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Research paper

Calu-3 cells grown under AIC and LCC conditions: Implications for dipeptide uptake and transepithelial transport of substances

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ABSTRACT

The aim of the present study was to investigate whether Calu-3 cell culture conditions influence drug and nutrient transport known to occur via carriers or transporters. Calu-3 cell layers, an *in vitro* model of the lung epithelium, were cultured using air interfaced culture (AIC) or liquid covered culture (LCC) on either polycarbonate or polyester as filter support material. We found that the development of the Calu-3 cell layer barrier function did not depend on the filter material but rather on the culture conditions as follows: (i) the apical uptake of Gly-Sar was significantly larger for cells grown in AIC compared to LCC, (ii) the TEER values for cells grown in LCC were approximately three times larger than for cells grown in AIC, (iii) the transepithelial transport in both AIC and LCC Calu-3 cells was polarized in the apical–basolateral direction of proline, glycine, α-methyl-D-glucoside, glipizide, taurocholic acid and estrone-3-sulfate, whereas inulin, mannitol and Gly-Sar showed no polarized transport. Etoposide showed polarized efflux (basolateral to apical transport) in AIC and LCC Calu-3 layers. These findings provide information about nutrient and drug transport in Calu-3 cells, and this may have implications for selecting culture conditions for transport studies in this *in vitro* model of the lung epithelium.

1. Introduction

The Calu-3 cell line is one of a few *in vitro* models of the human airway epithelial barrier [1]. Calu-3 cells grown on a permeable filter support have thus been used as an *in vitro* model to assess drug permeability in the lung [2]. In culture, Calu-3 cells appear as a differentiated functional epithelium with apical microvilli and tight junctions [3,4]. The Calu-3 cell line is cultured using different procedures, one being the conventional liquid-covered culture (LCC) used for many epithelia and another is the air-interfaced culture (AIC) that is specifically used to differentiate lung epithelial cell cultures. The latter culture method applies growth medium to the basolateral chamber of Transwell filters, while keeping the apical chamber free of medium. These culturing procedures yield markedly different epithelia [4]. The transepithelial electrical resistance is generally 2–3 times higher for LCC than AIC Calu-3 cell layers [4–7]. Furthermore, AIC Calu-3 layers are covered by more mucus than LCC cells [4]. Apparent permeability coefficients (P_{app}) for passively transported fluorescein sodium and mannitol are generally between 1–4 × 10^{-7} cm/s, which do not seem to correlated with the TEER values measured [2,4,6–10]. Less is known about permeability of solutes transported via carriers or transporters in LCC and AIC Calu-3 cell layers. In a recent study by Endter et al., the presence of a number of different membrane carriers from the SLC and SLCO families as well as active transporters from the ABC-family was detected at the mRNA level in 8- and 15-days-old Calu-3 layers [11]. We have shown that in Calu-3 cells, the transport protein hPEPT1, which is a di/tri-peptide transporter, is expressed and localized in the apical membrane of AIC cells [12]. Investigating the transepithelial transport of Gly-Sar, which is a substrate for both PEPT1 and hPEPT2, we found that PEPT1 rather than PEPT2 is responsible for the main fraction of the Gly-Sar permeability in Calu-3 cell layers grown under AIC [12]. The effect of AIC or LCC culture condition on peptide transport has not yet been investigated. As pointed out by Bosquillon in a recent review, less than 40 drug substances are administered via the pulmonary route and little is known about the role of carriers and transporters in the distributing of the drug substances in the lung tissue [13]). Some of these drug substances are, however, known to interact with transporters, e.g. corticosteroids such as budesonide, beclomethasone, and fluticasone, and β_2-agonists such as salbutamol, and formoterol interacts with organic cation transporters (OCT) and efflux transporters (MDR1 or BCRP) (see [13]). Even though it seems that the AIC Calu-3 cell layers are better than LCC cells in mimicking the bronchial epithelium

Abbreviations: AIC, air interface culture; A → B, apical is the donor side and basolateral is the receiver side; B → A, basolateral is the donor side and apical is the receiver side; CLSM, confocal laser scanning microscopy; Gly-Sar, glycyl-sarcosine; Gly-Pro, glycyl-proline; LCC, liquid-covered culture; PC, polycarbonate; PET, polyester; TEER, transepithelial electrical resistance.

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[4], the LCC cell layers could be useful for very lipophilic substances where the hydrated mucus layer produced by AIC cell layers could constitute a significant unstirred water barrier. The aim of the present study was thus to investigate whether the AIC and LCC conditions influence drug and nutrient transport known to occur via carriers or transporters, with special emphasis on dipeptide transport into the cells, in the Calu-3 cell *in vitro* model. We found that the culture conditions have a great impact on the development of the Calu-3 cell layers barrier function, regardless of the material used for the supports. These findings provide information about nutrient and drug transport in Calu-3 cells, and this may have implications for selecting culture conditions for transport studies in this *in vitro* model of the lung epithelium.

2. Materials and methods

2.1. Materials

Plasticware such as T-75 culture flasks and Transwell filters (1.12 cm^2, 0.4 μm pore size) made of polycarbonate (PC) or polyester (PET) was from Corning Costar, Fisher Scientific (Slangerup, Denmark). Collagen type I was from VWR (Albertslund, Denmark).

