Role of multidrug resistance-associated protein 1 in airway epithelium

van der Deen, Margaretha

CHAPTER SIX

Cigarette smoke extract affects functional activity of MRP1 in bronchial epithelial cells

Margaretha van der Deen[1], Elisabeth G.E. de Vries[1], Hylke Visserman[1], Wouter Zandbergen[1], Dirkje S. Postma[2], Wim Timens[3], Hetty Timmer-Bosscha[1]

Departments of Medical Oncology[1], Pulmonary Diseases[2] and Pathology[3], University of Groningen and University Medical Center Groningen, Groningen, the Netherlands

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Abstract

Cigarette smoke is the principal risk factor for development of chronic obstructive pulmonary disease (COPD). Multidrug resistance-associated protein 1 (MRP1) is a member of the ATP-binding cassette (ABC) superfamily of transporters which transport physiologic and toxic substrates across cell membranes. MRP1 is highly expressed in lung epithelium. This study aims to analyze the effect of cigarette smoke extract (CSE) on MRP1 activity. In the human bronchial epithelial cell line 16HBE14o$^-$ MRP1 function was studied flowcytometrically by cellular retention of carboxyfluorescein (CF) after CSE incubation and MRP1 down regulation by RNA interference (siRNA). Cell survival was measured by MTT assay.

Immunocytochemically it was shown that 16HBE14o$^-$ expressed MRP1 and breast cancer resistance protein. CSE increased cellular CF retention dose dependently from 1.7 fold at 5% CSE to 10.3 fold at 40% CSE (both $p<0.05$). SiRNA reduced MRP1 RNA expression with 49% and increased CF accumulation 67% versus control transfected cells.

CSE exposure further increased CF retention 24% ($p=0.031$). A linear positive relation between MRP1 function and CSE modulating effects ($r=0.99$, $p=0.089$) was shown in untransfected, control transfected and MRP1 down regulated 16HBE14o$^-$ cells analogous to blocking effects with MRP1 inhibitor MK571 ($r=0.99$, $p=0.034$). Co-incubation of CSE IC50 ($1.53\% \pm 0.22\%$) with MK571 further decreased cell survival 31% ($p=0.018$). In conclusion, cigarette smoke extract inhibits MRP1 activity probably competitively in bronchial epithelial cells. Inhibition of MRP1 in turn results in higher CSE toxicity. We propose that MRP1 may be a protective protein for COPD development.

Introduction

Cigarette smoking generates oxidative stress in the lung and is the principal risk factor for development of chronic obstructive pulmonary disease (COPD) and lung cancer [1, 2]. However, only 15 to 20% of all smokers will develop COPD [3]. We observed a reduced multidrug resistance-associated protein 1 (MRP1) expression in bronchial epithelial cells of COPD patients [4]. Proteins of the ATP-binding cassette (ABC) superfamily such as multidrug resistance associated protein (MRP1), P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) protect against oxidative stress, chemotherapeutic drugs or xenobiotics by extruding toxic substances out of cells [5-10]. These efflux pumps have drawn a lot of attention in cancer research as their overexpression can cause multidrug resistance (MDR) for chemotherapeutic drugs in tumor cells.

Organs that need a barrier function such as the lung and gastrointestinal tract express high levels of several ABC transporters [11]. Of all described MDR proteins, the expression of MRP1 is high in the lung, especially at the basolateral side of bronchial epithelium [12, 13]. MRP1 transports glutathione-, glucuronide-, and sulfate-conjugated organic anions [6, 14]. Physiological substrates for MRP1 are e.g. leukotriene C_4 and glutathione disulphide [15,16]. MRP1 can assist in the clearance of toxins entering from the lumenal or interstitial side of the airways into the interstitial fluid [17].

Cigarette smoke is a complex mixture of several thousands of compounds. Little is known about detoxification and elimination processes of noxious substances present in cigarette smoke. It can contain several compounds, or form metabolites, that are substrates for MRP1 and thus competitively affect MRP1 function. For instance, the tobacco-derived 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)-*O*-glucuronide has been identified as an MRP1 substrate that requires glutathione for transport [18].

