penicillin 100 UI/ml–streptomycin 100 μg/ml solution and 1% (v/v) nonessential amino acid solution. The media in both chambers was changed every two to three days. The cells were maintained in submerged culture conditions at 37°C in a humidified atmosphere of 95% air/5% CO_2 and were used for permeation studies after 21 days in culture.

Transepithelial electrical resistance of cell layers

The transepithelial electrical resistance (TER) value of the NHBE cell layers was measured using an Evom voltohmmeter with silver chopstick electrodes (Evom G, World Precision Instruments, Stevenage, UK). Prewarmed medium was added to the apical and basolateral side of the cells. The monolayers were equilibrated for 10 min in a humidified atmosphere of 95% air/5% CO_2 at 37°C, the chopstick electrodes were placed in the medium and TER values measured. The TER of the actual cell layers was calculated by subtracting the background caused by the blank Transwell insert and medium. The TER was corrected for the surface area of the cell support membrane (Ω cm^2).

Digoxin transport in cell layers

Transport experiments were performed in the apical to basolateral (A–B) and basolateral to apical (B–A) directions

in an atmosphere of 95% air/5% CO_2 at 37°C under stirring conditions in the presence or absence of P-gp inhibitors using Calu-3, Caco-2 and NHBE cells. The TER of each cell monolayer was measured after 30 min equilibration at 37°C in HBSS using an Evom voltohmmeter. The TER values of Calu-3 and NHBE cell layers at the beginning of the experiments were >350 Ω cm^2 and >400 Ω cm^2, respectively, and did not vary from the initial TER by >15% when measured at the end of the experiment. HBSS was aspirated from both compartments and 510 or 1510 μl of the test solution was added to the apical (for A–B transport) or basolateral (for B–A transport) side of the cells, respectively (designated as the donor solution). [^{3}H]Digoxin (± inhibitor) test solutions were prepared via dilution using HBSS supplemented with 0.1% BSA (w/v) to provide a final working concentration of 5.0 nM. GF120918A was added to the digoxin, when required, at a working concentration of 2.0 μM in 1% (v/v) DMSO. The DMSO was added to solubilise GF120918A and had no effect on cell layer permeability, digoxin transport or P-gp activity in control experiments, i.e. TER, mannitol permeability and digoxin permeability were unchanged compared with cells in HBSS alone ($P > 0.05$) and there was no effect of 1% DMSO in HBSS on the magnitude of the vectoral transport of digoxin in Caco-2 cells ($P > 0.05$). Cell layer permeability was verified using [^{14}C]mannitol as a paracellular marker at a concentration of 8.9 μM in test solutions. When used, GF120918A was added to both donor and receiver chambers to provide a constant concentration in all compartments.

Immediately after initiation of the transport experiments a 10 μl sample was taken from the donor solution to determine the initial donor concentrations (C_0). After 30, 60, 90 and 120 min, 500 μl (A–B transport) or 100 μl (B–A transport) samples were removed from the receiver solution and replaced immediately with prewarmed HBSS containing 0.1% (w/v) BSA to maintain a constant volume. At the final time point (120 min), 10 μl was removed from each donor solution to establish the final donor concentration. To assess any change in the monolayer integrity during the experiment, the TER was measured at the end of the study. The quantity of [^{3}H]digoxin and/or [^{14}C]mannitol in the collected samples was quantified by liquid scintillation counting using a 1209 Rackbeta dual scintillation counter (Beckman, UK) after addition of 5 ml Ready Protein$^+$ scintillant.

Isolated perfused rat lung

Male Wistar rats (approximately 300 g; Harlan UK Ltd, Oxon, Oxfordshire, UK) were fed with a SDS RM1(E) maintenance diet (Special Diets Services Ltd, Essex) and housed at 21°C, 45–60% humidity with a 12 h light/dark cycle. The surgical protocol was established under the UK Schedule 1 Programme (Animal Licence exempt). The lung was isolated using the simple set up described previously.[3] Briefly, rats were killed by an intraperitoneal injection of pentobarbital (130 mg/kg body weight; Vericore, Dundee, UK). As soon as the animals were unconscious, the main abdominal vessels were severed. A tight-fitting tracheal cannula consisting of 3 cm polyethylene tubing mounted on a blunt 21G needle was inserted caudally into a tracheal incision to a depth of 1 cm and secured with suture thread and a bulldog artery clip. The pulmonary artery was cannulated by introducing a polyethylene tube mounted on a blunt 21G needle 3 mm into the vessel. This was secured with a clip and the perfusion in single pass mode with peristaltic pump was initiated. The perfusion solution was a modified Krebs–Ringer buffer (in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2, HEPES 10, D-glucose 11, 4.5% (w/v) bovine serum albumin (BSA) and heparin 35 kU/ml) at pH 7.4, 37°C, saturated with 95% O_2 and 5% CO_2, at a constant flow rate of 8 ml/min.[24] The lungs were inflated with 1.5 ml of air using a 10-ml syringe connected to the intratracheal cannulae via silicone tubing. After blanching of the tissue the inflated lungs were dissected free from the chest cavity, suspended over a beaker by the trachea and maintained at room temperature during drug absorption studies.