The following reagents were used for cell culture: Dulbecco's modified Eagle's medium (DMEM) from Sigma–Aldrich (Broendby, Denmark), 10% fetal bovine serum (FBS) from Gibco, Fisher Scientific (Slangerup, Denmark), 90 U/ml penicillin and 90 μg/ml streptomycin from BioWhittaker, Cambrex (Vallensbaek, Denmark), Hanks balanced salt solution and phosphate-buffered saline (PBS) (without $CaCl_2$ and $MgCl_2$) from Invitrogen (Taastrup, Denmark), bovine serum albumin (BSA) and 2-(N-morpholino)ethanesulfonic acid (MES) from Sigma–Aldrich Danmark A/S (Broendby, Denmark), and N-[2-hydroxylethyl]piperazine-N′-[2-ethanesulfonate] (HEPES) from AppliChem GmbH (Darmstadt, Germany).

The isotopes [^{14}C]-mannitol (51 mCi/mmol), [^{3}H]-mannitol (17 Ci/mmol), [^{3}H]-taurocholic acid (5.0 Ci/mmol), [^{3}H]-proline (75 Ci/mmol), and [^{3}H]-estrone-3-sulfate (57.3 Ci/mmol) were from Perkin Elmer Life and Analytical (Boston, MA, USA). [^{3}H]-Etoposide (646 mCi/mmol) was from Moravek (Brea, CA, USA). [^{3}H]-inulin (1.67 Ci/mmol), [^{14}C]-Gly-Sar (56 mCi/mmol), and [^{14}C]-α-methyl-D-glucoside (286 mCi/mmol) were from GE Healthcare (Berkshire, UK). [^{3}H]-Gly-Sar (500 mCi/mmol) and [^{14}C]-glycine (87 mCi/mmol) were from Larodan Fine Chemicals (Malmoe, Sweden). [^{3}H]-Glipizide (6 Ci/mmol) was from Amersham Bioscience (Cardiff, UK).

Gly-Sar and Gly-Pro were from Sigma–Aldrich Danmark A/S (Broendby, Denmark).

Propidium iodide (1.0 μM), Alexa-488-phalloidin (0.066 mM), and Alexa-488-goat-anti-rabbit (10 μg/ml) were from Molecular Probes, Invitrogen (Eugene, OR, USA). Standard chemicals used were of analytical grade.

2.2. Methods

2.2.1. Cell culture and maintenance

Calu-3 cells were obtained from the American Type Culture Collection (ATCC) (HTB-55; LGC Promochem AB, Boraas, Sweden) at passage 31 and grown in T-75 culture flasks in an atmosphere of 5% CO_2 – 95% O_2 at 37 °C. Calu-3 cells were used in passage numbers 5–19 after the cells were received from the ATCC; for all experiments comparing different culture conditions, similar passage numbers were used. The cells were maintained in DMEM containing 10% FBS, 90 U/ml penicillin, and 90 μg/ml streptomycin. Calu-3 cells were sub-cultured when they reached approximately 80–90% confluence. Cells were seeded at a density of 10^5 cells/cm^2 onto collagen coated (0.29 μg/ml, 200 μl/insert) 12-well Transwell filters made of either polycarbonate (PC) or polyester (PET). Calu-3 cells were grown in an air-interfaced culture (AIC) or in a liquid-covered culture (LCC). For AIC, the cells were allowed to attach for 24 h before the medium in the apical compartment was removed. The cell layer was then allowed to differentiate under air-interface-feeding conditions by replacing the culture medium in the basolateral chamber every other day. For LCC, the culture medium was replaced in both the apical and basolateral chamber every other day. The studies were performed on polarized cell layers of Calu-3 cells between 7 and 17 days of growth.

2.2.2. Transepithelial electrical resistance (TEER)

TEER was measured in a tissue resistance measurement chamber (Endohm) (World Precision Instruments, Berlin, Germany) with a voltohmmeter (Millicell-ERS) (Millipore, Billerica, MA, USA). TEER was measured prior to the experiments at room temperature. The resistances of cell-free Transwell filters were minimal (PC filters, collagen coated: 22 Ω cm^2; PC filters, without coating: 19 Ω cm^2; PET filters, collagen coated: 27 Ω cm^2; PET filters, without coating: 27 Ω cm^2), and the TEER values reported were not corrected for the resistance of the cell-free filters.

2.2.3. Apical uptake of Gly-Sar in Calu-3 cell layers

Apical uptake of Gly-Sar in the absence or presence of Gly-Pro was measured in Calu-3 cells grown on PC or PET Transwell filters with AIC or LCC conditions on day 7, 10, 14, and 17. The buffers used were HBSS supplemented with 0.05% BSA and either 10 mM HEPES (pH 7.4) (HEPES-buffer) or 10 mM 2-(N-morpholino) ethanesulfonic acid (pH 6.0) (MES-buffer). Calu-3 cell layers were incubated for 10 min under LCC conditions and 30 min under AIC conditions with 0.5 ml MES-buffer at the apical side and 1.0 ml HEPES-buffer at the basolateral side. The cells were circularly rotated at 90 rpm on a KS10 DIGI shaker (Edmund Bühler) (Holm and Halby, Broendby, Denmark). Apical uptake of Gly-Sar was initiated by adding fresh MES-buffer containing 2.0 μM (1 μCi/ml) [^{14}C]-Gly-Sar or a combination of concentrations of 0–10 mM Gly-Sar and 1.0 μCi/ml [^{14}C]-Gly-Sar in the absence or presence of 20 mM Gly-Pro.

