CHAPTER SEVEN

Effect of COPD treatments on MRP1 mediated transport in bronchial epithelial cells

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Abstract

Background: Smoking is the principle risk factor for development of chronic obstructive pulmonary disease (COPD). Multidrug resistance-associated protein 1 (MRP1) is known to protect against toxic compounds and oxidative stress and might play a role in protection against smoke-induced disease progression. We questioned whether MRP1 mediated transport is influenced by pulmonary drugs that are commonly prescribed in COPD.
Methods: The immortalized human bronchial epithelial cell line 16HBE14o$^-$ was used to analyze direct *in vitro* effects of budesonide, formoterol, ipratropium bromide and N-acetylcysteine (NAC) on MRP1 mediated transport. Carboxyfluorescein diacetate (CF) was used as a model MRP1 substrate and was measured with functional flow cytometry.
Results: Formoterol had a minor effect, whereas budesonide dose-dependently decreased CF transport. Remarkably, addition of formoterol to the highest dose of budesonide increased CF transport. Ipratropium bromide inhibited CF transport at low concentrations and tended to increase CF transport at higher levels. NAC increased CF transport by MRP1 in a dose-dependent manner.
Conclusions: Our data suggests that besides their positive effects on respiratory symptoms, ipratropium bromide and NAC or the combination of budesonide and formoterol may be beneficial for long-term treatment of COPD via their stimulating effects on MRP1 functional activity.

Introduction

Smoking generates oxidative stress in the lungs and is the principal risk factor for development of lung cancer and chronic obstructive pulmonary disease (COPD). Detoxification and elimination processes of noxious substances present in cigarette smoke are important for both disease prevention and progression, yet little is known on these processes in the lung so far. Proteins of the ATP-binding cassette (ABC) superfamily such as the multidrug resistance-associated protein 1 (MRP1) may play a role, since they protect against oxidative stress and other xenobiotics. [1] Substrates for MRP1 are glutathione, glucuronate and sulfate conjugates and unconjugated compounds in presence of glutathione, e.g. tobacco specific nitrosamines. [2] Interestingly, the lung and trachea and other tissues with a barrier function highly express several ABC transporters. [3] Especially MRP1 is expressed at high levels in human lung tissue [4], mainly at the basolateral side of bronchial epithelium. [5, 6] We observed that MRP1 expression is diminished in bronchial epithelial cells of COPD patients [7] supporting the hypothesis that lower functional MRP1 activity is related to COPD development.

So far, COPD is recognized as a relentlessly progressive disease in which only smoking cessation reduces the accelerated lung function decline. There is no cure for COPD, yet recently it has been shown that some drugs are beneficial in the disease management. Inhaled corticosteroids and long-acting beta-agonists such as budesonide and formoterol reduce the number of exacerbations in COPD [8, 9] and their combination has been shown to be very effective. [10] Treatment of COPD patients with the anticholinergic drug ipratropium bromide results in a small improvement of lung function, yet does not influence the long-term decline in mild COPD. [11] Studies on oral use of the anti-mucolytic drug N-acetylcysteine (NAC) have provided contradictory results. [12, 13]

In contrast to the extensive knowledge on chemotherapeutic drugs as substrates for MRP1, limited data is available on the effect of pulmonary drugs on the functional expression of MRP1. [14] NAC induces higher cellular glutathione levels and in this way can protect against oxidative stress that may indirectly affect MRP1 function [15] but no information is available about budesonide, formoterol and ipratropium bromide in this respect.

In the present study, we questioned whether medications commonly prescribed to COPD patients affect MRP1 mediated transport. Therefore, we analyzed the direct *in vitro* effects of budesonide, formoterol, ipratropium

bromide and NAC on MRP1 by means of functional flow cytometry in immortalized human bronchial epithelial cells.

Material and Methods

Chemicals, media and reagents

Bovine serum albumin (BSA, fraction V), minimal essential medium (MEM, supplemented with Earle's salts and L-glutamine) and RPMI 1640 medium (supplemented with 25 mM Hepes and L-glutamine) were purchased from Invitrogen Life Technologies (Breda, The Netherlands). Carboxyfluorescein diacetate (CFDA), ipratropium bromide, NAC and propidium iodide (PI) were obtained from Sigma-Aldrich BV (Zwijndrecht, The Netherlands). Budesonide (Pulmicort, Turbuhaler) was obtained from AstraZeneca BV (Zoetermeer, The Netherlands), formoterol fumarate dihydrate from Astra Draco AB (Lund, Sweden), ethylenedinitrilo tetraacetic acid disodiumsalt dihydrate (EDTA) from Merck (Darmstadt, Germany), fetal calf serum (FCS) from Bodinco BV (Alkmaar, The Netherlands), Vitrogen from Nutacon (Leimuiden, The Netherlands) and MK571 from Omnilabo (Breda, The Netherlands).