Drug absorption in the isolated perfused rat lung

The test solutions were [^{3}H]digoxin and [^{14}C]mannitol, 45.8 nM and 65.6 μM, respectively, (±2.0 μM GF120918A) in 1% (v/v) DMSO in HBSS. After isolation, the lung preparation was allowed to stabilise for 2–3 min before test solution (100 μl) was instilled into the isolated perfused rat lung (IPRL) through the tracheal cannula using a calibrated 100-μl microsyringe (Hamilton Co. Bonadoz, Switzerland). The solution was applied 3 mm above the bifurcation of the trachea over a period of 1–2 s. Lungs were re-inflated with approximately 1.5 ml air using a 10-ml syringe connected to the tracheal cannula. Air volume in the lung was kept constant and perfusion flow was stable during the experiment and was not influenced by drug administration. The perfusion buffer dripping from the left atrium was sampled (0.5 ml) at predetermined time intervals for 90 min. The tissue viability was verified by visual inspection for signs of oedema, the ratio of dry vs wet weight after 90 min and the transfer profile of mannitol from airways to perfusate. Concentration of transported agents in perfusate was assayed by liquid scintillation counting using a 1209 Rackbeta dual scintillation counter after dilution in 5 ml of Ready Protein$^+$ scintillant. Each transport experiment was carried out using four IPRL preparations. The cumulative amount of the test compounds transferred from the airways to the perfusate in 90 min was calculated from the area under the perfusate concentration vs time plot and presented as the mass-fraction of the administered dose recovered in the perfusate.[24]

Calculations and statistical analysis

The apparent permeability coefficient P_{app} was calculated according to equation 1:

$$P_{app} = \frac{dQ/dt}{AC_0} \quad (1)$$

where dQ/dt (mol/s) was the transport rate, A (cm^2) was the surface area of the filter supporting the monolayer, and

C_0 (mol/cm^3) was the initial drug concentration in the donor fluid. Cell efflux was calculated according to equation 2.

$$Efflux = \frac{P_{app}\,(Basolateral - Apical)}{P_{app}\,(Apical - Basolateral)} \qquad (2)$$

Data were expressed as mean ± SD throughout. Statistical testing was performed using Minitab, version 10. The means of two sets of data were compared using a *t*-test, but when more than two sets of data were analysed one-way analysis of variance was used with Tukey's post-hoc test for significance between the groups. A significant difference was considered to be when $P \leq 0.05$.

Results

Normal human bronchial epithelial cells

In this study the cells grew to confluence within seven days irrespective of medium composition or the cell passage number (passage 2 or 3). Cells grown under all conditions generated a low, but measurable transepithelial electrical resistance (TER) after a three-day culture on Transwell inserts (71 ± 12–74 ± 14 Ω cm^2; Figure 1). Thereafter, TER was dependent on the medium composition and passage number. For passage 2 NHBE cells grown using BEBM/DME/F12 (50 : 50) the TER increased to 1117 ± 293 Ω cm^2 after 21 days in culture, whereas TER of passage three cells grown using BEBM/DME/F12 (50 : 50) reached a maximum (355 ± 68 Ω cm^2) after 15 days in culture. The acquisition of significant TER by passage 2 and 3 cells was in general accordance with previous studies, which reported different temporal profiles of TER acquisition and decline and different magnitudes of TER.[16,25,26] In contrast, the NHBE cells cultured using the NHBE cell culture medium did not significantly improve their TER after three days (i.e. >70 Ω cm^2). This suggested that the NHBE cell culture medium was unsuitable for generating air-interface NHBE cell layers for drug transport studies.

