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Lack of difference in pulmonary absorption of digoxin, a P-glycoprotein substrate, in *mdr1a*-deficient and *mdr1a*-competent mice

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Abstract

Although in-vitro experiments have suggested that P-glycoprotein (P-gp) may have an important influence on the disposition of inhaled drugs, the effect of P-gp on absorption from the lung in-vivo has not been reported previously. The aim of this study was to compare the pulmonary absorption of digoxin, a well-characterised substrate for P-gp, in *mdr1a* (–/–) (P-gp-deficient) and *mdr1a* (+/+) (P-gp-competent) mice. Digoxin was administered by intratracheal instillation over 3–4 s, a method demonstrated to result in dispersion of the dose to all regions of the lung. Drug distribution was determined in the lungs, plasma, brain, heart, liver and kidney of individual mice after 5, 10, 30, 60 and 90 min. Digoxin was cleared rapidly from the lung after intratracheal administration. No differences were observed in the maximum serum concentrations between *mdr1a* (+/+) and *mdr1a* (–/–) mice (37.8 ± 6.9 and 38.8 ± 15.8 ng mL^{-1}, respectively). The serum concentration versus time profiles were similar in both strains; the area under the drug serum concentration versus time curve (AUC) was 2010 and 1812 ng mL^{-1} min in *mdr1a* (–/–) and *mdr1a* (+/+) mice, respectively. For organs harvested at the end of the experiment (90 min), the only significant difference between the strains was the markedly elevated concentration of digoxin in the brains of *mdr1a* (–/–) mice. In conclusion, digoxin is rapidly absorbed from the mouse lung following tracheal instillation, with no difference in the rate or extent of absorption between *mdr1a*-deficient and -competent mice. This suggests that, in contrast to the scenario suggested by in-vitro data, P-gp in the respiratory epithelium may have little influence on the disposition of drugs that are well absorbed from the lung.

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Introduction

The mdr1 P-glycoprotein (P-gp) transporter is localised at the surface of the epithelium of the human lung (Cordon-Cardo et al 1990), and more specifically to the human airway (Lechapt-Zalcman et al 1997) and alveolar type I epithelial cells (Campbell et al 2003). Functional expression of this transporter has also been reported in the human airway epithelial cell lines Calu-3 (Hamilton et al 2001a) and 16HBE14o- (Ehrhardt et al 2003) and in primary cultures of type I-like pneumocytes (Campbell et al 2003; Endter et al 2007). This report describes the first attempt to evaluate the impact of P-gp on the absorption of inhaled substrates in-vivo.

P-gp is a permissive transporter (Stouch & Gudmundsson 2002), which means that many pharmaceutically relevant molecules are likely to be substrates, including compounds delivered by inhalation (Hamilton et al 2001b, 2002). Given the presence of P-gp in the lung and functional P-gp activity in respiratory epithelial cells in-vitro, it is logical to hypothesise that P-gp influences the pulmonary disposition of inhaled substrates, leading to the pulmonary retention of inhaled xenobiotics or pulmonary accumulation of systemically administered drugs. However, despite speculation that P-gp may influence pulmonary drug disposition (Campbell et al 2003), there is also evidence that, in fact, P-gp has little impact on pulmonary absorption (Tronde 2004), suggesting that further studies are required to clarify the role of P-gp.

The use of P-gp-deficient mice provides an opportunity to investigate the impact of the transporter on drug disposition in-vivo. Whereas human *mdr1* encodes for a single P-gp, in

mice two genes – *mdr1a* and *mdr1b* – encode two P-gps with partially overlapping distribution and substrate specificity. The CF-1 *mdr1a* (–/–) mouse is a subpopulation of the CF-1 mouse strain with deficient expression of *mdr1a* compared with the CF-1 *mdr1a* (+/+) mouse, which has normal expression of the protein. The spontaneously occurring deficiency in the *mdr1a* (–/–) mouse appears to have no harmful effects during normal animal maintenance but renders it more susceptible to the toxic effects of xenobiotics (Kwei et al 1999). P-gp *mdr1a* has been located in mouse lung immunocytochemically (Bonhomme-Faivre et al 2002), and quinidine is reported to distribute at higher concentrations into the lungs of *mdr1a* (–/–) mice compared with their *mdr1a* (+/+) counterparts following intravenous administration (Fromm et al 1999). In contrast, *mdr1b* P-gp has a more restricted tissue distribution (Leusch et al 2002).