Bronchial cells

The human bronchial epithelial cell line 16HBE14o$^-$, immortalized with pSVori$^-$ plasmid transfection, was kindly provided by Dr. D.C. Gruenert (California Pacific Medical Center Research institute; San Francisco, CA). [16] This cell line expresses MRP1 at relatively moderate levels. Cells were cultured in MEM supplemented with 10% heat inactivated FCS. Before trypsinization, cells were washed twice with phosphate buffered saline (PBS: 6.4 mM Na2HPO4, 1.5 mM KH2PO4, 0.14 mM NaCl, 2.7 mM KCl, pH=7.4) with 0.5 mM EDTA. 16HBE14o$^-$ cells were grown on tissue culture plastics coated with Vitrogen (30 µg/ml) and BSA (10 µg/ml).

Flow cytometry

To determine MRP1 mediated transport, cells were incubated with 0.1 µM CFDA as described previously [17] with slight modifications. CFDA is intracellularly converted to carboxyfluorescein (CF) which is a fluorescent MRP1 substrate. To establish the effect of COPD drugs on MRP1 mediated activity, 1 x 10^6 cells were incubated in 0.5 ml RPMI 1640 medium without FCS (37 °C, 5% CO_2, 1 hour) with CFDA and with or without the addition

of drugs. MK571 (20 μM) was used as a positive control for inhibition of MRP1 activity [17] and always showed strong inhibition (more than ten-fold) of MRP1 mediated efflux of CF. Budesonide and formoterol were added in concentrations of 10^{-8} M to 10^{-4} M. These drugs were dissolved in 96% ethanol and dimethyl sulfoxide (DMSO) respectively. In part of the experiments, cells were simultaneously incubated with budesonide and formoterol. Ipratropium bromide was added in concentrations from 10^{-7} M to $2x10^{-4}$ M and NAC from 10^{-4} M to $1.6x10^{-3}$ M and these drugs were dissolved in PBS and water respectively.

Cells were pelleted for 15 seconds at 12,000 g and resuspended in ice-cold RPMI medium without FCS. Drug or MK571 was added a second time in appropriate concentrations, this time without substrate CFDA (37 °C, 5% CO_2, 1 hour). After pelleting, cells were put on ice to stop efflux of substrate and were resuspended in 350 μl RPMI 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; Franklin Lakes, NY). We measured 10,000 events per sample (living cells). The Winlist 5.1 program (Verity Software House Inc.; Topsham, ME) was used to calculate mean fluorescence intensity (MFI) values. All measurements were corrected for the negative control (medium alone) and for incubation with the solvents 96% ethanol and DMSO if appropriate. The percentage of dead cells (PI positive population) did not increase with all tested drug concentrations. None of the tested drugs caused autofluorescence (fluorescence without CFDA and PI incubation).

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Each experiment was repeated 3 to 6 times independently. The paired Student's t-test (two-tailed) was used to calculate modulating effects of the drug under study compared to control. Differences were considered significant when $P < 0.05$. Statistical analyses were performed with SPSS 12 (SPSS Inc.; Chicago, IL).

