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Ciprofloxacin Is Actively Transported across Bronchial Lung Epithelial Cells Using a Calu-3 Air Interface Cell Model

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Ciprofloxacin is a well-established broad-spectrum fluoroquinolone antibiotic that penetrates well into the lung tissues; still, the mechanisms of its transepithelial transport are unknown. The contributions of specific transporters, including multidrug efflux transporters, organic cation transporters, and organic anion-transporting polypeptide transporters, to the uptake of ciprofloxacin were investigated *in vitro* using an air interface bronchial epithelial model. Our results demonstrate that ciprofloxacin is subject to predominantly active influx and a slight efflux component.

Ciprofloxacin is an established broad-spectrum fluoroquinolone antibiotic indicated for the treatment of exacerbations of respiratory tract infection in its oral and parenteral dosage forms. It has been shown to be particularly effective against *Pseudomonas aeruginosa*, which is a major cause of the severe decline in lung function in patients with cystic fibrosis and bronchiectasis (1–4). In general, the pharmacokinetics of fluoroquinolones in different tissues have been well characterized, with multiple transporters being implicated in contributing to the drug's deposition in the liver, kidney, and intestinal tract.

In the respiratory tract, studies have demonstrated that ciprofloxacin penetrates into the lung tissue through transepithelial permeation across the bronchial epithelium (5–7). Unlike aminoglycosides and β-lactams, which are excluded from intracellular infections, fluoroquinolones are efficient in treating respiratory tract infections (RTIs) caused by both intracellular and extracellular pathogens (8). Hence, inhalation could potentially be the preferred mode of delivery for this drug, as it would ensure direct deposition of the medication to the site of action and, at the same time, could reduce systemic toxicity. However, there is a lack of information regarding the mechanisms of ciprofloxacin transport across the lung epithelium.

At present, tobramycin (TOBI) and aztreonam (Cayston) are the only inhaled antibiotic treatments approved by the Food and Drug Administration (FDA). Although there is no regulatory approval for inhaled ciprofloxacin, current evidence demonstrates that it has the potential to be developed into an effective inhaled medication for RTIs (9–12).

To date, pulmonary transporters relevant to drug transport comprise three main groups: the ATP-binding cassette (ABC) efflux transporters (i.e., P-glycoprotein [P-gp] and multidrug resistance-associated protein 1 [MRP-1]); the solute carrier (SLC) transporters, which include members of the organic cation transporters (OCTs) and organic anion transporters (OATs); and finally, the organic anion-transporting polypeptides (OATPs) (13, 14). Very little is known about the potential impact of these lung epithelial membrane transporters on the absorption and disposition of antibiotics that are inhaled, which could alter their retention and accumulation within the lung, resulting in either suboptimal concentrations or toxicity. Thus, by understanding the mechanisms for ciprofloxacin transport across the lung epithelium, it may be possible to better predict the extent of pulmonary disposition required for optimal bacterial killing efficacy.

The aim of this study was to investigate the cellular mechanisms of ciprofloxacin transepithelial transport across human bronchiolar epithelial cells and examine the possible interactions of specific lung transporters on uptake and efflux. The air interface Calu-3 cell model has previously been established by us as an effective and predictive *in vitro* model for the study of drug delivery and toxicology (7, 15–18) and was thus used to study the mechanisms of ciprofloxacin transport. This model has physiological characteristics that are similar to those of native pulmonary epithelium in terms of differentiation, mucus production, transepithelial resistance, morphology, and, importantly, transporter expression (13, 17, 19).

Drug transport across a functional epithelium cell system could occur via the nonselective diffusion-driven paracellular route (i.e., a route with tight junctions and lateral interspaces) or via a transcellular route either by passive diffusion or mediated by specific transporters. Ciprofloxacin has been known to cross the epithelium primarily via transcellular routes, and this transport appears to be mediated by simple diffusion processes and facilitated mechanisms (7). However, the specific transporters involved in ciprofloxacin absorption have not yet been identified. To further understand the contribution of active transport processes in ciprofloxacin uptake across the lung epithelium, selective transport inhibitors were used to identify the ciprofloxacin transporters involved. Specifically, efflux transporters, including P-gp and the multidrug resistance-associated proteins (MRPs), together with influx transporters, including OCTs and OATPs, were studied. These transporters have been detected in healthy human lung cells and Calu-3 cells (13, 20–22).