In the present study, the role of MRP1 in the cytotoxicity of cigarette smoke extract (CSE) was evaluated in the human bronchial epithelial cell line 16HBE14o$^-$ by co incubation with an MRP1 inhibitor. The cellular retention of the commonly-used MRP1 substrate carboxyfluorescein (CF) combined with an MRP1 inhibitor or with MRP1 down regulation by RNA interference was used to study the effect of CSE on MRP1 function.

Material and Methods

Chemicals, media and reagents

Bovine serum albumin (BSA) fraction V, MEM supplemented with Earle's salts and L-glutamine, Oligofectamine, OptiMEM with GlutaMAX, RPMI 1640 medium (supplemented with 25 mM Hepes and L-glutamine) and Trizol were purchased from Invitrogen Life Technologies (Breda, The Netherlands). Carboxyfluorescein diacetate (CFDA), thiazolyl blue tetrazolium bromide (MTT) and propidium iodide (PI) were obtained from Sigma-Aldrich BV (Zwijndrecht, The Netherlands), DNase-I from Roche Diagnostics (Mannheim, Germany), doxorubicin-HCl from Pharmachemie BV (Haarlem, The Netherlands), EDTA from Merck, (Darmstadt, Germany) and foetal calf serum (FCS) from Bodinco BV (Alkmaar, The Netherlands). Fumitremorgin C (FTC) was kindly provided by Dr SE Bates (NCI, Bethesda, MD). MK571 was purchased from Omnilabo (Breda, The Netherlands), PSC833 from Novartis (Basel, Switzerland), the qPCR core kit from Eurogentec (Seraing, Belgium), the RNeasy kit from Qiagen (Venlo, The Netherlands) and Vitrogen from Nutacon (Leimuiden, The Netherlands).

The monoclonal antibodies MRPr1 (anti-MRP1) [19], M2I4, M2II12, M2III5, M2III6 (anti-MRP2) [19], M3II2, M3II18 (anti-MRP3) [20], M4I10 (anti-MRP4) [21], M5I1, M5II54 (anti-MRP5) [19] and BXP-21 (anti-BCRP) [22] were kindly provided by Dr G.L. Scheffer, VUMC, Amsterdam, The Netherlands). C219 (anti P-gp) was from Alexis (Kordia, Leiden, The Netherlands), biotinylated rabbit-anti-mouse or rabbit anti-rat secondary antibodies and streptavidin-peroxidase were purchased from Dako (Glostrup, Denmark).

Cell lines

The human bronchial epithelial cell line 16HBE14o$^-$ immortalized with pSVori$^-$ plasmid transfection, was kindly provided by Dr DC Gruenert (California Pacific Medical Center Research institute, San Francisco, CA) [23]. Cells were cultured on tissue culture plastics coated with Vitrogen (30 µg/ml) and BSA (10 µg/ml) in MEM supplemented with 10% heat inactivated FCS. Before trypsinisation, cells were washed twice with PBS (6.4 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 mM NaCl, 2.7 mM KCl, pH=7.4) with 0.5 mM EDTA. Controls consisted of the human small cell lung cancer cell lines GLC4 and GLC4/ADR [24], which were respectively weakly MRP1 positive and MRP1 overexpressing. Both were grown in RPMI1640 medium supplemented with 10% heat inactivated FCS. GLC4/ADR was *in vitro* selected for doxorubicin

resistance and maintained under continuous pressure of 1.2 µM doxorubicin [24]. Before every experiment, cells were cultured in the absence of doxorubicin for 3 weeks. All cell lines were passed twice a week and used in logarithmic growth phase, usually two or three days after subculturing.

Immunocytochemistry

In order to characterize the ABC transporter expression in 16HBE14o$^-$ cells cytospin slides were fixed in acetone for 10 min at room temperature. Monoclonal antibodies were incubated for 1 h in the recommended dilution, followed by incubation with biotinylated rabbit-anti-mouse or rabbit anti-rat secondary antibody (1/300) for 1 h and incubation with streptavidin-peroxidase (1/300) for 30 min. All incubation steps were carried out at room temperature. PBS/1% BSA or irrelevant isotype specific antibody was used as a negative control. Appropriate positive controls were included for each antibody tested.

Cigarette smoke extract

Mainstream smoke of four reference cigarettes (type 2R1, Kentucky Tobacco Research and Development Center, Lexington, UK) was bubbled through 50 ml MEM medium without FCS by means of a smoking device (Tobacco Health Research Institute). The smoking rate was 10 seconds/min (one puff), four puffs/cigarette. Within 30 min, CSE was adjusted to pH 7.4 and sterilized through a 0.22 µm filter. This solution was denoted as 100% CSE. Small aliquots of 1, 2 or 3 ml were stored at –80°C.