Apical uptake of [^{14}C]-Gly-Sar into Calu-3 cells was terminated after 5 min for cells grown under LCC conditions and 30 min under AIC conditions. The reason for the time difference was that the Calu-3 cells grown under AIC produce a mucus layer on top of the cell layer; however, after a short lag-phase (approximately 2 min), the uptake is linear up to 40 min (data not shown). The media was removed by gentle suction, followed by three washes of the cell layers with ice-cold HBSS. The filters were cut from the Transwell supports and placed into scintillation vials. Two milliliters of scintillation fluid (Ultima Gold, Perkin Elmer, Shelton, CT, USA) was added, and the radioactivity was counted in a liquid scintillation analyzer (Packard Tri-Carb 2100 TR, Canberra, Dreich, Germany). Specific cellular [^{14}C]-Gly-Sar uptake was corrected for the isotopes present in the extra-cellular space using 0.5 μCi [^{3}H]-mannitol per well as a marker of the extra-cellular volume as previously described by Bravo et al. [14]. Calu-3 cells grown under AIC or LCC conditions on PC or PET Transwell filters on day 10 and on PC Transwell filters on day 17 were measured for concentration-dependent apical Gly-Sar uptake, similarly to the above described.

2.2.4. Transepithelial transport of selected nutrients across Calu-3 cell layers

Transepithelial transport of selected compounds (18 μM [^{14}C]-Gly-Sar, 1.5 μM [^{3}H]-etoposide, 0.06 μM [^{14}C]-mannitol, 0.2 μM [^{3}H]-taurocholic acid, 11 μM [^{14}C]-glycine, 0.01 μM [^{3}H]-proline, 0.6 μM [^{3}H]-inulin, 0.2 μM [^{3}H]-estrone-3-sulfate, 3.5 μM [^{14}C]-α-methyl glucoside and 0.2 μM [^{3}H]-glipizide) was measured across Calu-3 cell layers grown on PC Transwell filters under AIC

or LCC conditions, respectively. Experiments were performed on day 16 ($n = 4$). The cells were incubated with buffers as described in the previous experiment. The concentration of the radioactive compounds in the donor solutions was 1.0 μCi/ml. For apical to basolateral transport studies, 10 μl samples were taken from the donor solution (0.5 ml) at $t = 0$, 20, 40, 60, and 180 min and 100 μl samples were taken from the receiver solution (1.0 ml) and replaced with fresh buffer ($t = 0$, 10, 20, 30, 40, 60, 120, and 180 min). For basolateral to apical transport studies, 10 μl samples were taken from the donor solution (1.0 ml) at $t = 0$, 20, 40, 60, and 180 min and 50 μl samples were taken from the receiver solution (0.5 ml) and replaced with fresh buffer ($t = 0$, 10, 20, 30, 40, 60, 120 and 180 min). Receiver samples were taken to measure the amount to compound moved across the cell layer. Donor samples were taken in order to monitor the sink condition and calculate recovery and mass balance. Samples were transferred to scintillation vials, where 2 ml of scintillation fluid was added and the radioactivity was counted in a liquid scintillation analyzer.

2.2.5. Immunohistochemistry

Assessment of cell morphology was investigated in Calu-3 cells grown on PC or PET Transwell filters with AIC or LCC conditions. Calu-3 cells were used on day 10 and 17 ($n = 3$). Immunohistochemistry was performed using indirect immunofluorescence staining followed by confocal laser scanning microscopy (CLSM). The cell layers were fixed for 10 min in 3% Para formaldehyde (Merck, Darmstadt, Germany) in HEPES-buffer. Cells were then permeabilized with 0.1% Triton X-100 (MP Biomedicals, Inc., Eschwege, Germany) in PBS for 5 min and rinsed twice in PBS. All preparation steps were performed on a shaking plate pre-heated to 37 °C. The filters were subsequently cut from the Transwell supports, the mucus layer was mechanically removed from AIC cells, and all filters were cut into six pieces and then blocked in PBS with 2% BSA for 30 min. AIC cells were first incubated at 37 °C on a shaking plate for 1 h, before being incubated together with LCC cells overnight at 4 °C. The following day, the cells were rinsed three times for 5 min in blocking solution. Bound antibodies were detected after incubation for 30 min for LCC cells and 75 min for AIC cells with the Alexa-488-conjugated goat-anti-rabbit immunoglobulin G (IgG) antibody, in order to get an even staining of the cell layers. This was then followed by three 5 min rinses of cell layers in blocking solution. Cell nuclei were visualized by counterstaining with 1 μM propidium iodide for 1 min for LCC cells and 2 min for AIC cells followed by two quick rinses of the cells with PBS. Supports, with the cells attached, were mounted on cover slips and sealed with nail polish. A preparation stained with secondary antibodies only was used as negative control. Morphology of the Calu-3 cell layers was visualized with Alexa 488-conjugated phalloidin that labels F-actin. The preparations were then kept at 5 °C. Confocal imaging was performed on a Zeiss LSM 510 confocal laser scanning microscope (CLSM), using a Zeiss plan apochromat 63× oil immersion objective with a numerical aperture of 1.4. Fluorophores were excited using an argon laser line at 488 nm and a HeNe laser line at 543 nm for the Alexa-488 (green) and the propidium iodide (red) fluorescent probes, respectively.