Based on the preliminary work, only the cells cultured in BEBM/DME/F12 (50 : 50) were suitable for P-gp transporter assays. Furthermore, the passage 3 cells declined in TER after day 15, when mannitol P_{app} became >1.0 × 10^{-6} cm/s (data not shown). However, NHBE cells at passage 2 cultured in BEBM/DME/F12 (50 : 50) medium produced acceptable values for TER, which were maintained within 10% of initial value over the course of transport experiments and had suitable mannitol permeability (P_{app} ranged from 0.35 ± 0.09 × 10^{-6} to 1.16 ± 0.38 × 10^{-6} cm/s) and thus this was the only condition in which the P-gp functional transporter experiments were undertaken for this cell line.

In experiments using NHBE cell layers at day 14 in culture, the P_{app} of digoxin was equivalent in A–B and B–A directions ($P < 0.05$), thus there was no suggestion of active transport (Figure 2a). Furthermore, there was no difference in A–B or B–A digoxin transport when GF120918A was included in the experiments, indicating no effect of P-gp on the permeability of digoxin (P_{app} under all conditions fell within the range 0.67–0.96 × 10^{-6} cm/s). Conversely, when NHBE cell layers were used after 21 days in culture,

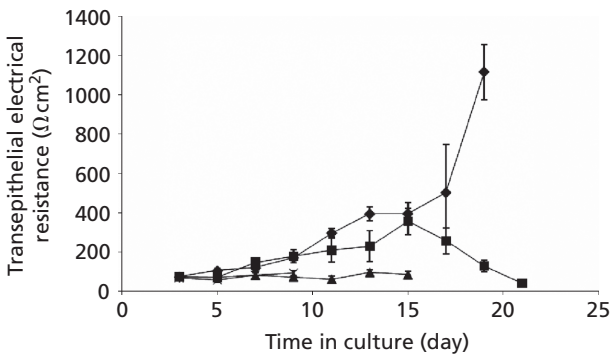

Figure 1 Transepithelial electrical resistance of normal human bronchial epithelial cell layers grown on Transwell supports as a function of days in culture. Cells (passage 2 and 3) were cultured in either normal human bronchial epithelial (NHBE) cell culture medium or a mixture of bronchial epithelial cell basal medium (BEBM) : Dulbecco's modified Eagle's medium/nutrient mixture F12 HAM (DME/F12) (50 : 50). (♦) BEBM : DME/F12 (50 : 50), passage 2; (■) BEBM : DME/F12 (50 : 50), passage 3; (▲) NHBE cell culture medium, passage 2; x NHBE cell culture medium, passage 3 (terminated after nine days). Data represent mean ± SD (n = 12).

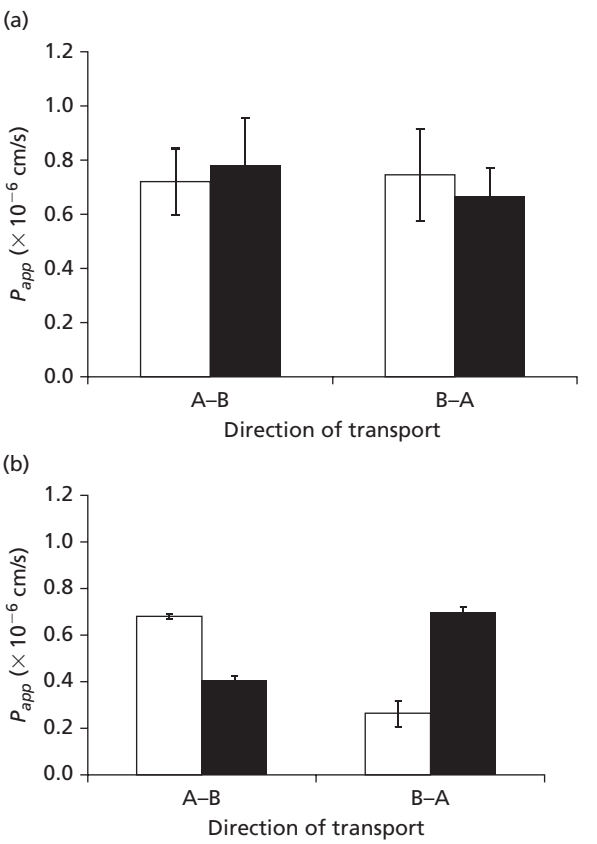

Figure 2 Apparent permeability coefficient (P_{app}) of [^{3}H]digoxin across normal human bronchial epithelial cells, passage 2. Transport after (a) 14 days and (b) 21 days in culture in the presence of GF120918A (2 μM) (■) and in the absence of this inhibitor (□). Data are expressed as mean ± SD, n = 6; A–B, apical to basolateral; B–A, basolateral to apical.