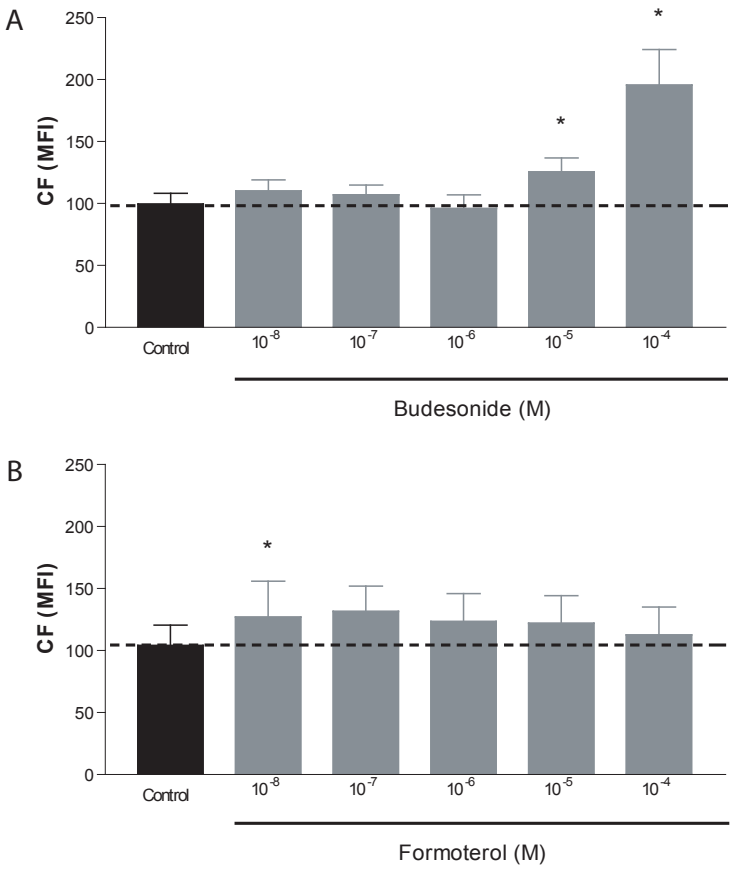

Figure 1. Modulation of MRP1 mediated activity with increasing concentrations of (A) budesonide or (B) formoterol. Dashed line indicates the MFI level of the control (incubation with vehicle). Data is shown of mean ± SEM of 3-4 independent experiments for budesonide and 5-6 independent experiments for formoterol. *P < 0.05 compared to control.

Results

Modulating effect of budesonide and formoterol on MRP1 mediated activity

Budesonide increased the accumulation of CF at concentrations of 10^{-5} M and 10^{-4} M with 26% and 97% respectively. Lower concentrations did not affect CF accumulation significantly (Figure 1A). Formoterol had a small, yet significant effect on the accumulation of CF at 10^{-8} M, but at higher doses this effect was not significant Figure 1B).

To address the question whether the effect of budesonide is altered by addition of formoterol, we incubated the highest concentration of budesonide (10^{-4} M) with increasing concentrations of formoterol. With this approach, the budesonide-induced CF accumulation decreased in a dose-dependent manner; with 10^{-6} M, 10^{-5} M, 10^{-4} M formoterol respectively 198%, 124% and 58% compared to the control (Figure 2).

Modulating effect of ipratropium bromide and N-acetylcysteine on MRP1 mediated activity

Ipratropium bromide increased CF accumulation in a dose-independent manner at lower concentrations (10^{-7} M to 10^{-5} M) with a maximum of 24% at 10^{-7} M (Figure 3A). Increasing the concentration of ipratropium bromide to $2x10^{-4}$ M potentiated the CF efflux (56% compared to control), suggesting that MRP1 mediated activity is stimulated rather than inhibited with ipratropium bromide. Similar results were obtained with NAC, i.e. CF accumulation was reduced in a dose-dependent manner (57% at $8x10^{-4}$ M) (Figure 3B). Increasing the NAC concentrations to $1.6x10^{-3}$ M potentiated the CF efflux even more (one observation).

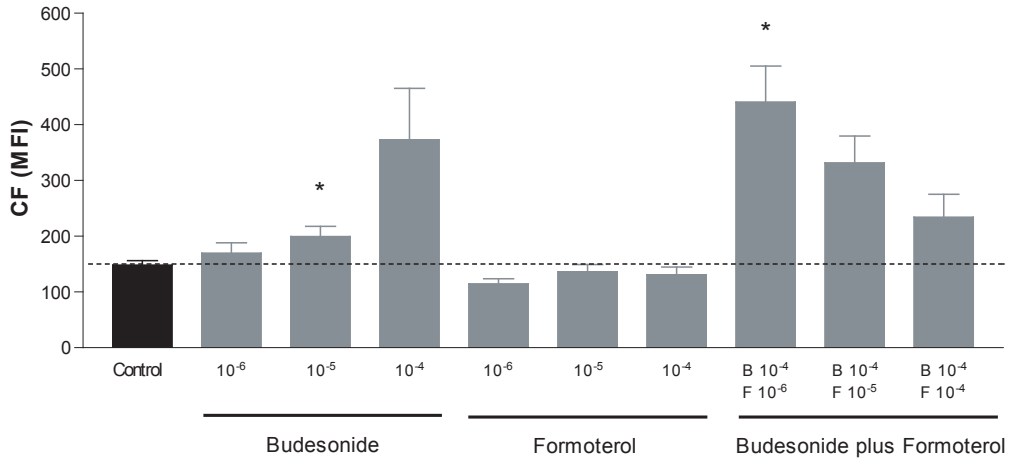

Figure 2. Modulation of MRP1 activity by budesonide, formoterol or their combination. Dashed line indicates the MFI level of the control (incubation with vehicle). Data is shown of mean ± SEM of 3 independent experiments. *P < 0.05 compared to control.