P-gp also has a major influence on the permeability of the blood–brain barrier and significantly reduces the access of P-gp substrates into the brain (Tsuji & Tamai 1997). Digoxin concentrations in the brain are increased approximately 35-fold in *mdr1a* (–/–) mice compared with *mdr1a* (+/+) mice, sampled 4 h after administration of an i.v. dose (1 mg kg^{-1}) (Schinkel et al 1995). Similarly, mdr1a P-gp has a significant influence on the efflux, or secretion, of P-gp substrates into the gastrointestinal tract (Schinkel et al 1995). In the present study, *mdr1a* (–/–) mice were used to evaluate whether *mdr1a* P-gp in the lung has a similar impact on the availability or absorption rate of pulmonary delivered compounds. Absorption of the P-gp-selective substrate digoxin was compared in *mdr1a* (–/–) and *mdr1a* (+/+) mice to quantify the effect of P-gp on the rate and extent of digoxin absorption from the lung.

Materials and Methods

Materials

[^{14}C]-Mannitol (2.18 GBq $mmol^{-1}$, 7.40 MBq mL^{-1}) was obtained from Amersham (Little Chalfont, UK); [^{3}H]-digoxin (1370 GBq $mmol^{-1}$, 37.0 MBq mL^{-1}) and Soluene-350 were obtained from Perkin Elmer, Beaconsfield, UK. Verapamil and urethane were obtained from Sigma (Poole) UK.

Animals

CF-1 *mdr1a* (+/+) mice, *mdr1a* (–/–) mice and CD-1 wild-type female and male mice, used to assess drug distribution in the lung after intra-tracheal (IT) dosing, were obtained from Charles River Laboratories (Margate, UK). Mice were 62–65 days old, with a weight range of 22–36 g.

Cage temperatures were maintained at 22°C ± 2°C with relative humidity at 45% ± 5%. There were 16–17 air changes per hour in the individually ventilated cages and a 12 h light cycle (08:00 to 20:00 h). The animals had ad-libitum access to water and food; food consisted of an irradiated standard maintenance mix (BeeKay, Hull, UK). The animals were provided with dust-free wood chips (BeeKay) in cages that had been autoclaved at 121°C for 20 min before animal housing.

All experiments were carried out in accordance with the Scientific Animal Procedures Act 1986 (United Kingdom) and the study was approved by the Ethical Committee of King's College London.

Drug administration

Mice were anaesthetised with urethane (2.5 g kg^{-1} i.p). A tracheotomy was then performed, with the mouse in a supine position, ensuring that the trachea was not completely severed, and a tight-fitting leur cannula was fitted. The mice was angled so as to minimise bending of the trachea, to provide a straight path for administration of drug. The drug solution was instilled slowly (over 4–5 s) into the lungs through a length of plastic tubing attached to the needle of a calibrated 25 μL Hamilton syringe. This was followed immediately by injection of a 20 μL air bolus to clear the cannula of residual fluid before the syringe was withdrawn completely. The IT dose was 20 μL saline containing the P-gp substrate digoxin 3.5 μg kg^{-1} (5920 kBq mL^{-1}) plus mannitol 123 μg kg^{-1} (43.9 kBq mL^{-1}), used as an indicator of respiratory epithelial permeability. The mice were maintained in a head-up position at an angle of 90° for 30 s after the administration of [^{3}H]-digoxin and [^{14}C]-mannitol solution, and placed on a heated blanket for the remainder of the experiment. In rare cases, animals had difficulty breathing and were connected via the tracheotomy cannula tubing to a ventilator until they could breathe unaided.