GF120918A reversed the predominant A–B digoxin influx (A–B P_{app} 0.68 ± 0.01 × 10^{-6} decreased to 0.41 ± 0.07 × 10^{-6} cm/s, B–A P_{app} of 0.26 ± 0.06 cm/s increased to 0.70 ± 0.07 × 10^{-6} in the presence of GF120918A; Figure 2b). The ratio of digoxin A–B to B–A transport after 21 days was 2.08, but the direction of polarised transport was reversed when GF120918A was applied to the cells. These results indicated the acquisition of P-gp activity by NHBE cells between 14–21 days in culture, with the polarised absorptive flux consistent with a low level of P-gp activity at the basolateral surface of the cell, although this requires confirmation by localisation studies.

Calu-3 cells

The permeability of digoxin across lower passage (~30) Calu-3 cell layers revealed that there was little or no polarisation of transport at 14 or 21 days in culture (Figure 3). P_{app} values at day 14 were in the A–B direction and 1.50 ± 0.40 × 10^{-6} cm/s in the B–A direction 1.89 ± 0.55 × 10^{-6} cm/s; $P > 0.05$. At day 21P_{app} A–B was 1.53 ± 0.05 × 10^{-6} cm/s and P_{app} B–A was 1.29 ± 0.07 × 10^{-6} cm/s; $P < 0.05$. The addition of GF120918A did not have a significant effect on digoxin transport in either A–B or B–A direction at day 14 or 21 in culture, indicating no influence of P-gp on the permeability of digoxin (Figure 3). These results concord with the absence of P-gp efflux in Calu-3 cells used at passages between 21–37 and after 14–21 days in culture, reported previously.[12]

The permeability of digoxin across higher passage (~50) Calu-3 cell layers revealed secretory efflux (ER = 2) at days 14 and 21 in culture (Figure 4). P_{app} value at day 14 for A–B transport was 0.59 ± 0.00 × 10^{-6} cm/s and for B–A transport was 1.23 ± 0.14 × 10^{-6} cm/s; $P < 0.05$. P_{app} A–B at day 21 was 0.76 ± 0.03 × 10^{-6} cm/s and P_{app} B–A was 1.58 ± 0.10 × 10^{-6} cm/s; $P < 0.05$. However, the addition of the GF120918A inhibitor had no effect on the cells after 14 days in culture, but it significantly suppressed the efflux processes

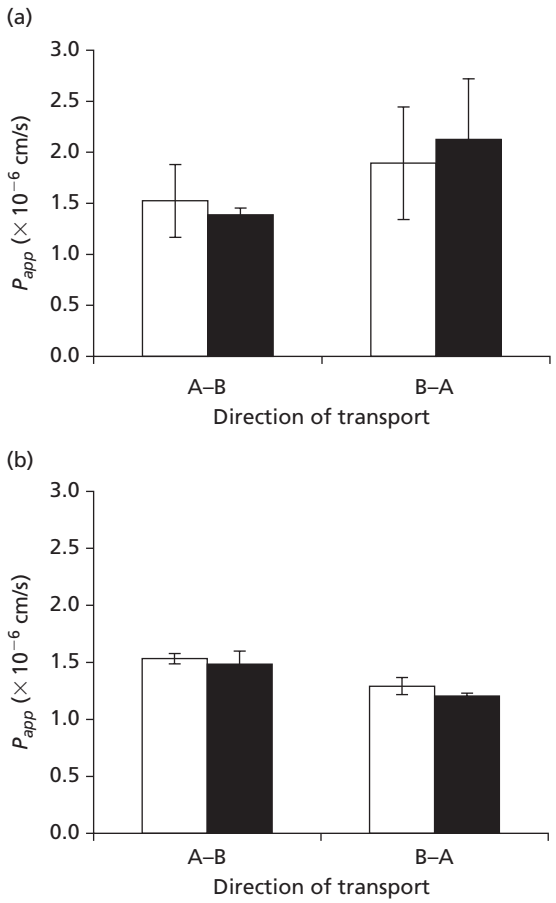

Figure 3 Apparent permeability coefficient (P_{app}) of [^{3}H]digoxin across Calu-3 cell layers (low passage). Transport across Calu-3 cell layers (low passage, ~30) in the apical to basolateral (A–B) and basolateral to apical (B–A) direction after (a) 14 days or (b) 21 days in culture on Transwell supports in the presence (■) and in the absence (□) of GF120918A (2 μM). Data represent mean ± SD (n = 6).