2.2.6. Data analysis

The apical uptake rate of Gly-Sar in Calu-3 cell layers as a function of apical Gly-Sar concentration was fitted to Eq. (1):

$$J = \frac{J_{max} \cdot [\text{Gly-Sar}]^n}{K_m^n + [\text{Gly-Sar}]^n} \quad (1)$$

where J is the uptake rate (nmol cm^{-2} min^{-1}), J_{max} is the maximal uptake rate (nmol cm^{-2} min^{-1}), K_m is the Michaelis constant (mM), and n is the Hill slope.

The apparent permeability coefficients (P_{app}) of the transepithelial transport of the investigated compounds were calculated using Eq. (2):

$$P_{app} = \frac{J}{C_0} = \frac{Q_t \cdot A^{-1}}{t \cdot C_0} \quad (2)$$

where J is the steady state flux (pmol cm^{-2} min^{-1}), C_0 is the compound concentration in the donor chamber at time 0 min, A is the area of the filter support (1.12 cm^2), and Q_t is the accumulated compound in the receiver chamber at time t.

2.3. Statistical analysis

Experiments in Calu-3 cell layers were performed using several passages ($n = 3$–4). Values are given as means ± standard error of the mean (SEM) unless otherwise stated. The statistical significance of the results was determined using two-tailed Students t-test or one-way ANOVA (Tukey–Kramer). $p < 0.05$ was considered significant.

3. Results

3.1. Culture conditions affect Calu-3 cells barrier properties and cell layer morphology

The barrier properties of Calu-3 cells, cultured under different growth conditions, were evaluated by measuring TEER values and by visualizing cell morphology using confocal laser scanning microscopy (CLSM). The TEER values of Calu-3 cells were significantly different between cells grown under AIC and LCC conditions (Fig. 1). However, by using either polycarbonate or polyethylene as the material for filter support, it does not affect the generation of TEER throughout the culture period for Calu-3 cells grown under AIC or LCC conditions (data not shown). The TEER values for Calu-3 cells grown under AIC conditions increased throughout the culture period from approximately 100 on day 7–237 ± 16 Ω cm^2 on day 17 (Fig. 1A). For Calu-3 cells grown under LCC conditions, the TEER values were 2–3 times higher, i.e., 576 ± 72 Ω cm^2 on day 17 (Fig. 1B). The major increase in TEER values of Calu-3 layers appears to happen before day 7, as the TEER did not change much over the investigated time period of day 7–17. Cells cultured in the presence of liquid on the apical side were morphologically quite different from cells cultured with an air interface. Whereas LCC Calu-3 cells had an average height of 25 μm and had a monolayer appearance, AIC layers had an average height of 80 μm and had a rugged and folded appearance (Fig. 2A–D).

3.2. Dipeptide uptake in Calu-3 cell layers grown under AIC or LCC conditions

The apical uptake of [^{14}C]Gly-Sar in Calu-3 cell layers was investigated in cells grown under AIC or LCC conditions after different days in culture. The apical uptake of 2 μM [^{14}C]Gly-Sar in Calu-3 cells grown under AIC conditions increased throughout the culture period (Fig. 3A). The apical [^{14}C]Gly-Sar uptake in Calu-3 cells grown under LCC condition did not increase with increasing time in culture (Fig. 3B). The choice of filter material, polycarbonate or polyethylene, did not affect the Gly-Sar uptake rates (data not shown). After 10 days in culture, there is a significantly higher apical uptake rate of Gly-Sar in Calu-3 cells grown under AIC conditions compared to cells grown under LCC conditions. On day 17, the apical uptake of Gly-Sar was 1.32 ± 0.12 pmol cm^{-2} min^{-1} for Calu-3 cells grown under AIC conditions, whereas for Calu-3 cells grown under LCC conditions, the uptake was only 0.24 ±

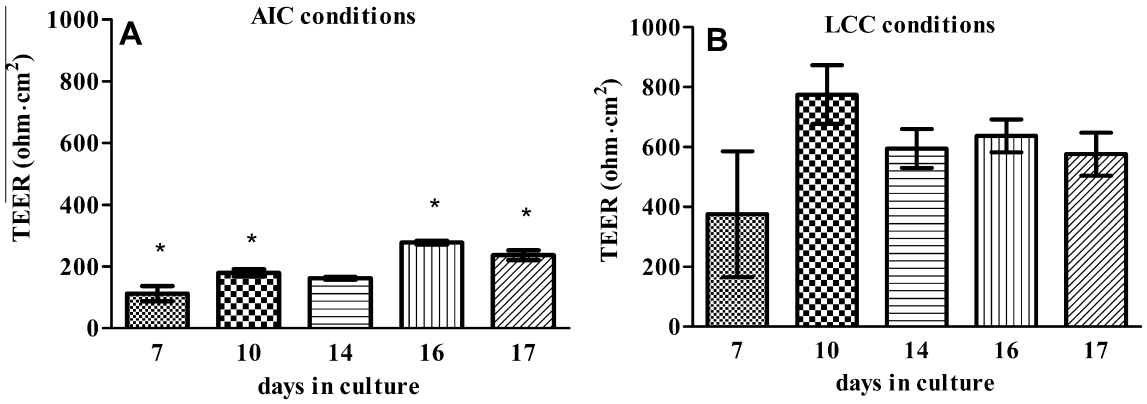

Fig. 1. The transepithelial electrical resistance (TEER) across Calu-3 cell layers as a function of days in culture. Calu-3 cells were grown on PC filters under AIC conditions (A) or LCC conditions (B). TEER was measured on five different days in the culture period (7–17 days). Data are shown as mean value ± SEM. (n = 4–10). $^{*}p < 0.05$.