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MATERIALS AND METHODS

Materials. Calu-3 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Nonessential amino acid solution, dimethyl sulfoxide (DMSO), tetraethyl ammonium chloride (TEA), verapamil HCl, ciprofloxacin, probenecid, and fluorescein sodium (flu-Na) were purchased from Sigma-Aldrich (Sydney, Australia). Other cell culture reagents, including trypsin-EDTA solution (2.5 g/liter trypsin, 0.5 g/liter EDTA), Dulbecco's modified Eagle's medium (DMEM; without phenol red and L-glutamine but including sodium bicarbonate and 15 mM HEPES), trypan blue solution (0.4%, wt/vol), phosphate-buffered saline (PBS), L-glutamine solution (200 mM), fetal bovine serum (FBS), and Hanks' balanced salt solution (HBSS), were obtained from Invitrogen (Australia). PSC833 was purchased from Tocris Bioscience (Bristol, United Kingdom). Transwell cell culture inserts (0.33 cm^2 polyester, 0.4-μm pore size) were purchased from Corning Costar (Lowell, MA), and all other sterile culture plasticware was from Sarstedt (Adelaide, Australia). All solvents used were of analytical grade and were supplied by Biolab (Victoria, Australia).

Cell culture. The Calu-3 cell line (HTB-55) was purchased from ATCC. Cells between passages 35 and 42 were maintained in tissue culture flasks of 75 cm^2 and cultured in prewarmed DMEM supplemented with 10% (vol/vol) fetal bovine serum, 1% (vol/vol) nonessential amino acid solution, and 1% (vol/vol) L-glutamine solution. Cells were incubated at 37°C in 5% CO_2 and 95% humidity until confluence was reached. Medium was exchanged every 2 to 3 days, and the cells were passaged weekly, according to ATCC-recommended guidelines.

Calu-3 cells were grown at the air interface to allow monolayer differentiation, and experiments were performed between days 11 and 14. These conditions have previously been established by Haghi et al. (17) and Grainger et al. (19). Briefly, cells were seeded at a density of 5×10^5 cells/cm^2 on 24 transwell cell culture inserts. The cells were introduced in 100 μl medium onto the apical surface of the transwell, and 500 μl medium was added to the basolateral chamber. Subsequently, the medium in the apical chamber was removed after 24 h, and medium was replaced only in the basolateral chambers every 2 to 3 days.

Transport inhibition studies. Selective pharmacological inhibitors were used to inhibit specific transporters and assess their contribution toward ciprofloxacin transport. Transport experiments were conducted in both the apical-to-basolateral (A-B) and basolateral-to-apical (B-A) directions. Solutions of ciprofloxacin (0.1 mM) were prepared in HBSS with or without the presence of the following inhibitors at the indicated concentrations: TEA (5 mM), estrone-3-sulfate (5 mM), verapamil hydrochloride (10 μM), PSC833 (3 μM), and probenecid (1 mM). Drug concentrations are based on previous literature to ensure effective inhibition, simultaneously taking into consideration the solubility and toxicity of the inhibitors (23–25). Cells were preequilibrated for 1 h with the respective inhibitors. Upon initiation of the transport studies, the volume of the donor compartment was replaced with the test solution (ciprofloxacin with the respective inhibitors). Samples from the acceptor compartment were taken every 30 min over a 4-h period and subsequently replaced with fresh inhibitor solutions. Control studies with ciprofloxacin (0.1 mM) alone were performed concurrently. Samples were analyzed using high-performance liquid chromatography (HPLC). Analyses were performed in triplicate.

Ciprofloxacin analysis. Ciprofloxacin was quantified using HPLC. This was achieved using a Shimadzu Prominence liquid chromatography system equipped with an SPD-20A UV-visible detector, an LC-20AT liquid chromatograph, an SIL-20A HT autosampler (Shimadzu Corporation, Japan), and a NovaPak C_{18} column (5 μm, 150 by 3.9 mm). The mobile phase was a mixture of methanol and 0.1 M sodium dihydrogen phosphate at a ratio of 30:70 (vol/vol), with the pH adjusted to 3.35 with phosphoric acid. HPLC was set according to the following conditions: a detection wavelength of 275 nm, a flow rate of 1.0 ml/min, and an injection volume of 100 μl. Linearity was obtained between 0.1 and 50 μg/ml ($R^2 = 0.99$), and the retention time was 7.85 min.