On each of the four study days, five normal and five P-gp-deficient mice were dosed. Blood was sampled from one mouse from each group at 5, 10, 30, 60 and 90 min, followed immediately by sacrifice and organ sampling. Male and female mice were distributed evenly between the study groups.

Drug distribution in the lung

Two instillation techniques were compared in wild-type CD-1 mice to ensure that adequate distribution of drug in the lung was achieved. Intratracheal (IT) instillation of 20 μL (digoxin 3.6 μg kg^{-1} and mannitol 123 μg kg^{-1}) was performed either rapidly or slowly. 'Rapid' instillation involved administration of the 20 μL instillate followed by 20 μL air bolus to the lungs within 1 s, whereas 'slow' instillation involved the same process over 4–5 s.

The lungs were isolated and removed immediately after instillation and dissected into four quadrants by single lateral and single longitudinal bisection. Drug concentrations were measured in the upper right, upper left, lower right and lower left quadrants.

Blood and tissue sampling

Approximately 300 μL of blood was withdrawn by cardiac puncture under anaesthesia. Samples were centrifuged at 180 *g* for 3 min to obtain serum (80–150 μL), which was stored at –20°C until analysis. The lungs and other organs were flushed with saline before removal. To flush the lungs, saline was introduced into the right ventricle and allowed to

flow under gravity (approximately 0.25 mL s^{-1}) through the pulmonary circulation. A small incision was performed in the left atrium to permit free efflux of the perfusate. The success of flushing was evident as the lungs turned from a light pink to a chalk white over 30–40 s. The systemic circulation was flushed by introducing the perfusate into the left ventricle with free efflux from the right atrium. This process was monitored by the blanching of the periphery of the liver lobes and the kidneys. The entire lungs, brain, heart, liver and kidneys were isolated at each time point, washed with saline and snap frozen in liquid nitrogen for 5 min. Samples were stored at −20°C until analysis.

Digoxin and mannitol analysis

[^{3}H]-Digoxin and [^{14}C]-mannitol were quantified simultaneously in each sample. Serum samples were thawed and 50 μL of serum was diluted in 900 μL Soluene-350 at 55°C and mixed on a shaker at 180 rpm for 90 min. H_2O_2 (0.2 μL) was added and the samples were heated at 55°C for a further 90 min, after which 5 mL of Ready Protein scintillant (Beckman Coulter, High Wycombe, UK) was added to the vial. Radioactivity was measured by liquid scintillation counting with internal quench correction (Rackbeta 1209, Wallac, Finland). The radioactivity of blank serum was measured to quantify background.

Sections of organs (approximately 100 mg) were transferred to vials and weighed accurately before undergoing the same sample preparation and assayed as described above for serum. Data were adjusted per gram of tissue.

Statistical analysis

The data were analysed for significance using the Mann–Whitney *U*-test. A P value below 0.05 was considered statistically significant.

Results

A series of preliminary experiments in wild-type CD-1 mice using different rates of instillation into the lung indicated that the solution distributed equally into all regions of the lung (Figure 1). The lower mean recovery of solution from the trachea after administration by slow instillation technique was not significantly different from that obtained using the 'rapid' instillation technique. The rapid installation was associated with a greater incidence of breathing difficulties, however, so slow instillation was used for the absorption experiments.

Rapid clearance of digoxin from the lung occurred after IT administration, with no difference in lung or serum levels of digoxin between *mdr1a* (–/–) and *mdr1a* (+/+) mice over the 90 min time period (Figure 2). However, a dramatic increase in the amount of digoxin in the brain was observed for *mdr1a* (–/–) mice compared with *mdr1a* (+/+) mice. The mannitol concentration in lung, serum and brain showed no difference between the two groups of mice. The ratio of digoxin to mannitol in the lungs of both *mdr1a* (–/–) and *mdr1a* (+/+) mice over 90 min was consistent, indicating that it is unlikely that the digoxin was undergoing any selective absorption or efflux process.