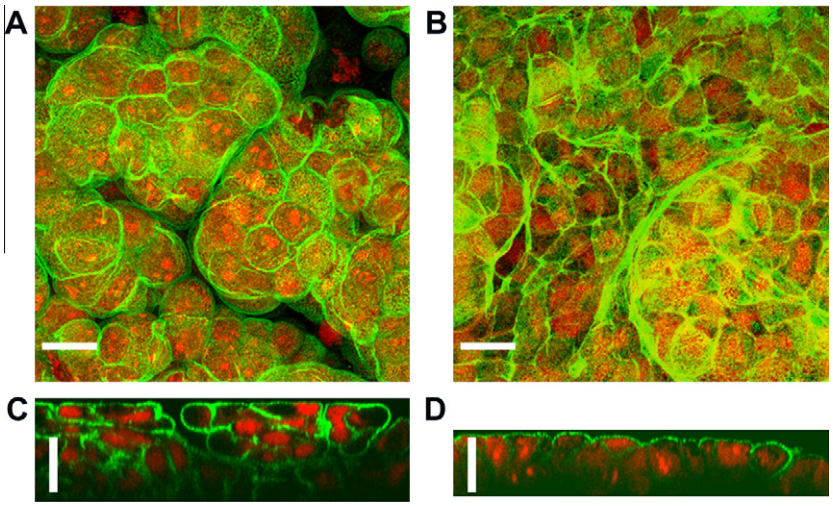

Fig. 2. Confocal images of Calu-3 cells grown for 16 days on collagen-coated polycarbonate filters. Left column; cells grown with an apical air interface (AIC). Right column; cells grown with culture media on the apical side (LCC). Figures A and B are maximal transparency projections from a stack of *xy*-sections, originating from the whole depth of the cell layers. Cell actin is stained with Alexa-488-phalloidin (green), and cell nuclei are stained with propidium iodide (red). Lower figures (C and D) are *xz* sections of the cell layers. Cell actin is stained with Alexa-488-phalloidin (green), and cell nuclei are stained with propidium iodide (red). The white bar represents a distance of 20 μm.

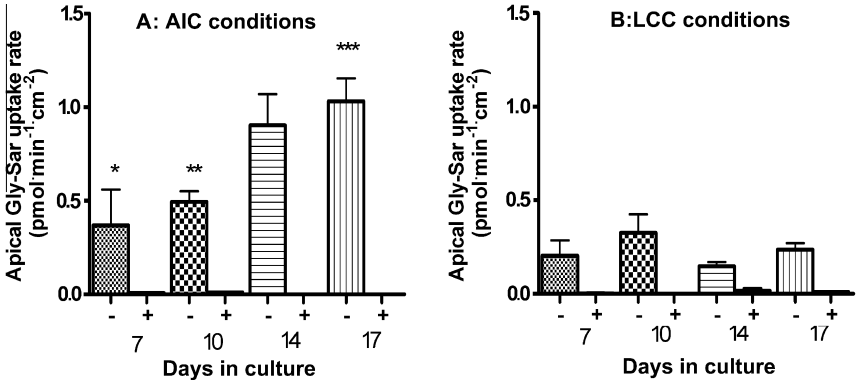

Fig. 3. The apical uptake rate of 2 μM [^{14}C]Gly-Sar in Calu-3 cell layers. –: Uptake of 2 μM [^{14}C]Gly-Sar in the absence of Gly-Pro. +: Uptake of 2 μM [^{14}C]Gly-Sar in the presence of 20 mM Gly-Pro. Cells were grown on polycarbonate filters under AIC conditions (A) or LCC conditions (B) for 7, 10, 14, or 17 days in culture. Data are shown as mean values ± SEM (n = 3–4). *: $p < 0.05$.

0.04 pmol cm^{-2} min^{-1}. For cells grown under AIC conditions, the apical uptake rates were significantly higher on day 17 compared to day 7, whereas under LCC conditions, the apical uptake rate of Gly-Sar was comparable for all four culture days investigated. In the presence of 20 mM Gly-Pro in the apical solution, the uptake of Gly-Sar in Calu-3 cells grown under both AIC or LCC conditions

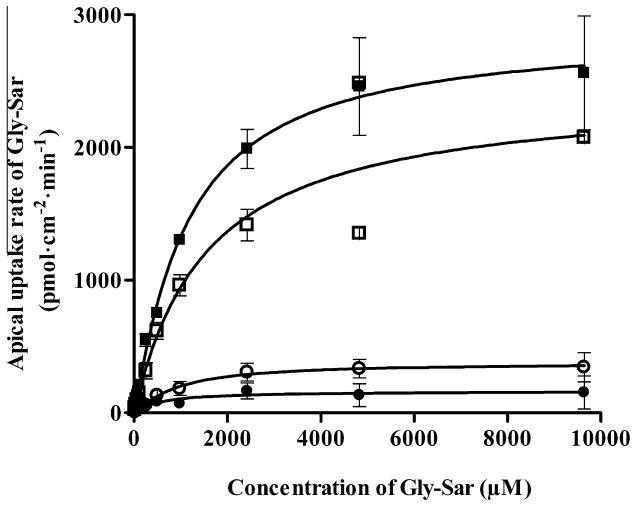

Fig. 4. The apical uptake rate of [^{14}C]Gly-Sar as a function of Gly-Sar concentration in Calu-3 cells. Cells were grown on polycarbonate filters under AIC (■,□) or LCC (●,○) conditions. The study was performed after 10 (○,□) or 17 (●,■) days of growth. Data are shown as mean values ± SEM (n = 3–4).

was reduced to less than 10% of the uptake observed in the absence of 20 mM Gly-Pro (Fig. 3a and b), illustrating that the dipeptide uptake is inhibitable, and thus it must be carrier mediated.

