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Research paper

Commonly used nonionic surfactants interact differently with the human efflux transporters ABCB1 (p-glycoprotein) and ABCC2 (MRP2)

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ABSTRACT

The efflux transporters ABCB1 (p-glycoprotein) and ABCC2 (MRP2) play an essential role in the limitation of oral bioavailability of drugs. In the last years, pharmaceutical surfactants like cremophor® EL or polysorbate 80 have been shown to interact with ABCB1. However, the knowledge about their influence on ABCC2 is still limited.

In this study, the interactions of the nonionic surfactants cremophor® EL, cremophor® RH 40, polysorbate 80, vitamin E TPGS 1000, pluronic® PE 10300 and sucrose ester L-1695 with both efflux transporters were investigated on cellular level.

Cell accumulation studies and transport studies were performed using transfected MDCK II cell models. The influence of ABCC2 inhibiting surfactants on the expression level of ABCC2 was also studied.

The investigations showed that cremophor® EL, vitamin E TPGS 1000 and higher concentrations of polysorbate 80 inhibit both transporters. Pluronic® PE 10300 and sucrose ester L-1695 inhibit ABCB1 but not ABCC2. Cremophor® RH 40 only shows inhibitory activity on ABCC2. During the investigated incubation period none of the inhibiting surfactants caused an alteration in *ABCC2*mRNA or protein expression. These findings indicate that the observed interactions are caused by specific inhibition of the transport activity of ABCC2.

1. Introduction

The number of poorly soluble and lipophilic drugs with limited oral bioavailability among the new drug entities entering galenic development has increased over the last years. In many cases lower oral bioavailability entails higher inter-individual variability in bioavailability [1]. This can result in an inadequate control of plasma concentrations and pharmacological effects [2].

Besides solubility and gastric stability, metabolic processes as well as transporter-mediated efflux play an important role in influencing oral bioavailability of drugs [3]. Chemical modification of substances by cytochrome P450 enzymes and subsequent conjugation result in many cases in compounds with increased hydrophilicity that are often excreted via efflux transporters such as ABCB1 (p-glycoprotein) [4]. ABCB1 belongs to the ATP-binding cassette (ABC) transporter subfamily that combines a wide range of proteins found in a number of different species. ABC transport proteins mediate the transport of a diversity of structurally different substances like amino acids, ions, peptides and also a variety of drugs through cell membranes [5].

A common method to improve the oral bioavailability of drugs is the use of solubilising agents like complex forming hydroxypropyl β cylcodextrin, co-solvents or surfactants in drug formulations [6–9].

Nonionic surfactants have been shown to inhibit the human efflux transporter ABCB1. So they may, additionally to their solubilising properties, influence the disposition of many drugs [10–14]. Another important ABC drug efflux transporter which is involved in limiting the oral bioavailability of many compounds is ABCC2 [4,15]. This transport protein is also known as the multidrug resistance associated protein 2 (MRP2). ABCB1 and ABCC2 share some similarities like their apical localisation in enterocytes, hepatocytes or the proximal tubular cells of the kidney [16,17] as well as their partially overlapping substrate specificity. ABCB1 preferentially transports neutral or weakly basic, large amphipathic compounds. ABCC2 particularly extrudes organic anions such as glutathione conjugates, sulfates or glucuronides [18]. Both transporters are furthermore involved in the efflux of different chemotherapeutics, protease inhibitors or antibiotics.

In the last two decades, interactions between nonionic surfactants and the efflux transporter ABCB1 have thoroughly been inves-

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tigated [10,19–21]. However, the knowledge about interactions between surfactants and ABCC2 is limited up to date. This discrepancy may be attributed to the later identification of ABCC2 (1996) compared to ABCB1 (1976) [22,23]. As ABCB1 is the efflux transporter which is understood best today, investigations on drug–drug interactions are recommended by the FDA especially for this transporter [24]. Nowadays, the importance of other drug transporters has gained increasing recognition.

Despite the increasing knowledge about these transporters and the mediated drug transport in vitro, the prediction of the impact on oral absorption in vivo is difficult. Particularly the identification of the specific impact of transporter-mediated lack of oral absorption remains an obstacle. One attempt to handle this problem is the correlation of results from in vitro assays such as permeability measurements in cell monolayers with in vivo data obtained from ABCB1 deficient and wild-type mice [25–27]. Although good correlations were shown to some extent it is still questionable if these correlations can be transferred to oral absorption in man. It has to be kept in mind that the type of in vitro assay used also influences the predicted in vivo behaviour of orally administered drug formulations. Collett et al. [28] showed that the correlation of in vitro permeability assessment using cell monolayers was less suitable to predict in vivo absorption of modified release formulations. In that study the usage of ex vivo colon preparations yielded data with much better correlation.

It was the aim of this study to investigate the interactions of commonly applied structurally different nonionic pharmaceutical surfactants (cremophor® EL, cremophor® RH 40, polysorbate 80, pluronic® PE 10300, vitamin E TPGS 1000 and sucrose ester L-1695) with ABCB1 and ABCC2. In this context it was of particular interest if the surfactant mediated effects are transporter-specific or not.

2. Materials and methods

2.1. Chemicals

Calcein acetoxymethyl ester (calcein-AM) was purchased from Mobitec (Goettingen, Germany), and stock solution (1 mM) was prepared in dimethylsulfoxide (DMSO). Calcein, probenecid (stock solution: 10 mM in DMSO), ivermectin (stock solution: 30 mM in DMSO) and resazurin (stock solution: 8.8 mM in phosphate-buffered saline (PBS)) were obtained from Sigma–Aldrich (Steinheim, Germany). PSC-833 (stock solution: 10 mM in ethanol 99% (v/v)) was kindly provided by Norvatis, Basel, Switzerland). Cremophor® EL (glycerol polyethylene glycol ricinoleate, critical micelle concentration (CMC) = 0.01%) was purchased from Synopharm (Barsbuettel, Germany), cremophor® RH 40 (glycerol polyethylene glycol hydroxy stearate, CMC = 0.039%) and polysorbate 80 (CMC = 0.005%) were obtained from Caelo (Hilden, Germany). Vitamin E TPGS 1000 (D-alpha-tocopheryl poly(ethylene glycol 1000) succinate, CMC = 0.02%) was kindly provided by Eastman (Llangefni, UK), pluronic® (polypropylene polyethylene block copolymer) PE 10300 (CMC = 0.003%) was supplied by BASF (Ludwigshafen, Germany), and sucrose ester L-1695 was obtained from Mitsubishi-Kagaku Foods Corporation (Tokyo, Japan). All cell culture reagents were purchased from Biochrom (Berlin, Germany). All other substances were of analytical grade and were obtained from commercial sources.