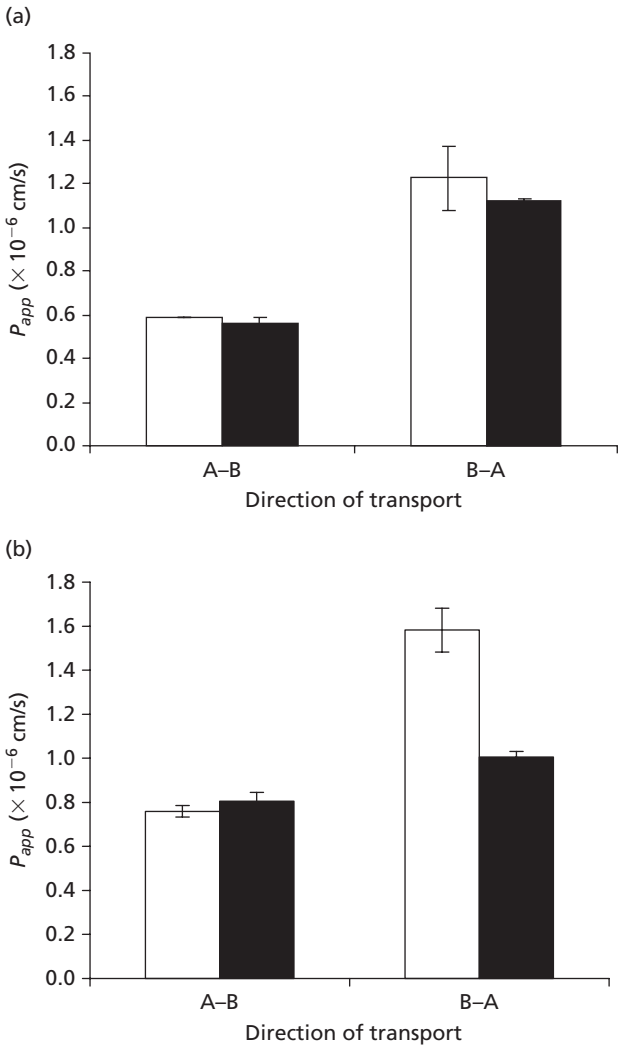

Figure 4 Apparent permeability coefficient (P_{app}) of [^{3}H]digoxin across Calu-3 cell layers (high passage). Transport across Calu-3 cell layers (high passage, ~50) in the apical to basolateral (A–B) and basolateral to apical (B–A) direction in the presence of GF120918A (2 μM) (■) and in the absence of this inhibitor (□) after (a) 14 days and (b) 21 days in culture. Data represent mean ± SD (n = 6).

in the cells cultured for 21 days, abolishing the polarisation of transport. The differences in results obtained using the lower and higher passages of cell lines supported the recognised view that the transport properties of cells may change during repeated passaging.[27]

Digoxin efflux in the three cell models

The primary aim of the study was to compare the P-gp activity that was found in Calu-3 and NHBE cell lines to the positive control Caco-2. As only low activity was found in the respiratory cells and these were influenced by the culture conditions and cell line passage number, the activity was compared using the conditions where the GF120918A had the largest inhibitory effect. Such analysis indicated that the efflux ratio produced by the respiratory cells never exceeded 2, which was 5-times less than P-gp mediated Caco-2 efflux (Figure 5).

Isolated perfused rat lung transport studies

A total of 41 ± 17% applied digoxin was transported into the perfusate over 90 min using the IPRL model in the absence of an inhibitor (Figure 6). The co-administration of GF120918A with digoxin did not have any significant impact ($P > 0.05$) on digoxin transport; the fraction of digoxin transported in 90 min was 46 ± 7% in the presence of GF120918A. The digoxin absorption profile was not significantly different at any of the time points when GF120918A was co-administered with digoxin. The cumulative amount of mannitol transported was 48.5 ± 5.0%, which was not affected by the presence of the inhibitor or digoxin indicating that the epithelial barrier was maintained (data not shown). No lung oedema was present during any of the experiments reported herein (determined visually and by weight).