Cytotoxicity assay

The micro culture tetrazolium test (MTT) was used to determine cytotoxicity of CSE in 16HBE14o$^-$ cells and performed as described earlier [25]. Cells were seeded at optimum density in order to test cell survival after at least 2 to 3 cell divisions had taken place in the control cells. 16HBE14o$^-$ cells were seeded in a 96-wells plate (5,000 cells/well) in quadruplicate for each CSE concentration (0 to 2.25% with 0.25% intervals) and cultured for 4 days. Assays were performed 4 times, and the CSE concentration that induced 50% cell growth inhibition (IC50) was determined. Results are expressed as mean ± SD. Toxicity of every new CSE batch was tested. In order to test whether MRP1 activity affects CSE cytotoxicity cells were co incubated with CSE and MRP1 inhibitor MK571 (50 µM). As controls, comparable experiments were performed with the P-gp inhibitor PSC833 (a cyclosporine

A-analogue, 1 μg/ml) and the BCRP inhibitor FTC (5 μM) [26]. This to asses whether CSE cytotoxicity was affected by MRP1 specifically or by ABC transporters activity more generally. The indicated inhibitor concentrations were combined with the IC50 of CSE. Inhibitor concentrations were chosen as described earlier [27] and incubations with inhibitors alone were used as controls.

RNA interference

Small interfering RNA (siRNA) duplexes directed against MRP1 (si-MRP1) and Luciferase (si-Luci, negative control) mRNA were purchased from Eurogentec (Maastricht, The Netherlands). The sense sequence for si-MRP1 was 5'-GGA GTG GAA CCC CTC TCT G-3' and the antisense sequence was 5'-CAG AGA GGG GTT CCA CTC C-3'. For si-Luci, the sense sequence was 5'-CUU ACG CUG AGU ACU UCG A-3' and the antisense sequence was 5'-UCG AAG UAC UCA GCG UAA G-3'. At day 1 after seeding, 16HBE14o$^-$ cells ($4x10^5$/well) were transfected in 6-well plates using 200 nM oligonucleotides with Oligofectamine reagent and OptiMEM with 10% FCS (according to the manufacturer's instructions). At day 2, the transfection medium was replaced by fresh culture medium. At day 3, the siRNA transfection procedure was repeated as repetitive transfection resulted in the highest MRP1 down regulation. Cells for flow cytometry and RNA extraction were harvested day 4.

Flow cytometric detection of MRP1 function

To determine MRP1 mediated efflux the cellular retention of the fluorescent MRP1 substrate CF was used as described previously [27] with slight modifications. Cells were incubated with CFDA (0.1 μM) which is intracellularly converted to CF. For measurements $1x10^6$ cells were incubated for 1 h (37°C, 5% CO_2) in 0.5 ml RPMI1640 medium without FCS, with CFDA and with, or without, the addition of 5%, 10%, 20% or 40% CSE. For siRNA transfected cells, only 1 h incubation with 20% CSE was used. This CSE concentration induced intermediate effect in untransfected cells so, after combination with siRNA further increased or decreased accumulation effects should be well detectable. MK571 (20 μM) was added to samples instead of CSE as a positive control for inhibition of MRP1 activity [27]. After incubation, cells were centrifuged for 15 seconds at 12,000 g. Cell pellets were resuspended in 0.5 ml ice-cold RPMI1640 medium without FCS and incubated again for 1 h (37°C, 5% CO_2) in the presence of the same CSE concentrations or MK571 concentrations but without CFDA. After pelleting,

cells were put on ice to stop efflux of substrate and were resuspended in 350 µl RPMI1640 medium with 0.1 µg/ml PI, to distinguish dead from living cells.