2.2. Cell lines

Polarized canine kidney cell lines (MDCK II) stably expressing human ABCB1 (MDCK-ABCB1) and human ABCC2 (MDCK-ABCC2) were used for all in vitro cell studies. The parental cell line (MDCK-par) [29,30] served as a control. The cell lines were kindly provided by A. Schinkel (The Netherlands Cancer Institute; Amsterdam, Netherlands) and were cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 2% stable glutamine and 1% penicillin/streptomycin.

2.3. Viability studies

Viability studies were performed for all cell lines with all surfactants using the resazurin assay [31]. A dilution of 1000 cells/200 μl medium was added to each well of a 96-well plate (TPP, Trasadingen, Switzerland) and cultured over night. On the following day, cells were treated with incubation buffer (IB) containing NaCl (142 mM), KCl (5 mM), KH_2PO_4 (1 mM), $CaCl_2{\cdot}6H_2O$ (1.5 mM), $MgSO_4{\cdot}7H_2O$ (1.2 mM), glucose (5 mM) and HEPES (12.5 mM) or solutions containing the surfactants in different concentrations (0.0005–0.5% in IB) for 1.5 h and 7 h, respectively, at 37 °C. Afterwards, cells were washed twice with warm PBS and cultured in fresh medium for three further days. On the day of investigation 20 μl resazurin (880 μM dissolved in PBS) were added to each well resulting in a final concentration of 80 μM/well. After incubation for 7 h, fluorescence was measured using the Fluoroskan II (Labsystems, Helsinki, Finland; wavelengths: excitation: 544 nm, emission: 590 nm). Because of low auto-reduction by resazurin each plate contained blank samples consisting of the medium and resazurin but without cells. The experiments were performed in quadruplicate on two different cell batches for each cell line.

2.4. Calcein-AM accumulation assay

To investigate the influence of surfactants on ABCB1-mediated transport, an in vitro accumulation assay was established using calcein-AM as a substrate for ABCB1. Easy penetration of cell membranes is assumed for this nonfluorescent precursor because of its lipophilicity [32]. Usually, calcein-AM is immediately eliminated from cells via ABCB1. In the presence of ABCB1 inhibitors, calcein-AM remains inside the cells where hydrolysis mediated by cellular esterases forms the highly hydrophilic and fluorescent dye calcein.

MDCK-par and MDCK-ABCB1 cells were seeded into black 96-well plates (BD, Heidelberg, Germany) at a density of 70,000 cells/well. After culturing for 3 days the cells were washed with warm IB and equilibrated for 10 min followed by preincubation with IB containing different concentrations of surfactants for 30 min. Then, calcein-AM stock solution was added to each well resulting in a final concentration of 1 μM/well. Calcein fluorescence was detected after 60 min using the Fluoroskan II (wavelengths: excitation: 485 nm; emission: 538 nm). Each experiment included a negative control (cells only treated with IB) as well as a positive control (3 μM ivermectin or 1 μM PSC-833). For both cell lines the experiments were performed in quadruplicate using at least two different cell batches.

Results are expressed as relative fluorescent units (RFU). These units are ratios of fluorescence signals from cells which were treated with ABCB1 inhibitors or surfactants versus cells which were only incubated with IB (negative control). In this study RFU values of two and higher indicate an interaction with ABCB1.

2.5. ABCC2 transport assay

Interactions with ABCC2 were investigated using a Transwell® assay model with parental and ABCC2-transfected MDCK II cells which were cultured as mentioned earlier.

For the ABCC2 transport assay the fluorescent anion calcein served as ABCC2 substrate [33,34]. Because of its inability to penetrate cell membranes the lipophilic nonfluorescent precursor calcein-AM was used to load the cells as described above for the

accumulation assay. Since the MDCK-ABCC2 cells also contain some level of native ABCB1 [35], all experiments were performed in the presence of PSC-833 (1 μM). This substance represents a potent inhibitor of ABCB1 with negligible effects on ABCC2 [33] and had to be used to ensure a sufficient amount of calcein-AM inside the cells.

For the investigations cells were seeded on polyester membranes (0.4 μm pore size, 12 mm diameter, Corning Life Sciences, Schiphol-Rijk, Netherlands) with a density of 300,000 cells/well (MDCK-par) and 500,000 cells/well (MDCK-ABCC2), respectively, and were cultured for 3 days. Monolayer integrity was confirmed by measuring transepithelial electrical resistance (TEER) using the EVOMX (WPI, Harry Fein, Berlin, Germany) throughout the assay.

In the beginning of the assay cells were preloaded with appropriate solutions of surfactants or probenecid (100 μM) in PSC-833 containing IB. Probenecid served as positive control in this assay [36,37]. After 1 h of preincubation, new test solutions additionally containing calcein-AM (1 μM) were added to both compartments. The final volumes in the apical and basolateral compartment were 0.5 ml and 1.5 ml, respectively. Aliquots of 100 μl were taken hourly for a total of 5 h either from the apical or basolateral compartment and replaced with preheated test solution containing calcein-AM. The collected samples were transferred into 96-well plates, and then calcein fluorescence was measured using the Fluoroskan II (wavelengths: excitation: 485 nm; emission: 538 nm). Calibration measurements were performed with calcein for each experiment and in the presence of each tested concentration of surfactant to avoid quenching effects.

2.6. Calculations

Apparent permeability coefficients (P_{app}) were calculated as follows:

$$P_{app} = \frac{dm}{dt} \cdot \frac{1}{A \cdot c_0} (\mathrm{cm/s}) \quad (1)$$

$\frac{dm}{dt}$ (nmol/s) represents the drug flux over time which was determined by linear regression analysis. A (cm^2) is the surface area of the membrane and c_0 (μmol/l) is the initial drug concentration [38].

Statistical analysis was performed using the Student's t-test; $p \leqslant 0.001$ was considered as statistically significant.

2.7. ABCC2mRNA analysis by Real-Time Quantitative Reverse Transcription (RT)-PCR

To evaluate whether the observed effects on ABCC2 are caused by the inhibition of transport activity rather than influences on the expression level of ABCC2 cells were cultured for 3 days in cell culture flasks (25 cm^2 or 75 cm^2). After washing the cells with PBS, they were incubated at 37 °C with test solutions containing IB and the respective surfactants in relevant concentrations for 7 h. As negative control IB without any surfactant was used. Thereafter, cells were washed twice with PBS and were then harvested with trypsin/EDTA solution. After centrifugation cell pellets were divided in two fractions to measure *ABCC2*mRNA by real-time RT-PCR and ABCC2 protein content using Western blot analysis.