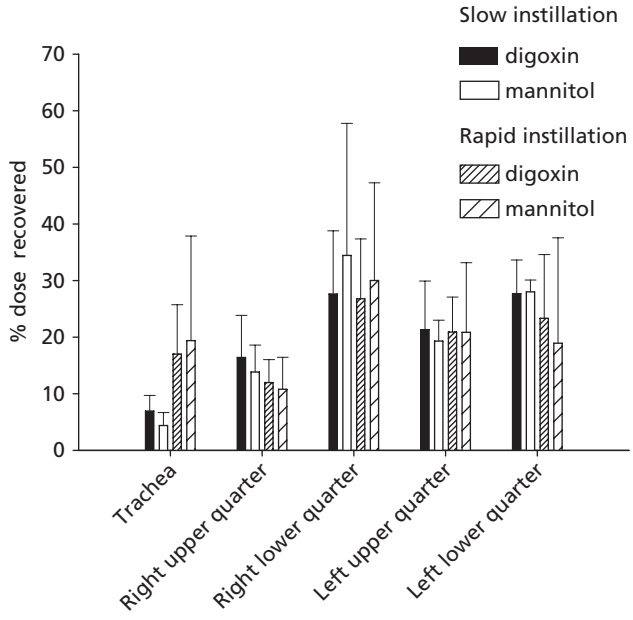

Figure 1 Distribution of digoxin and mannitol to different areas of the lungs following fast or slow instillation of 20 μL of a saline solution into the lung of CD-1 wild-type mice. Data represent mean ± s.d. (n = 4).

There was no difference between the two groups of mice in maximum serum concentration (C_{max}; Figure 2), with the time to reach C_{max} varying from 5 min in P-gp *mdr1a* (–/–) mice to 10 min in *mdr1a* (+/+) mice, although the concentrations at 5 and 10 min were not statistically different for the *mdr1a* (+/+) mice. For digoxin, C_{max} was 37.8 ± 6.9 ng mL^{-1} in *mdr*1a (+/+) mice and 38.8 ± 15.8 ng mL^{-1} in *mdr1a* (–/–) mice. For mannitol, C_{max} was 5641 ± 1219 ng $mL^{-1}$and 4873 ± 1079 ng mL^{-1} for *mdr1a* (–/–) and *mdr1a* (+/+) mice, respectively. The area under the serum concentration–time curve (AUC), calculated from 5 to 90 min using the linear/logarithmic trapezoidal rule, was not significantly different between *mdr1a* (–/–) and *mdr1a* (+/+) mice. For digoxin, the AUC was 2010 ng mL^{-1} min for *mdr1a* (–/–) mice and 1812 ng mL^{-1} min for *mdr1a* (+/+) mice. For mannitol, AUCs were 392 μg mL^{-1} min and 392 μg mL^{-1} min for *mdr1a* (–/–) and *mdr1a* (+/+) mice, respectively.

For organs harvested at the end of the experiment (90 min), the only significant difference between *mdr1a* (–/–) and *mdr1a* (+/+) mice was the 10-fold higher digoxin concentration in the brains of *mdr1a* (–/–) mice (Figure 3).

Discussion

There was no difference in the absorption of digoxin from the lungs of *mdr1a* (–/–) mice compared with *mdr1a* (+/+) mice following IT administration (Figure 2). A 10-fold higher digoxin level in the brain of *mdr1a* (–/–) mice indicated that systemic digoxin concentrations achieved in the study did