Discussion

Normal human bronchial epithelial cells

Primary normal airway epithelial cells were cultured *in vitro* to provide a more physiologically relevant airway model compared with immortalised cells. Like the airway epithelium *in vivo,* NHBE cell layers possess a mixture of cell types, which grow in a pseudostratified cell layer, including ciliated and goblet cells, which were clearly identifiable under microscopic examination.[25] Compared with Calu-3 cells, the primary systems were less readily available, more complex and expensive model to culture. NHBE cells have been used much less for drug transport applications and at present there is no universally accepted protocol for the culture of these cells.[28] Recently NHBE cells were evaluated as an in-vitro model for the human airway epithelium, including the efflux of rhodamine 123 which was attributed to P-gp, although this was not verified using a specific inhibitor such as GF120918A.[16]

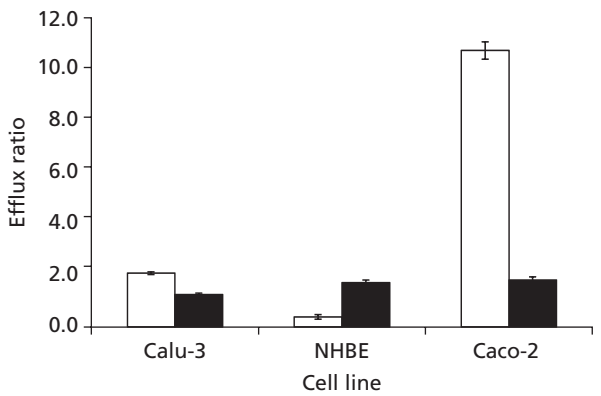

Figure 5 Efflux ratio of [^{3}H]digoxin across Calu-3 cells, normal human bronchial epithelial cells (NHBE) and Caco-2 cells. Transport was in the presence (■) and absence (□) of GF120918A (2 μM) after 21 days in culture on a Transwell support. The efflux ratio is the apparent permeability coefficient (P_{app}) (basolateral-to-apical) divided by the P_{app} (apical-to-basolateral). Data are expressed as mean ± SD, $n = 6$.

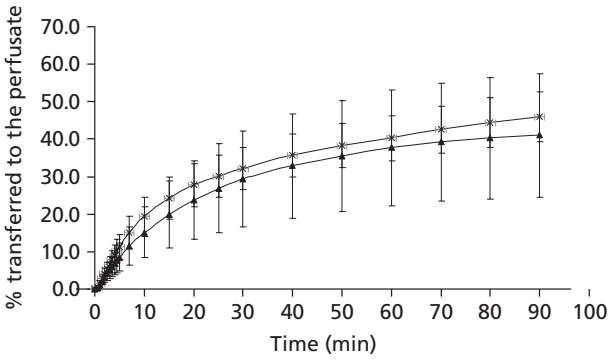

Figure 6 Cumulative percentage of [^{3}H]digoxin transferred to the perfusate vs time in the isolated perfused rat lungs [^{3}H]digoxin (45.8 nM) was administered intratracheally, with (x) or without (▲) the specific inhibitar GF120918A. Data are expressed as mean ± SD, $n = 4$.

Although NHBE cells grow readily as mixed cultures of goblet and ciliated cells, the generation of cell layers with barrier properties that are representative of the airway epithelium *in vivo* is a prerequisite before transepithelial drug transport can be measured. Therefore, the effect of the growth medium recommended by the supplier of the cells on cell barrier formation was compared with the effect of growth medium used recently to culture NHBE cells for P-gp transport studies.[16] The essential difference in medium composition was that the supplemented BEBM recommended by the supplier (referred to as NHBE cell culture medium) was diluted (50 : 50) with DME/F12 medium, but contained all the recommended supplements except for BPE.[16] In these studies the medium and time in culture influenced the epithelial cell layer permeability; other culture conditions not investigated herein may also influence transporter expression including seeding density, coating of the cell support with extracellular matrix proteins, and composition of the culture medium.