To quantify *ABCC2*mRNA total RNA was isolated from frozen cells using the RNAeasy® Mini extraction kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription of RNA was performed with random hexamer primers and the TaqMan reverse transcription kit (Applied Biosystems, Darmstadt, Germany). Expression analysis of *ABCC2* was quantified using the forward primer 5′-CTGGGAACATGATTCG GAAGC-3′, reverse primer 5′-GAGGATTTCCCAGAGCCGAC-3′ and the probe 5́-6FAM-CTGGGAACATGATTCGGAAGC-TAMRA-3′. For detection of *18S*rRNA, a pre-developed primer and probe mix was purchased from Applied Biosystems. PCR was performed using the real-time PCR cycler ABI prism 7700 Sequence Detector (Applied Biosystems). All reactions were performed according to the following protocol: heating at 50 °C for 2 min and at 95 °C for 10 min, followed by 40 cycles of heating at 95 °C for 15 sec and at 60 °C for 1 min. For quantification of *ABCC2*mRNA and *18S*rRNA signals were applied to a cloned standard (cloned PCR-fragments of the respective gene in pGem-Teasy, (Promega GmbH, Mannheim, Germany)) resulting in absolute copy numbers for the respective gene.

2.8. Western blot analysis

For Western blot analysis, frozen cell pellets containing 5 mM tris(hydroxymethyl)aminomethane (Tris)/HCl buffer supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche, Mannheim, Germany) were thawed on ice. Cell lyses was accomplished by repeated freezing in liquid nitrogen and thawing in a warm water bath. Afterwards, protein concentration was detected using bicinchoninic acid. About 50 μg (MDCK-par cells) or 20 μg (MDCK-ABCC2 cells) of total protein was loaded onto a 7.5% sodium dodecylsulfate–polyacrylamide gel after incubation in Laemli buffer at 37 °C for 30 min. Gel electrophoresis was performed at 100 V for 1.5 h.

Immunoblotting was performed for 2 h at 300 mA using a Tank Blotting System (BioRad, Munich, Germany) and an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, England). The monoclonal ABCC2 antibody M2III-6 (Alexis Biochemicals, Gruenberg, Germany) and the monoclonal mouse anti-rabbit glycerylaldehyde-3-phosphate dehydrogenase (GAPDH) antibody (HyTest, Turku, Finland) were diluted 1:500 and 1:2000, respectively, with Tris-buffered saline containing 0.05% Tween® 20 and 1% bovine serum albumin. Secondary horse-radish peroxidase-conjugated goat anti-mouse IgG antibody (BioRad, Munich, Germany) was diluted 1:2000. Human placenta served as positive control.

3. Results and discussion

3.1. Cell viability measurements

The influence of the tested surfactants on the viability of the used cell lines was investigated for different incubation periods (1.5 and 7 h, respectively) in order to reflect the residence time of the surfactants in the two different cell assay designs.

The results are presented in viability (%) in relation to the results of cells which were treated with IB, only. For this study a decrease in viability below 50% was considered as a profound inhibitory potency on cell viability.

As shown in Fig. 1, an incubation period of 1.5 h was well tolerated for polysorbate 80, vitamin E TPGS 1000, cremophor® EL and cremophor® RH 40 from all cell lines. Only a concentration of 0.5% cremophor® EL resulted in a reduction in cell viability below 50%. Pluronic® PE 10300 and the sucrose ester L-1695 showed a stronger cytotoxicity with concentrations above 0.01% leading to a complete inhibition of viability (Fig. 1). The extended exposure time of 7 h (Table 1) generally resulted in an increased reduction in cell viability below 50% in the presence of surfactants. Especially, in the case of pluronic® PE 10300 and the sucrose ester L-1695 concentrations above 0.005% led to a complete inhibition of cell viability. The cytotoxic effects of vitamin E TPGS 1000, polysorbate 80 and cremophor® EL started at concentrations of 0.025% and 0.05%, respectively. Cremophor® RH 40 did not influence cell viability up to concentrations of 0.05% (Table 1).