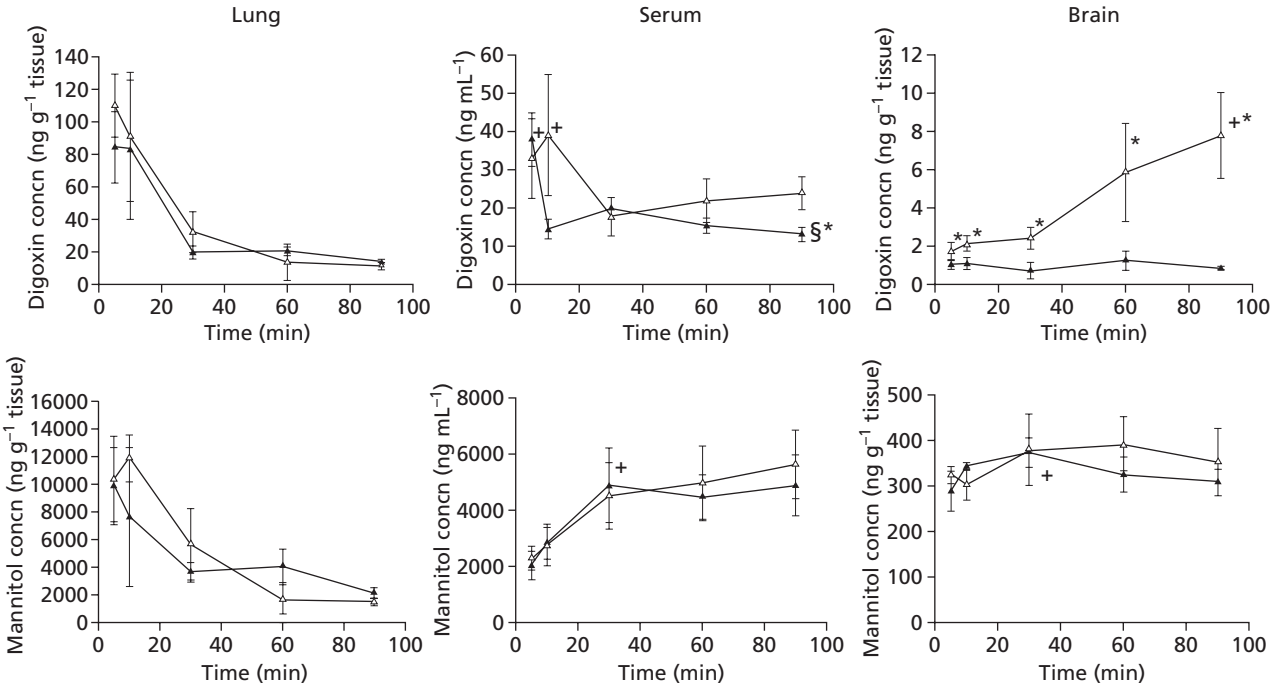

Figure 2 Digoxin (top row) and mannitol (bottom row) levels in lung, serum and brain following intratracheal administration to *mdr*1a (+/+) (triangles) and *mdr*1a (–/–) (circles) CF-1 mice. Data are mean ± s.d. (n = 4; 2 male, 2 female), $^{+}$n = 3 ± s.d., §n = 2 ± range. *$P < 0.05$.

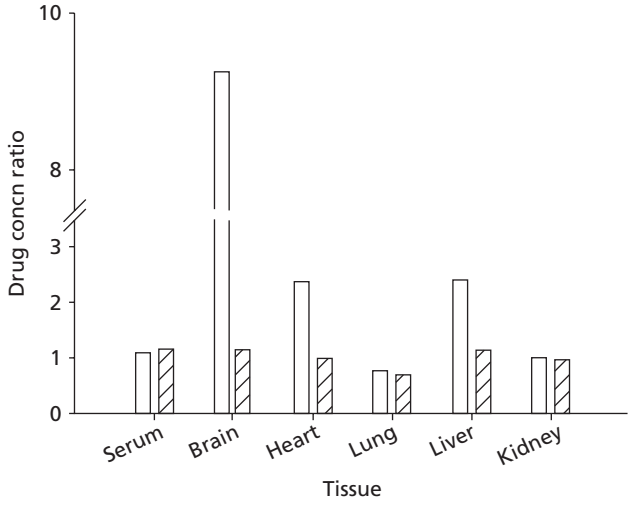

Figure 3 Ratios of drug concentration in *mdr*1a (–/–)/*mdr*1a (+/+) CF-1 mice obtained for serum, brain, heart, lung, liver and kidney at t = 90 min following intratracheal administration with digoxin (open bars) and mannitol (hatched bars). Data are means (n = 6–8).