Fluorescence of CF was analyzed with a FACSCalibur flow cytometer (Becton Dickinson Medical Systems, NJ). Per sample, 10,000 events were measured. The Winlist 5.1 program (Verity Software House Inc., Topsham, ME) was used to calculate mean fluorescence intensity (MFI) values. The efflux blocking factor (BF) was defined, as described earlier [27], as the ratio between MFI of CF plus modulator and MFI of CF alone. Each measurement was corrected for background (auto) fluorescence measured in samples incubated without CFDA and PI but with CSE, MK571 or medium alone. In order to determine whether the modulating effect of CSE was related to MRP1 function the BFs obtained with CSE incubation for untransfected 16HBE14o-, si-Luci and si-MRP1 transfected cells were plotted against the inverse of the CF retention (MFI^{-1}) of the cells at each condition. The latter as a measure for MRP1 function at each condition. As a control, the same plot was made for BFs obtained with MK571.

RNA isolation and quantitative RT-PCR

Cells transfected with siRNA duplexes were resuspended in 1 ml of Trizol. After 5 min incubation at room temperature, the lysate was stored at –80°C until use. RNA was isolated according to standard manufacturer protocols followed by a DNase-I treatment. For RNA purification, the RNeasy kit was used and RNA (800 ng) was subjected to a cDNA synthesis reaction.

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using the ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA) as described [28]. In brief, a qPCR core kit was used and the PCR mixture contained 900 nM of sense and antisense primers, 200 nM of fluorogenic probe (labeled by a 5' FAM reporter and a 3' TAMRA quencher). Each sample was analyzed in duplicate. Sequences for MRP1 primers were 5'-GGT GGG CCG AGT GGA ATT-3' for the sense strand, 5'-TTG ATG TGC CTG AGA ACG AAG T-3' for the antisense strand and 5' FAM-CTG CCT GCG CTA CCG AGA GGA CCT-TAMRA 3' for the probe strand. Sequences for the housekeeping gene GAPDH primers were 5'- GGT GGT CTC CTC TGA CTT CAA CA-3' for the sense strand, 5'-GTG GTC GTT GAG GGC AAT G-3' for the antisense strand and 5' FAM-ACA CCC ACT CCT CCA CCT TTG ACG C-TAMRA 3' for the probe strand. Cycle numbers at which the sample fluorescence signal increases above a fixed threshold level (C_T value) correlate inversely with initial mRNA levels.

Statistical analysis

The paired Student's t-test or the independent samples t-test was used to calculate statistical differences. Correlations between BFs and CF retention were calculated using non linear regression analysis and statistically validated by analysis of variance (sum of square reduction test). Differences were considered significant when $P<0.05$. Statistical analyses were performed with SPSS 10 (SPSS Inc., Chicago, IL).

Results

Immunocytochemistry

16HBE14o$^-$ cells were characterized for presence of MRP1, 2, 3, 4 and 5, P-gp and BCRP. MRP1 staining was clearly present and predominantly membrane associated, whereas also organelles in the cytoplasm stained positive. In general, staining for the other ABC transporters was negative (MRP2, 3, 5) or weak (MRP4, P-gp) in 16HBE14o$^-$ cells. Staining for BCRP was strongly positive and mainly cytoplasmatic with some membranal staining.

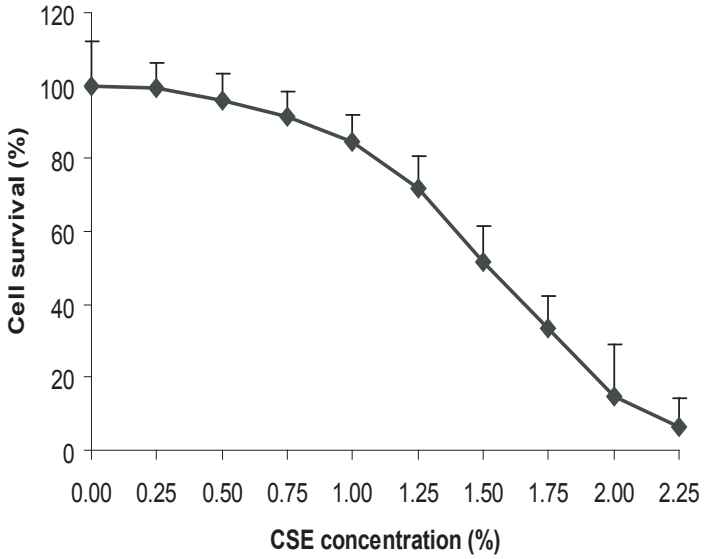

Figure 1. Cell survival assay (MTT) with CSE in 16HBE14o$^-$ cells. 16HBE14o$^-$ cells were incubated with 0.25% to 2.25% CSE for 4 days. The IC50 concentration was 1.53 ± 0.22% (mean ± SD, n=4).