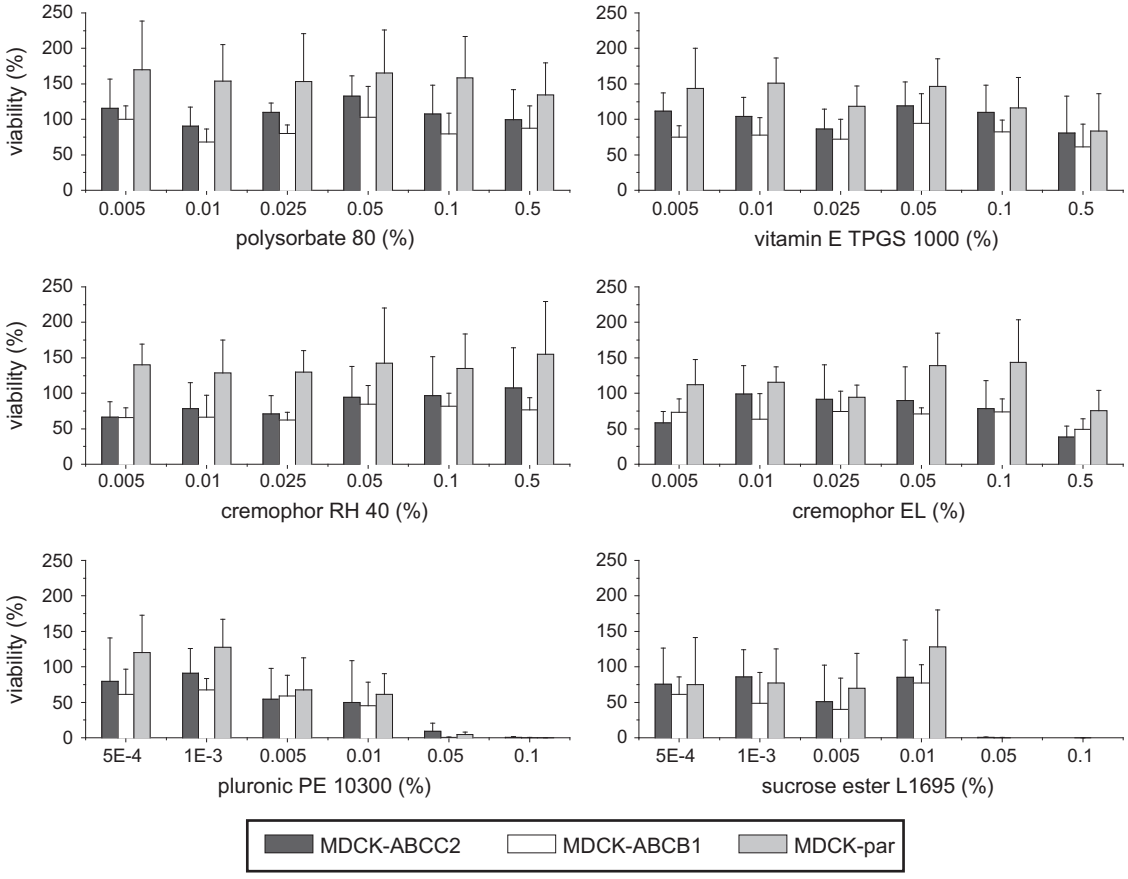

Fig. 1. Presented are results for viability measurements of MDCK-ABCC2, MDCK-ABCB1 and MDCK-par cells after incubation of cells with different concentrations of surfactants for 1.5 h. Given are means + SD of eight experiments.

Table 1
Given are the results for the viability studies of MDCK-ABCC2, MDCK-ABCB1 and MDCK-par cells after incubation with different concentrations of surfactants for 7 h. Presented are results of selected concentrations of surfactants, $n = 8$, mean ± SD, n.d. – not detectable.

Surfactant	Conc. (%)	Viability (%) mean ± SD		
		MDCK-ABCC2	MDCK-ABCB1	MDCK-par
Polysorbate 80	0.005	99.6 ± 60.8	109.6 ± 50.9	64.2 ± 39.4
	0.025	64.2 ± 18.1	89.1 ± 42.8	48.8 ± 25.2
	0.05	49.6 ± 13.9	80.3 ± 22.1	28.6 ± 9.6
Vitamin E TPGS 1000	0.005	77.1 ± 25.6	74.0 ± 8.7	62.9 ± 14.3
	0.025	39.6 ± 17.7	33.9 ± 11.9	27.7 ± 8.6
	0.05	37.1 ± 11.6	28.9 ± 4.8	26.3 ± 6.2
Cremophor® RH 40	0.005	112.7 ± 42.7	107.8 ± 11.4	85.1 ± 13.9
	0.025	134.8 ± 42.8	102.6 ± 11.7	91.3 ± 21.7
	0.05	96.6 ± 22.4	105.7 ± 10.1	65.5 ± 25.4
Cremophor® EL	0.005	104.5 ± 27.2	78.2 ± 40.6	79.5 ± 27.7
	0.025	100.4 ± 48.1	76.9 ± 31.0	64.5 ± 26.3
	0.05	44.7 ± 32.3	91.8 ± 23.8	39.0 ± 9.5
Pluronic® PE 10300	0.005	30.7 ± 8.6	10.5 ± 7.4	40.4 ± 11.9
	0.025	n.d.	0.6 ± 0.5	0.3 ± 0.3
	0.05	n.d.	0.4 ± 0.3	0.4 ± 0.4
Sucrose ester L-1695	0.005	79.2 ± 21.0	76.7 ± 16.9	64.9 ± 17.3
	0.025	n.d.	0.6 ± 0.5	n.d.
	0.05	n.d.	0.4 ± 0.5	n.d.

Generally, pronounced variations in viability between the different cell lines were observed. This might be due to the cultivation of the cell lines on different well plates and the potentially variable proliferation times of the different cell lines. These divergences may have led to different results compared to cells incubated with IB which were used as 100% reference.

The findings of these viability studies were taken into account when appropriate surfactant concentrations for the cell assays were selected.

During the different investigations, it was noticed that concentrations of all surfactants, which showed viabilities below 50% in the resazurin assay, were well tolerated in both assays, e.g. indicated by acceptable TEER values. A possible explanation for these findings is the better toleration of cytotoxic effects caused by the surfactants by confluent monolayers compared to single cells. This leads to the assumption that the viability assay as it was used in this study was not very applicable. In further studies it is advisable to study the acute toxicity of the surfactants on confluent cell monolayers rather than the long term toxicity on single cells in terms of comparability.

3.2. Interactions with ABCB1

The calcein-AM accumulation assay was used to investigate interactions of pharmaceutical surfactants with ABCB1. In this assay the cells were first loaded with surfactants for 0.5 h before the ABCB1 substrate calcein-AM was added. Fluorescence was measured after 1 h of incubation. In preliminary experiments we observed that longer incubation times up to 120 min resulted in higher background fluorescence in both untreated and treated cells. This consequently led to lower RFU values (data not shown). The term background fluorescence is used to describe the calcein fluorescence of the cells after treatment with calcein-AM. It is caused by the limited capacity of ABCB1 for transporting calcein-

AM out of the cells. A small amount of calcein-AM remains inside the cells which causes the low fluorescence of untreated cells. Increased incubation times result in increasing amounts of calcein-AM inside the cells yielding higher background fluorescence values.

To verify the assay the known inhibitors ivermectin and PSC-833 were used as positive controls. Ivermectin (3 μM) provided maximal RFU values of 4.56 ± 2.0 for the MDCK-ABCB1 cells and 1.87 ± 0.2 for the parental cells (Fig. 2). These results are comparable to the data obtained for PSC-833 (1 μM, MDCK-ABCB1: 4.12 ± 1.01 and MDCK-par: 1.01 ± 0.21). For this assay RFU values of ⩾ 2 were considered as an indicator for an interaction with ABCB1. The RFU values for the transfected cells were obviously higher. Accordingly, both substances could be verified as inhibitors of ABCB1. The low RFU values in the native cells due to the absence of ABCB1 demonstrate the specific interaction of both substances with the human ABCB1. These findings show the functionality of the assay.

After incubation with pluronic® PE 10300 and the sucrose ester L-1695, the RFU values reached maxima of 6.38 ± 2.39 and 6.29 ± 0.49, respectively, in transfected cells as shown in Fig. 2.