Calu-3 epithelial cell integrity. The flu-Na (molecular mass, 0.367 kDa) uptake experiments were performed to measure the barrier integrity of the Calu-3 epithelial cell after the transport experiments, as previously described (17). Flu-Na was first dissolved in prewarmed HBSS to a concentration of 2.5 mg/ml, and to initiate the experiment, 200 μl of this solution was added to the apical surface of the cells. Six hundred microliters of HBSS was placed in the basolateral chamber, where 100 μl of sample was drawn from the chamber every 20 min over a 1-h period. An equal volume of fresh HBSS was added to replace the sample that was withdrawn. Samples were placed in a black, 96-well plate, and fluorescence readings were taken using a POLARstar Optima fluorescence plate reader (BMG Labtech, Offenburg, Germany) with excitation and emission wavelength settings of 485 and 520 nm, respectively.

Cell viability. The cytotoxicity profiles of ciprofloxacin in the presence and absence of the transport inhibitors were assessed using a CellTiter 96 AQ_{ueous} One Solution cell proliferation assay (Promega). Calu-3 cells were seeded into sterile 96-well microtiter plates at a density of 5×10^4 cells/well and were left overnight to allow cell attachment. Cells were treated with increasing concentrations (up to 50 mM) of ciprofloxacin and other transporter inhibitors in complete DMEM at a final volume of 200 μl. Control wells contained cells and medium without the addition of drugs. Plates were incubated at 37°C in 5% CO_2 and 95% humidity for 72 h. The cells were analyzed for viability following the addition of 20 μl of AQ_{ueous} One Solution to each well and incubation for 3 h at 37°C in 5% CO_2 and 95% humidity. The absorbance of the wells was measured at 490 nm using a fluorescence plate reader (POLARstar Optima; BMG Labtech, Offenburg, Germany). The cell viability (in percent) was calculated on the basis of the following formula: (average absorbance of treated cells/average absorbance of control cells) × 100.

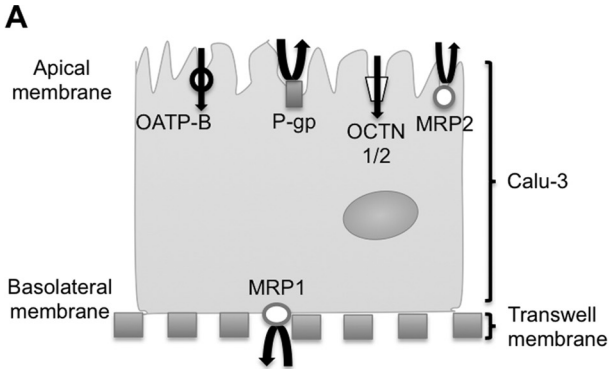
Data analysis. The apparent permeability coefficient (P_{app}) across Calu-3 epithelial cells was calculated using the following equation: $(V/AC_0)(dC/dt)$, where V is the volume of solution in the basolateral chamber, A is the surface area of the transwell membrane (cm^2), C_0 is the initial drug concentration in the apical compartment (μg/ml), and dC/dt is the flux (rate of change in cumulative mass transport, where C is concentration and t is time) of flu-Na.

All results are expressed as means ± standard deviations of at least three separate determinations. To determine significance between groups and the control, the unpaired 2-tailed t test was performed (quoted at the level of $P < 0.05$).

RESULTS AND DISCUSSION

The current results indicate that active transport mechanisms are involved in ciprofloxacin transepithelial absorption. Figure 1 presents a schematic diagram of the Calu-3 cells, showing the expression and localization of the various types of transporters (Fig. 1A), and a list of the selective inhibitors and the drug transporters that they inhibit (Fig. 1B).