Cytotoxicity assay

In the MTT assay, the IC50 (mean ± SD) of CSE was 1.53 ± 0.22% (n=4) in 16HBE14o$^-$ cells (Figure 1). The addition of MRP1 inhibitor MK571 to the IC50 concentration of CSE further decreased cell survival by 31% (p=0.018, n=3) (Figure 2), while the P-gp inhibitor PSC833 and the BCRP inhibitor FTC did not affect CSE cytotoxicity. The inhibitor concentrations used in the combination assays exhibited less than 8% cytotoxicity when used as a single drug (survival after MK571, 96 ± 9%; PSC833, 92 ± 6%; FTC, 103 ± 8%).

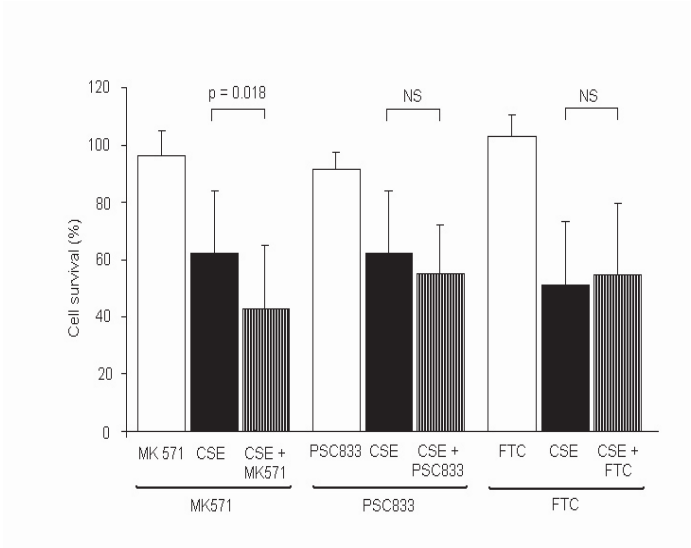

Figure 2. Cell survival assay (MTT) with CSE in 16HBE14o$^-$ cells. 16HBE14o$^-$ cells were incubated with the IC50 concentration of CSE with or without inhibitors or with inhibitor alone. Cytotoxicity of CSE increased with MRP1 inhibitor MK571 (p=0.018, n=3) but not with P-gp or BCRP inhibitors PSC833 or FTC respectively. Inhibitors alone were non-toxic in these concentrations.

Flow cytometric detection of MRP1 function and RNA interference

Cellular CF increased with increasing CSE concentrations in untransfected 16HBE14o$^-$ cells ($p<0.05$) at all tested CSE concentrations (5-40%; Figure 3a). The calculated BF ranged from 1.7 ± 0.4 at 5% CSE to 10.3 ± 3.3 at 40% CSE (n=5). 16HBE14o$^-$ cells transfected two times repetitively with si-MRP1 showed a reduction of 49 ± 9% in MRP1 mRNA levels compared to cells transfected with si-Luci (at day 4, p=0.002, n=5)**.** CF retention was increased in cells transfected with si-MRP1 compared to control cells transfected with si-Luci (67% p= 0.03, n=3; Figure 3b).

After incubation with 20% CSE cells transfected with si-MRP1 had

an increased CF retention compared to control cells transfected with si-Luci (24% increase, p=0.031, n=3) but not compared to untransfected cells. In transfected as well as non-transfected cells CF was highly increased after coincubation with the MRP inhibitor MK571 compared to their respective controls (p<0.05) (Figure 3). Statistical analysis of the BFs calculated for CSE in untransfected 16HBE14o$^-$, si-Luci transfected and si-MRP1 transfected cells plotted against the inverse of CF retention (MFI^{-1}) as a measure of MRP1 function at each condition (Figure 4) revealed a linear positive relation between these parameters (r=0.99, p=0.089). This relation was analogous to the relation found for the BFs calculated for MK571 and MFI^{-1} (Figure 4, r=0.99, p=0.034).