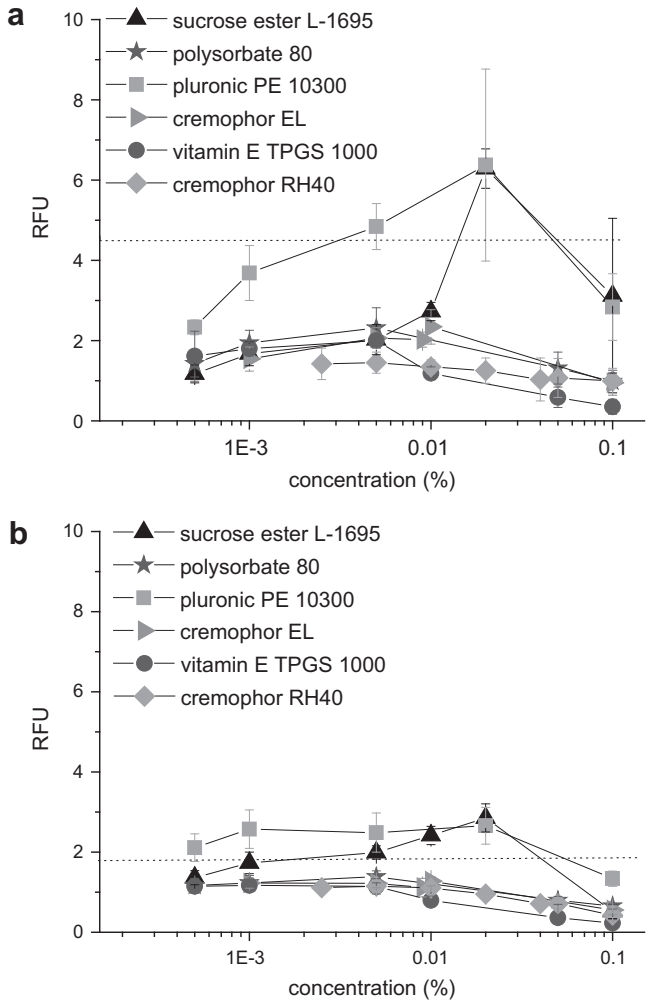

Fig. 2. Presented are the results for the calcein-AM accumulation assay for MDCK-ABCB1 cells (a) and MDCK-par cells (b) after treatment with calcein-AM (1 μM) and different concentrations of surfactants. Depicted are relative fluorescence units (RFU) as ratios of treated vs. nontreated (incubation with incubation buffer only) cells after 1-h incubation (excitation: 485 nm; emission: 538 nm). Given are means ± SD of at least six experiments, the dotted lines represent results for the incubation with the positive control (ivermectin, 3 μM: RFU: 4.56 ± 1.99 (MDCK-ABCC2), 1.87 ± 0.20 (MDCK-par)).

Vitamin E TPGS 1000, cremophor® EL and polysorbate 80 resulted in maxima with RFU values of approximately 2. Cremophor® RH 40 showed only a marginal increase in RFU values with maxima of 1.45 ± 0.26 in MDCK-ABCB1 and 1.15 ± 0.13 in MDCK-par cells (Fig. 2).

The results indicate a strong inhibitory effect on ABCB1 function for pluronic® PE 10300 and the sucrose ester L-1695 because of the high RFU values which were even higher than the results for the positive controls. In contrast, cremophor® EL, vitamin E TPGS 1000 and polysorbate 80 exhibited only a slight modulation of ABCB1 with RFU values about 2. No inhibition was observed for cremophor® RH 40. In the parental cells no effect was observed that could be attributed to ABCB1. Because interferences of the assay due to auto-fluorescence of surfactants could be excluded (data not shown), the resulting RFU values can clearly be related to interaction with ABCB1.

For cremophor® EL, vitamin E TPGS and polysorbate 80 our findings are in good agreement with the results obtained by Bogman and co-workers using murine monocytic leukaemia cells overexpressing ABCB1 [39]. Rege et al. also demonstrated a modulating effect of these surfactants in Caco-2 cells [14]. In addition, Bogman et al. showed that two different pluronics® (PE 8100 and PE 6100) inhibited ABCB1-mediated rhodamine123 transport [39]. The inhibition of ABCB1 in the presence of different pluronics® was also observed by Batrakova et al. [10].

It cannot be excluded that interactions with other transport mechanisms can influence the results of this study. For example Rege et al. showed that polysorbate 80 specifically interacts with the peptide transporter PepT-1 whereas cremophor® EL inhibited the monocarboxylic acid transporter [14]. For pluronic® P85 and polysorbate 20 interactions with the breast cancer resistance protein were shown [40]. The inhibition of enzymes like CYP3A4 was also demonstrated for polysorbate 20 and 80 [13].

However, for this assay, cells transfected with human ABCB1 were used and the results were always compared to data obtained for native cells. Using this assay design the observed effects can mainly be attributed to interactions with ABCB1.

In the presence of higher concentrations of surfactants (especially ⩾ 0.05%) a remarkable reduction in the RFU values was observed throughout the experiments. This reduction is most likely due to micellar trapping of calcein-AM outside the cells. Surfactant concentrations above the CMC result in the formation of micelles. To our opinion calcein-AM is trapped inside the micelles and therefore not longer able to penetrate the cell membrane. This results in a loss of interaction with ABCB1 expressed in a reduction in RFU values. Reduced interaction of substrates with ABCB1 due to micellar trapping was shown for higher concentrations of cremophor EL, polysorbate 80 and pluronic® P85 in different studies [41–43]. In this study especially the results for polysorbate 80 and cremophor® RH 40 confirm this assumption. For polysorbate 80 an increase in RFU values up to a concentration of 0.005% was observed. Above this concentration, which represents the CMC for polysorbate 80, an obvious reduction in the RFU values was detected. For cremophor® RH 40 a decrease in the RFU values with increasing surfactant concentration was also observed. Interestingly, this surfactant caused neither any cytotoxicity over the complete concentration range nor inhibited ABCB1.

3.3. Interactions with ABCC2

Besides the determination of the influence of pharmaceutical excipients on ABCB1, it was of main interest whether the surfactants modulate ABCC2 function as well. The partly overlapping substrate specificity and localisation of both transporters led to the presumption that the surfactants might interact in the same way with both transporters.

The assay was performed for a total of 5 h after loading the cells with surfactants for 1 h as reported in literature for study designs using the Transwell® system [21,33].

The functionality of the transport assay is shown in Fig. 3. The apparent permeability coefficients (P_{app}) of calcein in MDCK-ABCC2 cells are significantly higher in the secretory transport direction than in the absorptive direction indicating a directed apical transport of calcein via ABCC2. The presence of the positive control probenecid caused a significant reduction in the P_{app} values of calcein representing inhibition of the transport activity of ABCC2. In the parental cells significant differences in the P_{app} values of calcein between both transport directions were not observed. Concluded from the observed increase in absorptive transport and decrease in secretion and the inability of probenecid to influence the calcein transport a directed apical transport does not occur in the parental cells.