The apparent permeability coefficient (P_{app}) of ciprofloxacin over 4 h in the presence and absence of transporter inhibitors is shown in Fig. 2 and Table 1. There is an evident dissymmetry of P_{app} values in the secretory (B-A) and absorptive (A-B) directions and an active net absorption of ciprofloxacin across the Calu-3 epithelial cells. The results indicate for the first time that influx transporters, the OCTs, and, more specifically, OATPs are involved in the absorption of ciprofloxacin across the lung epithelium. The presence of TEA (OCT competitive substrate) (20) and estrone-3-sulfate (OATP2B1 inhibitor) (25) significantly reduces the absorption of ciprofloxacin in the apical to the basolateral direction. Additionally, these inhibitors reduce the net ciprofloxacin absorption, which is consistent for a carrier-mediated process in part contributing to the absorption of ciprofloxacin across the lung epithelia. This is not surprising, as ciprofloxacin is a zwitter-



B

Inhibitor	Affected transporter
Tetraethyl ammonium chloride (TEA)	OCT
Estrone-3-Sulfate	OATP2B1
Verapamil	P-gp, MRPs
PSC833	P-gp
Probenecid	MRPs

FIG 1 (A) Schematic diagram of Calu-3 epithelial cells showing the expression and localization of different transporters; (B) the different inhibitors used in this study and the transporter proteins that they inhibit (13, 20, 24, 25, 32, 33).

ion at physiological pH (26), and thus, solute carriers are expected to contribute to the absorption, distribution, and elimination of ionic compounds.

For the efflux pathways, verapamil has been known to inhibit both P-gp and MRPs (24). In the presence of the inhibitor, the P_{app} of ciprofloxacin was significantly increased and decreased in both the absorption and secretory pathways, respectively. This is indicative of the presence of these efflux transporters. However, the low permeation of ciprofloxacin only in the secretory direction suggests that these efflux transporters have only a minor contribution to the transport of ciprofloxacin. The significantly higher permeation of ciprofloxacin could have been contributed by the influence of verapamil on the physical properties of lipid bilayers by altering the membrane fluidity and subsequently increasing membrane permeability, in addition to its modulating effects on these ABC transporters (27–29). Additionally, other studies have also demonstrated that verapamil may also have an effect on uptake carriers such as OCTs (30, 31), which could be explored in the future.

To further investigate the specific transporter involved, PSC833 was selected since it is a highly effective and relatively more specific inhibitor of P-gp (24) but did not significantly affect the absorption of ciprofloxacin in the A-B direction. Hence, there are only slight contributions of P-gp to ciprofloxacin efflux. In comparison, the presence of probenecid, a known MRP transporter inhibitor (32, 33), significantly decreased the permeation of ciprofloxacin in both the absorptive and secretory directions. In addition, statistical analysis of the ciprofloxacin P_{app} in the presence of probenecid showed no significant difference in the absorptive (A-B) and secretory (B-A) directions ($P = 0.07$), suggesting MRP involvement in the active transport of ciprofloxacin across the Calu-3 lung epithelial model. However, there is conflicting evidence suggesting that probenecid may also interact with not only MRPs but also OAT and OATP due to its anionic nature (34, 35). Hence, the involvement of these influx transporters could also contribute to the decrease in ciprofloxacin permeation when probenecid is used.

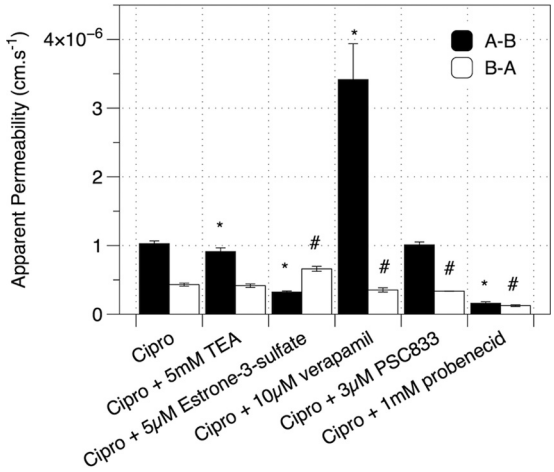


FIG 2 P_{app} of 0.1 mM ciprofloxacin (Cipro) in both the A-B and B-A directions across Calu-3 epithelial cells. Data are presented as means ± SDs ($n = 3$). *, significantly different from ciprofloxacin in the A-B direction; #, significantly different from ciprofloxacin in the B-A direction.