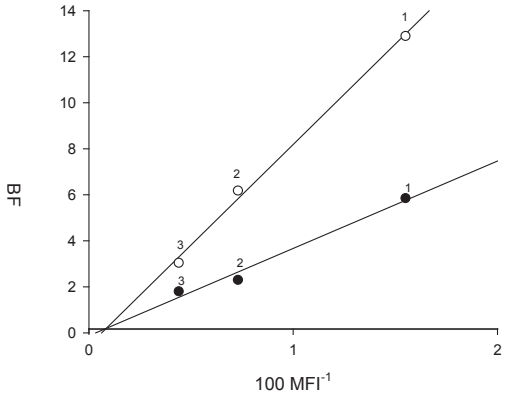

Figure 3. CF retention as measure for MRP1 function in 16HBE14o$^-$ cells with or without CSE incubation and /or specific MRP1 RNA down regulation. **A,** 16HBE14o$^-$ (control) cells were incubated with CF alone or with CF plus increasing concentrations of CSE (5-40%) for 1 h. **B,** 16HBE14o$^-$ cells transfected with siRNA against luciferase (si-Luci) or transfected with siRNA against MRP1 (si-MRP) were co-incubated with 20% CSE for 1 h. CF retention was measured by flow cytometry. MK571 was used as positive control of MRP1 inhibition. MFI=mean fluorescence intensity, CF=carboxyfluorescein, *p<0.05 CSE treated versus untreated control; **p= 0.03 si-MRP versus si-Luci

Discussion

This is the first study demonstrating that CSE affects the activity of MRP1 in the MRP1 expressing bronchial epithelial cell line 16HBE14o$^-$. The survival of 16HBE14o$^-$ cells following exposure to CSE was significantly lower in the presence of the MRP1 inhibitor MK571. This suggests that CSE is more cytotoxic when the function of MRP1 is blocked. The CF retention, which served as an inverse measure of MRP1 function, increased with increasing

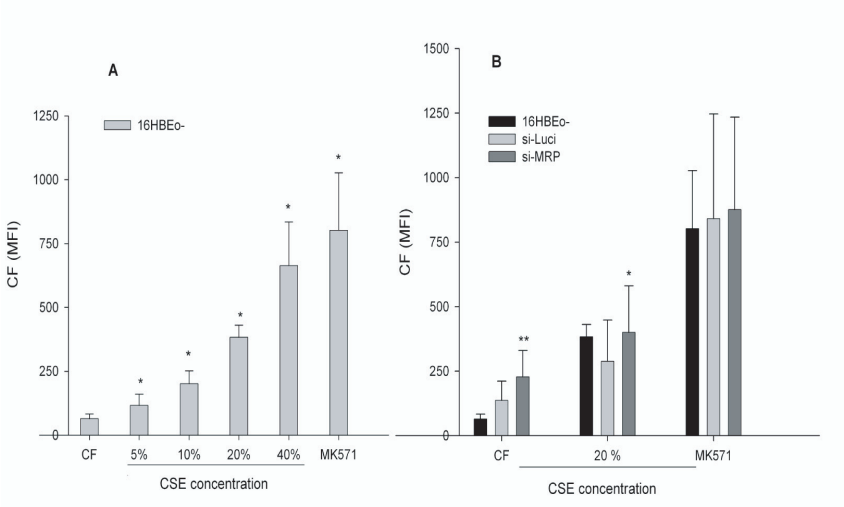

Figure 4. BF after co-incubation of 16HBE14o$^-$, 16HBE14o$^-$ cells transfected with siRNA against luciferase (si-Luci) or transfected with siRNA against MRP1 (si-MRP) with CF and 20% CSE or MK571 expressed relative to MRP function expressed as MFI-1 after incubation with CF alone. Correlations for CSE (●—●) r=0.99, p=0.089, for MK571 (○—○) r=0.99, p=0.034; [1] 16HBE14o$^-$, [2] si-Luci, [3] si-MRP

CSE concentrations. Specific down regulation of MRP1 with RNA interference reduced MRP1 RNA expression by 49% and increased CF retention by 67%. Addition of CSE further increased this CF retention by 24%. A linear relation between MRP1 function and CSE blocking effects was shown in analogy to the blocking effects with MK571 in untransfected, si-Luci control transfected and MRP1 down regulated 16HBE14o$^-$ cells.

Smoking generates oxidative stress in the lungs and is the principal risk factor for COPD. The MRP1 level in normal human bronchial epithelium is very high [11, 13]. We have previously detected lower MRP1 expression in bronchial epithelium of COPD patients compared to healthy controls [4]. Surprisingly, almost no information is available concerning the effects of tobacco smoke on the function of MRP1.