The addition of different concentrations of cremophor® RH 40 caused a concentration dependent reduction in the calcein permeability in MDCK-ABCC2 cells, whilst there were no remarkable differences between both transport directions in MDCK-par cells (Fig. 4a and b). A concentration of 0.04% cremophor® RH 40 led to a significant decline in secretory permeability of MDCK-ABCC2 cells comparable to the results obtained for probenecid. In the presence of cremophor® EL the apparent permeability coefficient of calcein was significantly decreased in MDCK-ABCC2 cells (Fig. 5a). Even concentrations below the CMC of approximately 0.01% [14] resulted in a reduction of the calcein transport rate. Concentrations above the CMC were even more potent than the used standard probenecid (100 μM). As shown in Table 2, the permeability of calcein was concentration dependently reduced in the presence of vitamin E TPGS 1000 as well, but to a lesser extent than observed for cremophor® EL. For polysorbate 80 a significant reduction in the secretory calcein permeability in ABCC2-transfected MDCK cells was only investigated for a concentration of 0.05%. In contrast to these findings the addition of pluronic® PE 10300 and sucrose ester L-1695 resulted in increased permeabilities of calcein in the transfected cells (Table 2).

In the parental cells apical directed transport of calcein in the presence of the different surfactants was mostly negligible (Fig. 5b, Table 2). Thus, it can be concluded that the observed inhibitory effects of cremophor® EL, cremophor® RH 40, vitamin E TPGS 1000 and high concentrations of polysorbate 80 are due to selective inhibition of ABCC2. A contrary result has previously been reported for the surfactants cremophor® EL, vitamin E TPGS 1000 and polysorbate 80 by Bogman et al. [36]. Even though the authors also used MDCK-ABCC2 cells, they did not observe an inhibitory effect. The cause of this discrepancy is not clear; it might be due to differing substrates and incubation times.

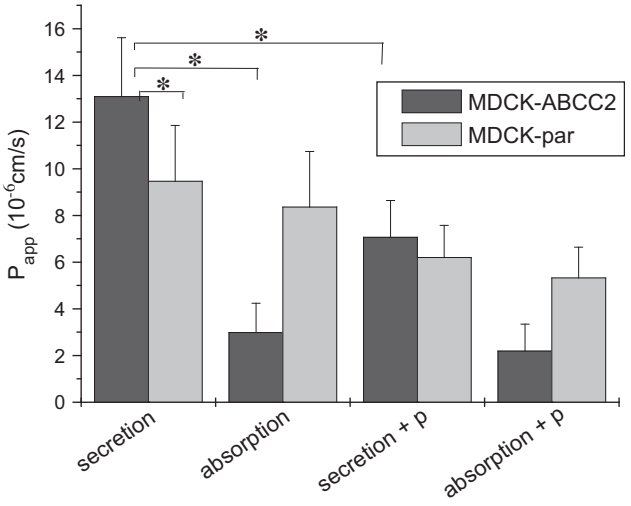

Fig. 3. Given are the apparent permeability coefficients (P_{app}) of calcein of MDCK-ABCC2 and MDCK-par cells in the secretory and absorptive transport direction without inhibitor and in the presence of probenecid (+p, 100 μM = 0.00285%). Depicted are means + SD of at least 17 experiments, $^{*}p \leqslant 0.001$ (Student's *t*-test).

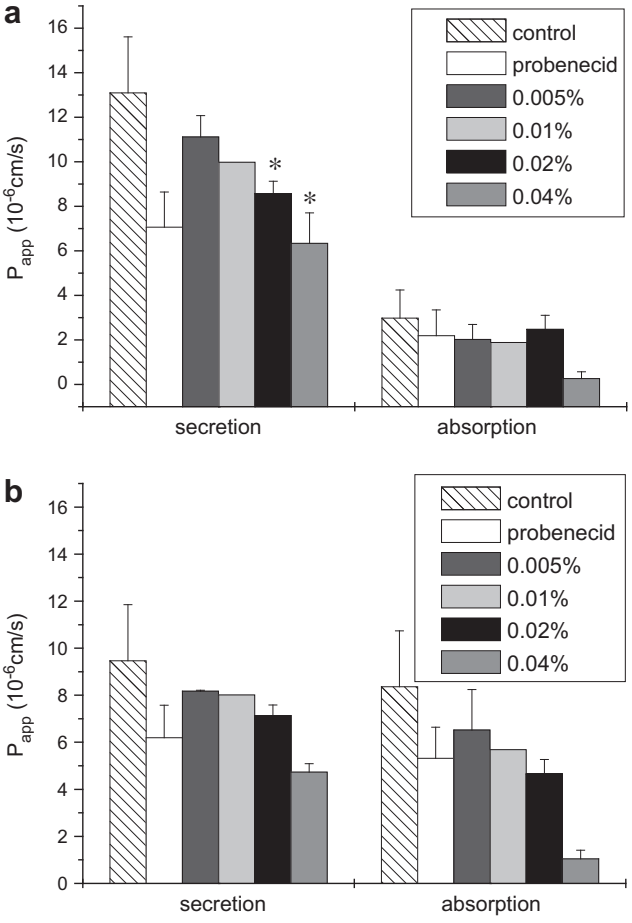

Fig. 4. Presented are the apparent permeability coefficients (P_{app}) of calcein without inhibitor (negative control, $n = 17$; striped column) and in the presence of probenecid (positive control, 100 μM = 0.00285%, $n = 18$; white column) and cremophor® RH 40 (0.005%, 0.02% and 0.04%, $n = 4$; 0.01%, $n = 2$; gray scale columns) of MDCK-ABCC2 (a) and MDCK-par (b) cells in the secretory and absorptive transport direction. Given are means + SD, $^{*}p \leqslant 0.001$.

Pluronic® PE 10300 and the sucrose ester L-1695 caused an increase in calcein permeability in our study. Evers et al. observed an increased export of calcein in MDCK-ABCC2 cells in the presence of pluronic® L 61, an analogue of PE 10300 [33]. They postulated that this surprising effect was not likely to be due to real stimulation of ABCC2 but to a strong inhibition of native ABCB1. In agreement with this understanding, Batrakova et al. showed that ABCC2-transfected cells contain increased levels of native ABCB1 [35]. In order to verify a possible effect of ABCB1 in the present transport assay, the cells were incubated with pluronic® PE 10300 in the presence of 5 μM PSC-833 instead of 1 μM. Indeed, now a reduction in the calcein permeability to values that are close to those of untreated cells (data not shown) was observed which supports the postulated effect of ABCB1 in ABCC2-transfected cells. The concentration of PSC-833 used by Evers (0.1 μM) as well as the concentration used for the experiments presented here (1 μM) was not sufficient to completely inhibit native ABCB1 in contrast to the tested pluronic® PE 10300. Additionally, Batrakova et al. stated that the inhibitory effect of pluronic® block copoly-