To further investigate the apical uptake of Gly-Sar in Calu-3 cell layers, the concentration-dependent Gly-Sar uptake was investigated at two different days of culture for both AIC and LCC cells (Fig. 4). The concentration-dependent apical uptake of Gly-Sar was saturable in the concentration range investigated after both 10 and 17 days in culture (Fig. 4). The apical Gly-Sar uptake could be characterized as an expression off substrate interaction with only one transport system as described by Eq. (1). The derived kinetical parameters K_m, J_{max}, and the Hill coefficient (n) are given in Table 1. The Hill coefficients were all approximately 1, and the K_m values were approximately 1 mM, indicating that the uptake of Gly-Sar in Calu-3 cells is due to hPEPT1-mediated transport. The maximal apical uptake rates were significantly larger for cells grown under AIC conditions than for cells grown under LCC conditions. For cells grown under AIC conditions, the maximal apical uptake rate was significantly larger on day 17 than on day 10 (Fig. 4).

3.3. Nutrient transport in Calu-3 cell layers grown under AIC or LCC conditions

To further investigate bidirectional transport across Calu-3 cells, the transport of 10 compounds across Calu-3 cells was measured. In Fig. 5, the apparent permeability coefficients of all compounds measured under AIC or LCC conditions are shown.

Taurocholic acid, proline, estrone-3-sulfate, glipizide, glycine, and α-methyl-D-glucoside all showed polarized transport from the apical (A) to the basolateral (B) side under both LCC and AIC conditions, with significantly greater permeability constants (P_{app}) in the A–B direction than in the B–A direction. The permeability of proline in the B–A direction was greater for cells grown in AIC than cells grown in LCC. Similarly, the permeability of glycine and α-methyl-D-glucoside in the A–B direction is greater for cells grown in LCC than cells grown in AIC. The greatest influx ratio (J_{A-B}/J_{B-A}) was measured for glycine: 79.2 in LCC (when compared to 47.3 in AIC) and the second largest for α-methyl-D-glucoside: 50.4 in AIC and merely 8.8 in LCC. The transport of glipizide was substantial as it had the largest apparent permeability coefficients (P_{app} = 19.0 10^{-6}cm s^{-1} in LCC and P_{app} = 16.6 10^{-6}cm s^{-1} in AIC) of all the compounds investigated here. The difference between the permeability from A–B and B–A of glipizide was small; the influx ratios were 6.0 and 5.2 for cells grown under LCC and AIC, respectively. The transport of etoposide was polarized from the basolateral to the apical side under both growth conditions. The permeability of etoposide was larger for cells grown under AIC than LCC, regardless of the direction. Transport of the paracellular markers (mannitol and inulin) was low in both directions, with a maximum of 1.2% of mannitol and 3.8% of inulin transported to the receiver chamber after 180 min. The influx ratios were between 0.66 and 2.20, and no significant difference was found in the apparent permeability coefficients (P_{app}) for both the direction, A–B and B–A, and the growth conditions, LCC or AIC. Surprisingly, Gly-Sar did not show significant difference in P_{app} regardless of the growth conditions or direction. Collectively, Calu-3 cells' transport proteins cause polarized transport of a number of the nutrient or drug substances investigated.

Table 1
Kinetic parameters for Gly-Sar uptake in filter-grown Calu-3 cells (n = 3–4). Values are derived from the data shown in Fig. 4 using Eq. (1).

		K_m (μM)	J_{max} (pmol cm^{-2} min^{-1})	Hill coefficient
AIC	Day 10	1584 ± 229	2414 ± 125	0.9 ± 0.1
	Day 17	1138 ± 318	2868 ± 289	1.1 ± 0.2
LCC	Day 10	890 ± 340	376 ± 54	1.2 ± 0.4
	Day 17	557 ± 515	163 ± 47	1.0 ± 0.6

4. Discussion

The impact of transporters when defining the pharmacokinetic profile of drug substances has been increasingly recognized over the last years. For absorptive transporters, one example is the oral absorption of the GABA-A receptor agonist gaboxadol, which is a substrate of the proton-coupled amino acid transporter PAT1 [15]. In rat and dog studies *in vivo*, the absorption of gaboxadol is altered by co-administration of PAT1 inhibitors tryptophan and 5-hydroxy-trytophan, indicating that the oral absorbed of gaboxadol occurs via PAT1 [15,16]. The lung also represents a potential administration route for drugs substances, but compared to oral administration, less is known about the role of transporters in lung tissue [13]. As *in vitro* models often provide the first insights into transport mechanisms, the aim of the present study was to investigate whether the AIC and LCC conditions influence drug and nutrient transport known to occur via carriers or transporters, in particular dipeptide transport into the cells, in the Calu-3 cell *in vitro* model.