not saturate the P-gp transporter in the blood–brain barrier of *mdr1a* (+/+) mice. No other organ examined showed such a marked difference in digoxin distribution between *mdr1a* (–/–) and *mdr1a* (+/+) mice. No differences between the two groups of mice in the disposition of mannitol was found in any organ, including the brain.

A significant clinical effect of P-gp on pulmonary absorption would have been indicated by retention of digoxin in the airways of *mdr1a* (+/+) mice, and faster systemic absorption in *mdr1a* (–/–) mice. However, clearance profiles of digoxin from the lung were indistinguishable in the two groups of mice, suggesting a negligible contribution of P-gp to the disposition of inhaled digoxin.

IT instillation was used to avoid the complications of aerosolisation, such as quantifying the dose to the lung, drug loss in the delivery device, effect of particle size, swallowing of orally deposited drug and drug stability to processing. The drug was administered in a single instillation, with the only loss being the small proportion of dose that remained in the syringe. Despite the difficulty of standardising administration by syringe (Byron & Niven 1988), the method has proven suitable for absorption studies (Adjei & Garren 1990; Bennett et al 1994). It is important that the instilled dose does not obstruct the airways, which would disturb the breathing pattern and could increase swallowing of the instilled drug. The use of a cannula and tracheotomy in this study ensured that the whole dose was delivered directly to the lungs. Although such administration may result in a tendency towards central deposition and patchy distribution compared with that achieved by aerosol delivery (Colthorpe et al 1992; Folkesson et al 1992; Niven et al 1995), the technique was demonstrated to provide suitably even and reliable dosing to the lung (Figure 1).

In-situ perfusion was used to flush the organs of blood and ensure that only tissue digoxin was measured in each organ. Although perfusion extends the time taken for organ harvesting, it is important when assaying vacuous organs such as the heart or well-perfused organs such as the lungs. Aside from the brain, little difference was seen in digoxin concentrations in the different organs. Apparently elevated levels in the liver and heart in *mdr1a* (–/–) mice at early

sample times (data not shown) were only statistically significant at t = 90 min in the heart (Figure 3). Digoxin undergoes little metabolism in-vivo (Fromm et al 1999; Kawahara et al 1999), thus there were no concerns over substrate stability over the 90-minute study.

A comparison of the pharmacokinetics of digoxin in *mdr1a* (–/–) and *mdr1a* (+/+) mice has been reported previously, with by far the most striking effect being in brain concentration (Kawahara et al 1999). In the current study, the brain digoxin concentration–time profile in *mdr1a* (–/–) mice also showed marked accumulation with time, in contrast to the lower levels and lack of accumulation in *mdr1a* (+/+) mice, due to the absence of P-gp efflux at the blood–brain barrier in *mdr1a* (–/–) mice. Similar findings have been reported for the P-gp substrates ivermectin and ciclosporin in the brains of *mdr1a* (–/–) mice (Kwei et al 1999). Digoxin is a reliable probe for P-gp in-vivo (Schinkel et al 1995; Mayer et al 1996) and even at much higher doses than those used in this study – 500 μg kg^{-1} (Fromm et al 1999) and 1000 μg kg^{-1} (Kawahara et al 1999), P-gp in the blood–brain barrier of *mdr1a* (+/+) mice excludes digoxin.