CSE is a mixture of many substances. The metabolite of one of the nitrosamines present in tobacco smoke, NNAL-*O*-glucuronide, has been shown to be transported by MRP1 in the presence of glutathione [18]. Presence of MRP1 substrates in CSE or intracellular generation of MRP1 substrates by CSE can inhibit the efflux of MRP1 substrate CF. This could either be a result of substrate competition with CF, an inhibitory effect of metabolizing enzymes (e.g. glutathione S-transferases), or a direct effect on

the activity of the MRP1 protein (e.g. the ATPase activity) as was described for other inhibitors of ABC transporters [29, 30]. Kamp *et al.* [31] have shown that CSE decreases ATP and glutathione levels in the alveolar epithelial cell lines A549 and WI-26 in a concentration-dependent manner after 4 h incubation. In our experimental setting only MK571, the inhibitor of MRP1, increased the epithelial cytotoxicity as induced by CSE, whereas this was not the case for the inhibitors of P-gp and BCRP, even though BCRP was highly expressed in these 16HBE14o$^-$ cells. Since P-gp and BCRP also depend on ATP for their function, it is unlikely that the observed effects were caused by a general effect of CSE on cellular ATP. In flow cytometric analyses of MRP function glutathione addition or depletion did not affect CF retention after co-incubation with CSE in 16HBE14o$^-$ cells (data not shown) indicating that glutathione depletion by CSE was not the limiting factor in CF transport in our model.

CF and MK571 are good probes for MRP1 function [32] one might anticipate that other MRPs contribute to the transport of CF [33] and its inhibition by MK571 [34] or CSE. We showed that MRP2, 3 and 5 were not detected by immunocytochemistry in 16HBE14o$^-$ cells and MRP4 staining was very weak. Therefore, in 16HBE14o$^-$ MRP1 is the most predominantly expressed of all investigated MRPs. This indicates that the increased retention of CF after co incubation with CSE in this model is mainly due to reduced transport by MRP1. Since reduced efflux of CF after incubation with CSE was also observed in the lung cancer cell line GLC4 and the MRP1 overexpressing cell line GLC4/ADR (data not shown), the observed effect is not cell line but MRP1 specific.

Successful down regulation of mRNA expression with RNA interference by 49% in 16HBE14o$^-$ cells resulted in 67% higher CF accumulation. Co-incubation with CSE in si-Luci control transfected and MRP1 down regulated 16HBE14o$^-$ cells revealed an increased CF retention in the MRP1 down regulated cells versus the si-Luci transfected cells. The modulating capacity (expressed as BF) of CSE is linearly related to MRP1 function expressed as the inverse of CF retention. The relation obtained with CSE is analogous to the relation obtained with MK571. In this model CSE shows a modulation pattern comparable with the strong MRP1 inhibitor MK571, indicating that CSE hits the same target, MRP1. The difference in slope between the 2 lines (8.73 for MK571, 3.80 for CSE) suggests that MK571 has a higher MRP1 modulating efficacy than the MRP1 substrates in CSE for instance due to a difference in substrate affinity or to a difference in

blocking efficiency.

Interestingly, our study shows that inhibition of MRP1 activity increases CSE cytotxicity in lung epithelial cells *in vitro* presumably by a diminished clearance of toxins present in cigarette smoke. Smoking is the principal risk factor for COPD, but only a subset of smokers develops COPD. Recently, a polymorphism at the *MRP1* gene locus was identified in the human population [35]. Functional studies revealed that this polymorphism reduces MRP1 activity. The presence of reduced MRP1 activity due to such a genetic polymorphism combined with our observation that MRP1 may protect epithelial cells against CSE toxicity may contribute to the explanation why only a subset of smokers is susceptible to the development of COPD.

In conclusion, in bronchial epithelial cells MRP1 mediated activity is competitively inhibited by cigarette smoke extract. In a model of the same cell line with different MRP1 activities a linear relation between CSE modulation and MRP1 function is found. Inhibition of MRP1 with MK571 makes bronchial epithelial cells more susceptible to CSE cytotoxicity. These results indicate a functional role of MRP1 in relation to the handling of cigarette smoke in human lungs.

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