4.1. Basic characteristics of AIC- and LCC-cultured Calu-3 cell layer

The Calu-3 cell line is cultured using different culture conditions and filter support. Here, we found that using polycarbonate or polyester as filter support material does not affect the generation of layer TEER, apical dipeptide uptake, or general cell layer morphology. A pronounced effect on TEER and apical dipeptide uptake was observed for the different culture procedures used, i.e. the conventional liquid-covered culture (LCC) and the air-interfaced culture (AIC). Our results are in agreement with previous studies reporting TEER values of 300–550 Ω cm^2 and 800–1000 Ω cm^2 for AIC and LCC layers, respectively [4,5,10,17]. Morphological differences were quite apparent for AIC and LCC cell layers. Whereas LCC Calu-3 cells had an average height of 25 μm and had a monolayer appearance, AIC layers had an average height of 80 μm and had a rugged and folded appearance suggesting a pseudostratified epithelium. Cell layers from both growth conditions displayed

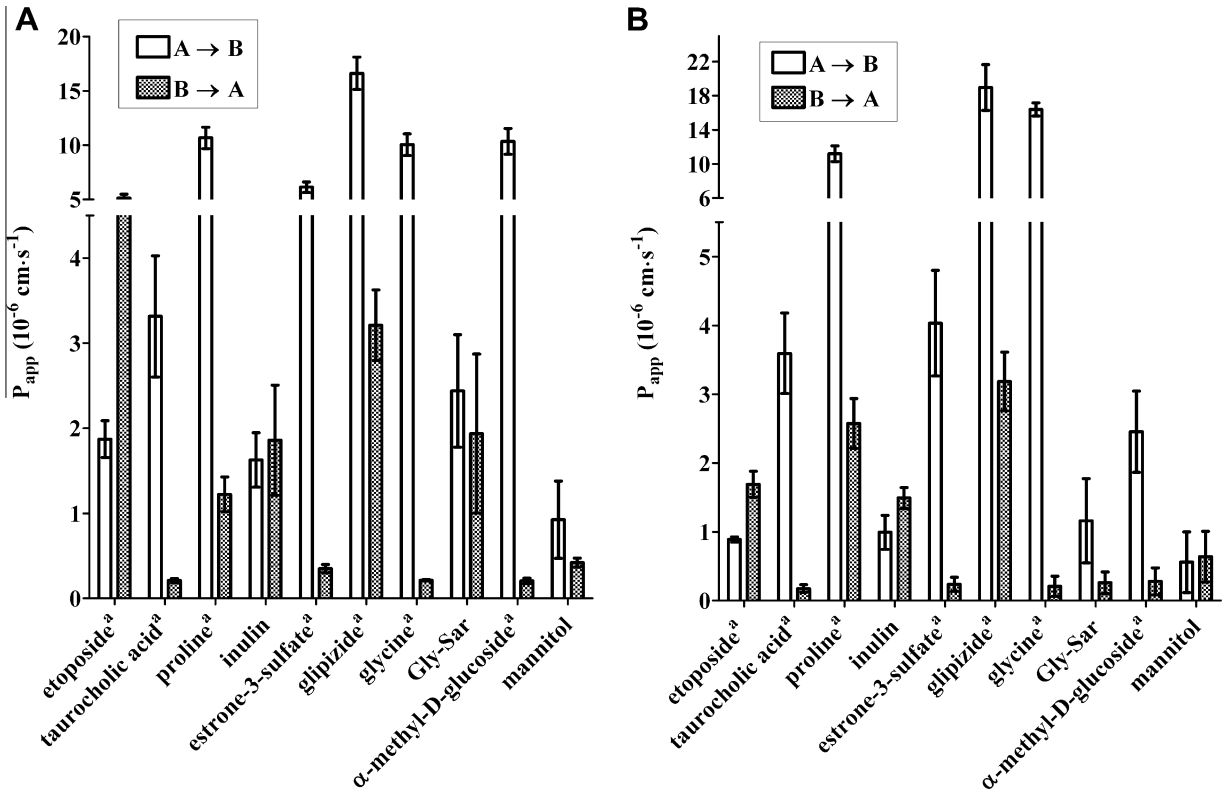

Fig. 5. Bidirectional transport of selected nutrients and drug substances across Calu-3 cells. (A) Calu-3 cells grown under AIC conditions. (B) Calu-3 cells grown under LCC conditions. The experiments were performed after 16 days in culture. Apical pH was 6.0, and basolateral pH was 7.4. A–B: the apical chamber is the donor side, and the basolateral chamber is the receiver side; B–A: the basolateral chamber is the donor side, and the apical chamber is the receiver side. Data shown as mean values ± SEM (n = 3–4). [a]Indicates that the A–B transport is different from the B–A transport of the indicated substance ($p < 0.05$).

occludin staining, indicating formation of tight junctions. Calu-3 cells grown under LCC have previously been reported to have height of 15–30 μm with defined tight junctions [4,18], whereas AIC cell layers are 20–45 μm high [2,4]. The present results are thus quite comparable with previous studies.