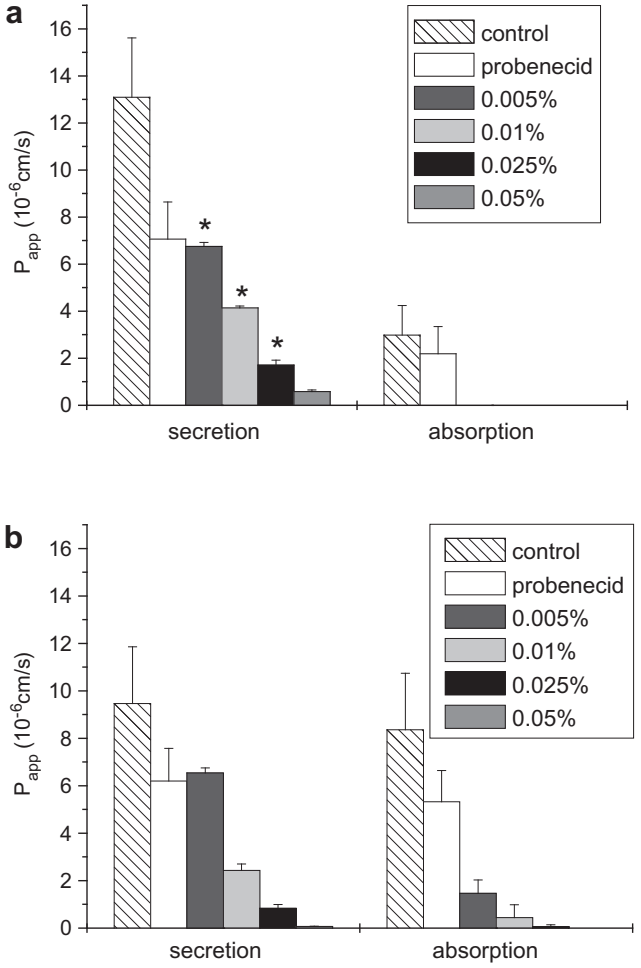

Fig. 5. Given are the apparent permeability coefficients (P_{app}) of calcein without inhibitor (negative control, $n = 17$; striped column) and in the presence of probenecid (positive control, 100 μM = 0.00285%, $n = 18$; white column) and cremophor® EL (0.005–0.025%, $n = 4$, 0.05%, $n = 2$; gray scale columns) of MDCK-ABCC2 (a) and MDCK-par (b) cells in the secretory and absorptive direction. Presented are means + SD, $^{*}p \leqslant 0.001$.

mers (in their case pluronic® P85) on ATPase activity of ABCC2 is much lower than on ABCB1 ATPase activity [44]. So far, we consider pluronic® PE 10300 and sucrose ester L-1695 not to inhibit ABCC2.

The use of the ABCC2 substrate calcein represents an easy and fast analytical method to investigate interactions with ABCC2 but is limited by its dependency on the formation by intracellular esterases. Another fluorescent dye that can serve as substrate of ABCC2 is sulforhodamine 101 [45]. Advantageously, this substance possesses auto-fluorescence and is independent of esterases. In preliminary tests using our assay system a directed apical transport of sulforhodamine 101 was shown for a concentration of 1 μM which resulted in a very low fluorescence signal, only. Furthermore, our positive control probenecid was not able to inhibit the transport of sulforhodamine 101 but stimulated sulforhodamine transport (data not shown). These findings are in accordance with results from Zelcer et al. who investigated drug interactions with ABCC2 in membrane vesicles from transfected insect cells. They reported that probenecid strongly stimulates the transport of estradiol-17-β-glucuronide but inhibits the transport of methotrexate, both substrates of ABCC2. The authors postulated that the effect of an ABCC2 modulator depends on the substrate transported. The interactions between each substrate–modulator pair depend on the complexity of the drug binding sites of ABCC2 [46].

Table 2
Given is the influence of different surfactants on the apparent permeability coefficients (P_{app}) of calcein for MDCK-ABCC2 cells and MDCK-par cells in the presence of 1 μM PSC-833. Presented are means ± SD (calcein as calcein-AM 1 μM, $n = 18$, probenecid, $n = 17$, surfactants, $n = 4$), n.d. – not detectable, Student's t-test, compared to secretory P_{app} of calcein in MDCK-ABCC2 cells.

Transport direction	MDCK-ABCC2 P_{app} (10^{-6} cm/s)		MDCK-par P_{app} (10^{-6} cm/s)	
	Secretory	Absorptive	Secretory	Absorptive
Calcein	13.09 ± 2.52	2.98 ± 1.26*	9.46 ± 2.39*	8.36 ± 2.38
Probenecid (100 μM)	7.06 ± 1.58*	2.19 ± 1.15	6.20 ± 1.38	5.32 ± 1.32
Vitamin E TPGS 1000				
0.005%	13.97 ± 1.74	0.54 ± 0.13	10.20 ± 0.90	5.67 ± 0.60
0.01%	10.28 ± 2.42	n.d.	6.58 ± 1.45	2.39 ± 0.69
0.02%	8.03 ± 1.03	n.d.	5.78 ± 1.16	2.50 ± 0.87
0.04%	4.85 ± 1.40	n.d.	3.18 ± 1.19	0.94 ± 0.24
Polysorbate 80				
0.001%	15.58 ± 0.66*	3.68 ± 0.40	12.28[a]	7.50[a]
0.005%	16.72 ± 0.44*	3.53 ± 0.65	13.11[a]	6.78[a]
0.01%	14.59 ± 1.13	2.65 ± 0.32	12.50[a]	6.49[a]
0.05%	7.53 ± 1.40*	1.64 ± 0.21	6.39[a]	4.08[a]
Pluronic® PE 10300				
0.002%	19.95[a]	3.26[a]	10.82[a]	14.81[a]
0.005%	24.21 ± 0.74*	3.92 ± 0.84	10.41 ± 1.34	9.38 ± 2.60
0.01%	15.89 ± 2.20	3.71 ± 1.64	8.69 ± 1.81	13.01 ± 2.75
Sucrose ester L-1695				
0.001%	17.74 ± 2.51*	3.13 ± 1.01	10.84 ± 2.36	11.07 ± 9.54
0.0025%	19.69 ± 1.19*	3.45 ± 0.76	10.41 ± 1.06	8.50 ± 2.44
0.005%	19.52 ± 6.69	3.56 ± 0.42	11.65 ± 3.53	9.81 ± 2.46

[a] $n = 2$.
* $p \leqslant 0.001$.