Currently, there are only a few studies on the role of transporters and the transport mechanisms for ciprofloxacin in organs such as the intestine and liver but none on their role in the lung. In terms of active influx transport, a study by Hirano et al. (36) found that ciprofloxacin inhibits L-carnitine transport across a placental cell line through the inhibition of the OCTN2 transporters. This was subsequently proposed to be the potential mechanism of its toxicity toward fetuses, as L-carnitine has an important role in generating energy in the placenta for the developing fetus. On the other hand, earlier studies have shown preferential efflux of ciprofloxacin in Caco-2 cells, which may explain the transintestinal elimination of ciprofloxacin (37, 38). However, P-gp was excluded as a cause of its secretion, and it was suggested that the transport mechanism is sensitive to organic anion exchange and verapamil (39). Similar findings were observed in kidney epithelial cells (40), and this is in good agreement with findings in the current study.

On the basis of the results, both active and passive transport contributed to the uptake of ciprofloxacin through the pulmonary epithelial cells. This has significant implications with respect to the delivery of ciprofloxacin antibiotics for treatment of RTIs. The low permeation of ciprofloxacin through the secretory pathway suggests that high doses of orally or intravenously administered ciprofloxacin would be required to ensure that sufficient concentrations of antibiotics reach the lungs via systemic absorption, which would result in potential toxicity. Hence, an inhalation route to treat RTIs would be favorable. Conversely, the high rate of

TABLE 1 P_{app} of ciprofloxacin in both the A-B and B-A directions across Calu-3 epithelial cells

Inhibitor	Concn (μM)	P_{app}[a] (cm · s^{-1} [10^{-7}])		Net absorption[b] (cm · s^{-1} [10^{-7}])
		A-B	B-A	
Control (ciprofloxacin base)	100	10.27 ± 0.39	4.31 ± 0.22	5.96
TEA	5,000	9.12 ± 0.55[c]	4.15 ± 0.26	4.96
Estrone-3-sulfate	5	3.22 ± 0.16[c]	6.60 ± 0.36[d]	−3.38
Verapamil	10	34.15 ± 5.22[c]	3.53 ± 0.3[d]	30.62
PSC833	3	10.11 ± 0.41	3.36 ± 0.02[d]	6.75
Probenecid	1,000	1.60 ± 0.21[c]	1.25 ± 0.13[d]	0.53

[a] Data are presented as mean ± standard deviation ($n = 3$).
[b] Net absorption is absorption in the A-B direction minus absorption in the B-A direction.
[c] Significantly different from ciprofloxacin in the A-B direction.
[d] Significantly different from ciprofloxacin in the B-A direction.

clearance of inhaled ciprofloxacin (10, 16) is due to the high permeation of this drug through the absorptive pathway. This was evident in two recent studies looking at administration of ciprofloxacin through the pulmonary and gastrointestinal routes in a rat and mice, respectively (41, 42). Of note, the MIC for *Pseudomonas aeruginosa* was found to be between 0.25 and 1.0 μg/ml (10, 43). The study by Chono et al. (42) showed that a dose of 5,000 μg/kg of body weight of ciprofloxacin intragastrically instilled into the mice resulted in a ciprofloxacin level of 0.45 μg/ml in the lungs at 30 min, indicative of its low permeation into lung tissues. Conversely, a dose of 200 μg/kg ciprofloxacin intranasally instilled into a rat resulted in approximately 18 μg/ml of ciprofloxacin in the epithelial lining fluid at 30 min after administration, which was much higher than the required MIC (41). Hence, the inhalation route will be more favorable because it will provide a high local concentration in the lung using lower doses of ciprofloxacin and will avoid systemic exposure. However, this concentration rapidly diminishes to half its initial concentration within 1 h, demonstrating its rapid clearance from the lungs. Formulation strategies can therefore be directed toward increasing its residency time in the lungs or enhancing penetration of ciprofloxacin into the lung tissue. Moreover, drug-drug interaction could occur at the lung level, resulting in a subsequent change in the ciprofloxacin distribution when it is combined with other substrates or modulators of the different transporters involved.