Although digoxin has been well characterised as a substrate for P-gp, it should be noted that digoxin is also a substrate for organic anion transporting polypeptide (OATP) transporters (Yao & Chiou 2006). There is little data regarding the activity of OATP transporters in the respiratory epithelium, but their effect on digoxin transport in the gastrointestinal epithelium and blood–brain barrier is eclipsed by the effect of P-gp. It is unlikely that OATP transport is altered in the *mdr1a* (–/–) mice strain in such a way as to confound the aims of this study.

In this study we used the lowest practicable digoxin concentration, to minimise the possibility of the local digoxin concentration in the lung saturating the P-gp transporter in the respiratory epithelium. The low dose of digoxin used and good regional distribution achieved by IT administration minimise the risk of saturating the transporter. However, even if high local concentrations did occur, the rapid clearance from the lung would have quickly reduced the excess to a level at which the transporter effect would have become apparent (Figure 2). The *mdrlb* P-gp is upregulated in the liver and kidney of *mdr1a* (–/–) mice (Schinkel et al 1994), and this may also occur in the lung. Although it has been suggested that *mdr1b* is upregulated in excretory organs in *mdr1a* (–/–) mice, this does not occur at the intestinal epithelium or blood–brain barrier, the major P-gp-expressing barriers to drug delivery. Thus, although activity of *mdr1b* in the respiratory epithelium cannot be excluded, in mice *mdr1a* is the principal transporter in the barriers where P-gp has a significant effect on drug absorption in man, and upregulation of *mdr1b* has not compensated for *mdr1a* deficiency in these tissues (Schinkel et al 1995; Leusch et al 2002).

The feasibility of using verapamil as a competitive P-gp inhibitor to allow studies in animals expressing both *mdr1a* and *mdr1b* proteins was investigated. However, our attempts to achieve systemic P-gp inhibition by administering 20 mg kg^{-1} verapamil i.p. 30 min before IT administration of digoxin and mannitol proved unsuccessful (data not shown). The alternative strategy of co-administering verapamil (5 mg kg^{-1}) with digoxin and mannitol via the IT instillation in an attempt to inhibit any P-gp activity locally within the lung was also unsuccessful, as this IT dose of verapamil was not tolerated. To use P-gp inhibition as a strategy to show an effect of P-gp in the respiratory epithelium would require the use of a more specific and less pharmacologically active inhibitor such as GF120918 (Hyafil et al 1993).

Although the impact of P-gp on pulmonary drug absorption has not previously been studied directly, the absorption of two P-gp substrates, losartan and talinolol, from rat lung in-vivo has been reported to be rapid and extensive (Tronde et al 2003) and the clearance of losartan from the isolated perfused lung was similarly rapid. Relatively high doses were used in these studies, but were argued to have pharmaceutical relevance. In the present study the dose was sufficiently low that the absence of a significant impact of P-gp on the absorption of digoxin is clear. However, digoxin is rapidly absorbed from the lungs; P-gp efflux may have greater impact on the absorption of more hydrophilic compounds, which generally have slightly slower absorption rates.

The physiological role for P-gp in the respiratory epithelia is unclear, although a protective function in limiting xenobiotic absorption or promoting xenobiotic secretion may be envisaged. As inhalation is being developed increasingly as a route for the systemic delivery of peptides and proteins, the effect of transporters on inhaled compounds will attract greater attention. It has already been demonstrated that certain peptides are substrates for P-gp (Chikhale et al 1995; Gao et al 2001). If P-gp activity is lower in the lung than in the intestine (or less effective), this may be advantageous for systemic peptide delivery via the lung.

Spontaneously deficient and knock-out *mdr1a* (–/–) mice have been used to demonstrate P-gp-mediated efflux in the brain (Schinkel et al 1994, 1995; Fromm et al 1999; Yokogawa et al 1999) and gastrointestinal tract (Leusch et al 2002). This is the first report of the use of *mdr1a* (–/–) mice to evaluate the contribution of P-gp to pulmonary drug efflux and disposition. Whilst P-gp activity has been reported in respiratory epithelial cells, the results of this study question the widely held assumption that P-gp is important in determining the disposition of inhaled drugs.

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