3.4. ABCC2mRNA analysis and Western blot analysis

In order to evaluate whether the observed effects on ABCC2 are due to real inhibition of transport activity or not it should also be investigated if the relevant surfactants can influence the regulation of mRNA levels or protein levels of ABCC2 within the incubation period of the transport assay. For this purpose *ABCC2*mRNA expression and ABCC2 protein content were determined after incubation with the ABCC2 inhibiting surfactants cremophor® RH 40, cremophor® EL, vitamin E TPGS 1000 and polysorbate 80 in relevant concentrations for 7 h. No considerable alteration of *ABCC2*mRNA expression was observed in the transfected cells as indicated by the ratios of treated versus nontreated transfected cells in the range of 1 (Table 3).

In the native cells, only very low quantities of *ABCC2*mRNA were detected (data not shown). This is most probably due to the specificity of the primer for human *ABCC2*mRNA with only marginal cross-reactivity with *ABCC2*mRNA from dog as the native origin of the cells.

Table 3
Presented are *ABCC2*mRNA levels in MDCK-ABCC2 cells and MDCK-par cells as ratios of treated vs. nontreated cells after incubation with buffer or different surfactants for 7 h. Given are means of two separate experiments.

Tested surfactant	Concentration of surfactant (%)	MDCK-ABCC2 (ratio of treated vs. nontreated cells)
Cremophor® RH 40	0.02	1.0
	0.04	1.0
Polysorbate 80	0.01	1.4
	0.05	0.9
Vitamin E TPGS 1000	0.02	1.0
	0.04	1.3
Cremophor® EL	0.01	1.8
	0.025	0.6

As the *ABCC2*mRNA expression does not necessarily reflect altered protein levels [38], the protein levels were also investigated. In Figs. 6 and 7, representative Western blots are depicted. The MDCK-ABCC2 cells revealed pronounced ABCC2 bands at 190 kDa with almost similar intensity of the different bands after incubation with IB and the tested surfactants (Fig. 6). Parental cells showed only slight band intensity at 190 kDa (Fig. 7). The GAPDH concentrations were well comparable for the different cell lines. The results indicate that no obvious differences in the protein expression of ABCC2 after incubation with the surfactants in different concentrations were observed. The possibility that the observed effects on ABCC2 reflect alterations of the ABCC2 expression level can therefore be excluded. This result can be supported by findings from Trauner et al. and Kubitz et al. [47,48] who have reported that longer incubation times (12–24 h) are necessary to activate regulatory mechanisms on the expression level of the transporters. A short-term removal of active ABCC2 from the apical membrane by encapsulation of ABCC2 in vesicles was reported, too. The vesicle mediated transport into the cytosol occurred within 3–12 h in the presence of endotoxin lipopolysaccharide [48]. Based on this a feasible surfactant mediated encapsulation of ABCC2 in vesicles resulting in a reduction in active ABCC2 in the apical membrane which consequently leads to a loss of transport activity cannot completely be excluded so far.

Based on our findings we conclude that the observed inhibiting effects of the commonly used surfactants cremophor® RH 40, cremophor® EL, vitamin E TPGS 1000 and polysorbate 80 on ABCC2 are most likely due to direct interactions with this transporter.

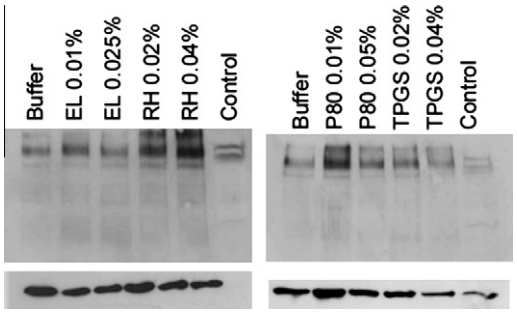

Fig. 6. Presented are two exemplary Western blots of ABCC2 (190 kDa) and GAPDH (36 kDa) in MDCK-ABCC2 cells (5 μg protein in each case) after 7-h incubation with buffer, positive control (protein from human placenta (30 μg/μl)) and different concentrations of polysorbate 80 (P80), vitamin E TPGS 1000 (TPGS), cremophor® EL (EL) and cremophor® RH 40 (RH).

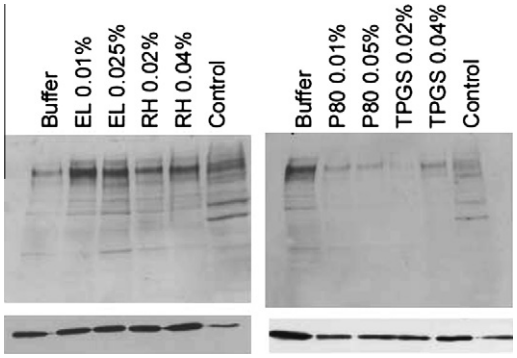

Fig. 7. Presented are two exemplary Western blots of ABCC2 (190 kDa) and GAPDH (36 kDa) in MDCK-par cells (10 μg protein in each case) after 7-h incubation with buffer, positive control (protein from human placenta (30 μg/μl)) and different concentrations of polysorbate 80 (P80), vitamin E TPGS 1000 (TPGS), cremophor® EL (EL) and cremophor® RH 40 (RH).

4. Conclusion

In conclusion the present study demonstrates that different nonionic surfactants possess differential modulating effects on the efflux transporters ABCB1 and ABCC2. Pluronic® PE 10300 and the sucrose ester L-1695 caused a strong inhibition of ABCB1 whereas they had no effect on ABCC2. Cremophor® EL moderately inhibited ABCB1 but showed strong inhibition of ABCC2 which was more effective than probenecid in concentrations above the CMC. Vitamin E TPGS 1000 and polysorbate 80 slightly inhibited both transporters. In contrast, cremophor® RH 40 only interacted with ABCC2 in a concentration dependent manner. There was no influence on *ABCC2*mRNA and protein expression within the trial period. These findings indicate a direct interaction of the surfactants with the efflux transporters rather than alterations of membrane integrity or expression levels of the transporters.

However, the crucial mechanisms of interaction between surfactants and ABCB1 or ABCC2 remain to be further investigated.

Furthermore, not only the efflux and consequently elimination of drugs should be observed. The uptake of compounds is of substantial interest, too. The observed effects on the efflux transporters might partly be masked by a regulated uptake of the tested substrates or the compounds themselves. In the future the influence of pharmaceutical excipients on uptake transporters like organic anion transporters will have to be investigated as well.

Acknowledgements

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