Fluorescein sodium (flu-Na) was used to assess paracellular transport as a measure of Calu-3 epithelial cell integrity after the transport experiments, and results obtained with flu-Na were subsequently compared to those obtained with the control. Ciprofloxacin with and without the inhibitors had no detrimental effects on the integrity of the epithelial cells under the conditions or time scale studied, as shown in the results of the flu-Na permeation assay in Fig. 3. No significant difference was found between the results for cells treated with ciprofloxacin and their respective inhibitors and those for control cells ($P > 0.05$). Additionally, a toxicity assay was used to measure the viability of Calu-3 cells (liquid interface model) exposed to increasing concentrations of ciprofloxacin and other transport inhibitors over a 72-h treatment period (Fig. 4). The viability of the cells was calculated with reference to that of untreated cells, where the average absorbance value was normalized to 100% viability. Both ciprofloxacin and inhibitors were found to be not toxic to the lung epithelial cells over the concentration range used in this study, except for verapamil (50%

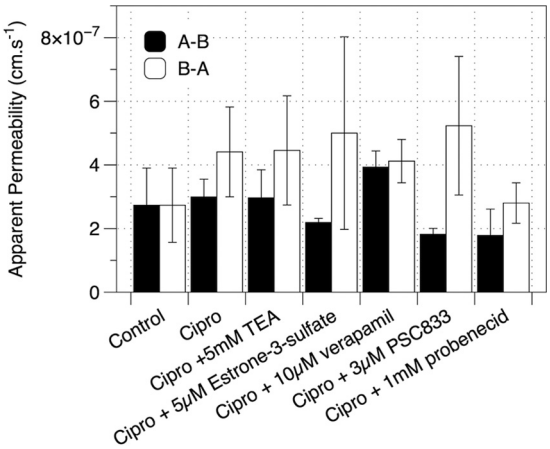


FIG 3 Transport of flu-Na across the Calu-3 epithelial cell layer after 4 h of ciprofloxacin transport with the presence of various transport inhibitors (mean ± SD, $n = 3$).

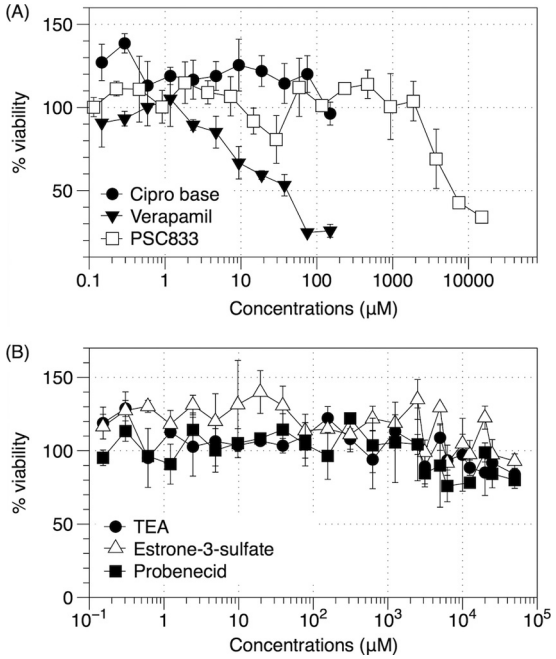


FIG 4 Cytotoxicity of ciprofloxacin, verapamil, and PSC833 (A) and TEA, estrone-3-sulfate, and probenecid (B) for Calu-3 cells after 72 h (mean ± SD, $n = 3$).

inhibitory concentration = 14.12 ± 1.15 μM). Hence, the concentration of verapamil was limited to 10 μM in the transport study to prevent any cell toxicity. However, it needs to be noted that the cytotoxicity experiments were performed over a 72-h period, whereas the transport studies were performed over 4 h on air interface Calu-3 cells, which have morphologies and biochemical responses different from those of cells in liquid-covered cultures. Nevertheless, the experiments demonstrate the range of drug concentrations that these cells could tolerate.

Conclusion. This study has clearly shown that ciprofloxacin is a substrate for active transport in the air interface Calu-3 cell model and suggests the involvement of OCTs, OATP2B1, and MRPs as transporters. Importantly, this has implications with respect to the delivery of ciprofloxacin antibiotics by inhalation treatment and also possible drug-drug interactions. Hence, further studies on expression levels and functional properties are required to better understand the clinical relevance of these drug transporter interactions.

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