4.2. Dipeptide transport in Calu-3 cells

The first indication of carrier-mediated transport via peptide transporter present in Calu-3 cells was published by Mathias et al., who showed that the uptake of Gly-Sar, a substrate of PEPT1 and PEPT2, into AIC cultured Calu-3 cells was decreased in the presence of 25 μM ouabain or 20 mM unlabeled Gly-Sar [2]. Subsequently, it was showed that both hPEPT1 and hPEPT2 mRNAs are expressed in Calu-3 cells and that the hPEPT1 protein localizes to the apical membrane [12]. Expression of peptide transporters in native human lung tissue has been confirmed in a number of studies [12,19]. In the present study, we found that Gly-Sar uptake increases during the culture time under AIC conditions, whereas Gly-Sar uptake in LCC cells was markedly lower. In AIC Calu-3 cells, the Gly-Sar uptake is saturable and the K_m values for Gly-Sar uptake are similar to the ones reported for hPEPT1 [20,21] which is similar to previous findings in Calu-3 cells [12]. This indicates that AIC Calu-3 cells are suitable for investigating the impact of peptide transporters in mediating drug uptake. We have previously shown that the apical to basolateral Gly-Sar transport in Calu-3 cells is pH-dependent and reduced in the presence of δ-aminolevulinic acid, cephalexin, or captopril [12]. However, in this series of experiments, we were not able to significantly show that the apical to basolateral transport of Gly-Sar exceeds the basolateral to apical transport. Even though the AIC cells have a saturated Gly-Sar uptake, which may be inhibited by Gly-Pro, this does not translate into polarized transepithelial transport. The lack of polarization may be due to variations in peptide transporter expression or the lack of a transport protein mediating the basolateral exit of Gly-Sar. Similar observations have been made in SKPT cells that express PEPT2. SKPT cells are SV-40 transformed proximal tubule cells from the rat kidney, where the apical uptake of Gly-Sar is saturable. When SKPT cells are in the presence of Gly-Pro, the A–B transport is decreased, but no polarization is detectable for the transepithelial transport when comparing the A–B transport with the B–A transport [22].

4.3. Transepithelial transport in AIC and LCC Calu-3 cells

For the comparison of transepithelial transport in AIC and LCC Calu-3 cells, nine additional compounds were chosen in addition to the dipeptide Gly-Sar. Inulin and mannitol were chosen as paracellular markers for which no polarized transport was observed. The permeabilities of mannitol and inulin were comparable when measured under AIC and LCC culture conditions. The permeability of mannitol was approximately 5×10^{-7} cm/s, which is comparable to the previously obtained values for paracellular markers such as fluorescein sodium and mannitol ($1–4 \times 10^{-7}$ cm/s) [2,4,6–10]. Similar to other studies, the permeabilities do not seem to be directly correlated to the TEER values measured [2,4,6–10]. Two compounds showed markedly different transport permeabilities between LCC and AIC conditions, i.e. etoposide and α-methyl-D-glucoside. For etoposide, the A–B and B–A transport, in Calu-3 cells grown in AIC, were approximately twice as high as in cells grown under LCC. This results in a polarized B–A transport of etoposide in AIC and LCC Calu-3 cells. Etoposide is a substrate of several

efflux transporters such as P-gp, MRP2, and MRP3 [23–25], and it now seems that AIC conditions are required for measuring higher etoposide B–A polarized transport. The transport of α-methyl-D-glucoside, used to study SGLT-mediated glucose transport, is polarized in the A–B direction under both AIC and LCC conditions. However, the A–B transport is approximately three times higher in AIC Calu-3 cells than in LCC cells. Taurocholic acid is a substrate of a number of transporters such as NTCP, ASBT, and OSTα/β [26,27]. Taurocholic acid has polarized A–B transport in Calu-3 cells under both AIC and LCC conditions, suggesting that one or more transporters of taurocholic acid are present in the apical membrane of Calu-3 cells. Proline and glycine are amino acids that are transported by several transporters such as PAT1, PAT2, and SIT1 which have been described in different epithelia (for a review, see Broer [28]). Polarized A–B transport of proline and glycine was identified under both AIC and LCC conditions, and the permeabilities of proline and glycine are among the largest values measured in the present study. Based on our functional transport studies, Calu-3 cells have amino acid transporters for proline and glycine in the apical membrane and the overall permeability is quite comparable between LCC and AIC conditions. Glipizide is another compound with a high permeability measured and a polarized A–B transport under both LCC and AIC conditions. Part of the polarization is likely to be due to the pH gradient applied in the transport experiments, as glipizide is an acid drug substance with a pK_a value between 5 and 6 [29]. In Caco-2 cell monolayers, glipizide is suggested to be a substrate for the basolateral OSTα/β and a low-capacity apical efflux transporter [29]. Estrone-3-sulfate is another acid substance that has been identified as a substrate of several carriers such as OATP2B1, MRP1, BCRP, and OSTα/β [26,30–32]. In Calu-3 cell layers, estrone-3-sulfate transport is polarized in the A–B direction under both LCC and AIC conditions and the permeability values determined are similar under LCC and AIC conditions. These results are in contrast to estrone-3-sulfate transport studies in intestinal Caco-2 cells, where the transport is polarized in the B–A direction [29]. As estrone-3-sulfonic acid has a pK_a value of 2.2, differences in uncharged species of estrone-3-sulfate between buffers with pH 6.0 and 7.4 are not likely to account for the polarization in transport observed in Calu-3 cells. It seems possible that a different expression pattern of transport proteins in the apical and basolateral membranes of Calu-3 and Caco-2 cells may be the cause of these observations.

Based on the functional studies presented, it appears that Calu-3 cells express a range of transporters mediating the transepithelial transport nutrient and drug substances. We have shown that Calu-3 cells express PEPT1 and PEPT2, but for other transporters, identification of the molecular nature of the transporters is required. Overall, the present study serves as a starting point for further work on functional consequences of transporters expressed in Calu-3 cell layers.

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