Calu-3 cells

The Calu-3 cell line, derived from a human bronchial adenocarcinoma, is widely used for drug transport studies in the airways. When cultured *in vitro*, Calu-3 cells differentiate and cell layers exhibit good barrier properties. However, many different protocols for culture of these cell layers have been described in the literature and there are contradictory reports regarding the expression of P-gp in this cell line

(Table 1).[23] The Calu-3 cell layers were cultured under well characterised air interface growth conditions, generating cells suitable for transport studies (TER 396 ± 18 Ω cm^2; mannitol P_{app} between 0.13–0.28 × 10^{-6} cm/s).[23]

The difference in susceptibility of the secretory efflux to GF120918A observed at 14 and 21 days in high passage Calu-3 cells suggested that different transport mechanisms dominated at these points in culture. The expression of P-gp has been reported to be dependent on time in culture.[29,30] It was likely that the efflux observed at 14 days was mediated via non-P-gp transporters, such as the multi-resistant proteins which were not inhibited by the highly selective GF120918A. It then appeared that P-gp activity developed with time in culture for Calu-3 cells, as observed for to NHBE cells. Thus, at 21 days, P-gp was active and responsible for the observed polarisation of efflux (as shown by the inhibition by GF120918A), with the influence of other transporters either absent or net neutral.

Digoxin efflux in the three cell line models

The low efflux ratio produced by the respiratory cell models was consistent with NHBE and Calu-3 cells expressing P-gp to a lower extent than the carcinoma cell line, Caco-2.[5,31,32] In Calu-3 cells, the digoxin transport was polarised in the B–A direction, suggesting the presence of P-gp in the apical cell membrane after 21 days in culture, a result that concorded with the data generated by Hamilton *et al.*[13] In contrast, the efflux observed in NHBE cells appeared to result from active transport at the basolateral plasma membrane. This was in contrast to the conclusions of Lin *et al.*[16], who reported apical membrane localisation based on their observations of nonspecific inhibition of secretory efflux. These results indicated the need for more selective substrate–inhibitor combinations and for careful immunochemical cyto-localisation of specific transporters.

Isolated perfused rat lung transport studies

The simplicity of cell models offers many advantages when investigating drug transport mechanisms, but this is at the expense of preserving the organ architecture and the full range of cell types that are present *in vivo*: over 40 cell types in the lung. Therefore, an IPRL was used to investigate the P-gp activity in the more complex environment of the intact ex-vivo lung, which possesses a viable airway epithelium. The presence of the P-gp and other transporters, e.g. MRP1 and major vault protein (also called lung resistance-related protein; LRP) in normal human lung tissues has previously been reported in the literature.[4,33–35] Despite the high number of in-vitro studies, the functional effect of efflux transporter activity on the transport of drugs across the lung barrier in animal models has not been extensively investigated. The P-gp substrate losartan has been reported to be highly transported accross the IPRL.[36] This finding was interpreted to suggest that any effect of P-gp in limiting the absorption of losartan was insignificant and, like the finding reported herein for digoxin, implies that rapid passive absorptive transfer from the lung dominates over any active absorption mechanism.

In the experiments reported herein, it cannot be excluded that P-gp activity in the isolated lung was undetected because of excessive local levels of digoxin or insufficient GF120918A concentration in the lung tissue. Although aerosol administration was not used to ensure an even and deep distribution of the administered compounds into the lung, the lowest practicable amount of digoxin was administered to avoid saturating the transporter (the digoxin concentration was much lower than that used successfully to probe P-gp activity in other tissues). Full inhibition of P-gp in the lung may require the inclusion of the inhibitor in the perfusate and/or pre-administration of the inhibitor. However, the limited availability of the GF120918A precluded this; instead the inhibitor and substrate were co-administered in solution to ensure that they were delivered to the same regions of the lung.

Conclusions

The relatively low levels of P-gp expression in the lung compared with those in other tissues has been well documented.[5,31,32] This study has shown that although there was evidence of nominal P-gp activity in respiratory cells *in vitro*, this was not found in the ex-vivo lung and was at a much lower level compared with P-gp activity in the intestinal epithelial cells. The sensitivity of the NHBE and Calu-3 cells to culture conditions showed that, like Caco-2 cells, they required careful characterisation before being used to study drug disposition, and the interpretation of any activity found required verification in more complex models of drug absorption. The P-gp inhibitor GF120918A provided a highly selective reversal of a marked cell efflux from Caco-2 cells, but it had only a limited effect on the airway cell lines which possessed limited P-gp-mediated digoxin efflux.

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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