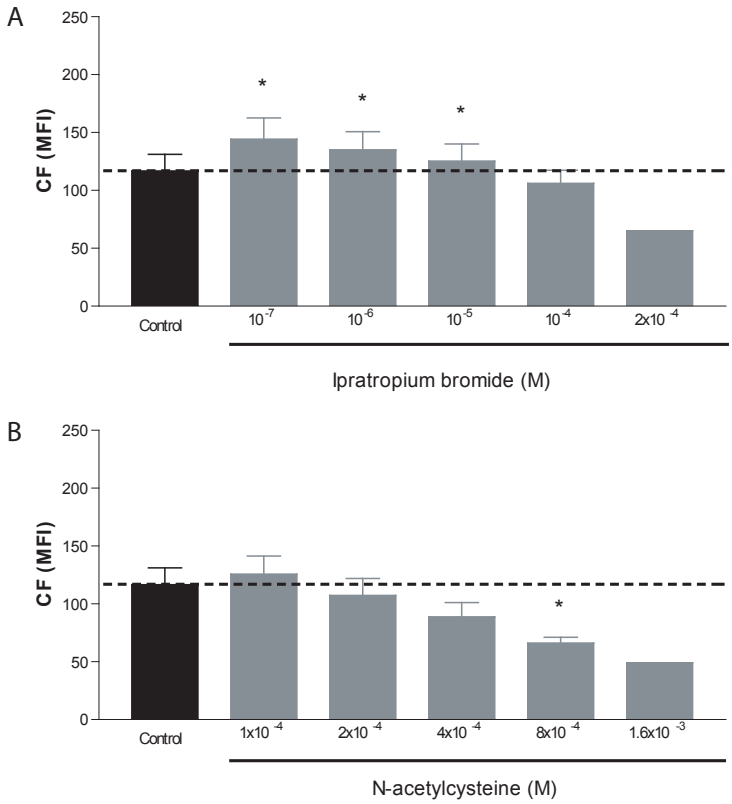

Figure 3. Modulation of MRP1 mediated activity with increasing concentrations of (A) ipratropium bromide or (B) N-acetylcysteine. Dashed line indicates the MFI level of the control (incubation with vehicle). Data is shown of mean ± SEM of 5 independent experiments for ipratropium bromide and 3-6 independent experiments for N-acetylcysteine. *P < 0.05 compared to control.

Discussion

The membrane protein MRP1 is highly expressed in human lung tissue and may protect the airways against damage induced by cigarette smoking. [6] This study shows for the first time the direct influence of pulmonary drugs on MRP1 functional activity, drugs that have already proven their effectiveness in the clinical management of COPD.

Since MRP1 function is most likely cytoprotective in lung cells, we argued that modulation of its function with pulmonary drugs might induce either a positive or negative effect in COPD with long term treatment. We observed that budesonide interfered with MRP1 mediated transport by

increasing CF retention. This implies either that budesonide is an MRP1 substrate or that it acts on MRP1 function in another way. Budesonide is intracellularly esterified to a fatty acid which is thought to be the mechanism of its prolonged activity after a single dose. [18] MRP1 has been shown to be capable of rather slow outward transport of phospholipids, phophatidylcholine and sphingomyelin, and phospholipid analogues in the presence of oxidized glutathione and ATP in human erythrocytes and epithelial cells. [19] It is tempting to speculate that esterified budesonide could behave like a phospholipid analogue with a polar head and two fatty acid tails and is then outwards transported by MRP1. As such, budesonide can compete with other MRP1 (physiological) substrates and in that way affect the functional activity of MRP1.

We measured the direct effect of budesonide on MRP1 mediated transport. In addition, budesonide is also able to affect the transcriptional regulation of MRP1, as shown by diminished expression of MRP1 in the lung epithelial cell line Calu-1 after long-term incubation with budesonide (10^{-5} M). [20] Taken together, the data suggest that treatment with budesonide could potentially have a negative effect in COPD, given the cell protective properties of MRP1. Our observation does not distract from the well known beneficial effects of budesonide in diseases like asthma, since steroids suppress virtually every step of the inflammatory cascade. However, our findings may contribute an alternative hypothesis why inhaled steroids are not capable to alter the long-term course of COPD, particularly in smokers with COPD. [9]

In combination with formoterol, the direct inhibitory effect of budesonide on CF accumulation of epithelial cells was reduced, whereas formoterol as such had only minor effects on MRP1 mediated transport. This indicates that budesonide or its esterified derivatives is less available as an MRP1 substrate/inhibitor when combined with formoterol or that formoterol interferes with the interaction between budesonide (esters) and MRP1. Although formoterol is moderately lipophilic, it has a high affinity for cellular membrane lipid bilayers. [21] High concentrations of formoterol might interfere with the function of membrane proteins as this depends on the lipid environment of the protein. Since phospholipid transport by MRP1 seems to have very slow kinetics [19] compared to the transport of smaller substrates like CF, MRP1 transport of budesonide (esters) may be more affected than the transport of CF. This could explain the relative improvement of CF transport in our model after combined incubation with budesonide and formoterol.

This effect may also indirectly run via adenosine 3',5'-cyclic monophosphate (cAMP) since formoterol stimulates cells via the beta(2)-adrenergic receptor [22] resulting in higher intracellular levels of cAMP. However, it is not clear why this occurs in the presence of budesonide while formoterol alone had no effect. The here proposed model for budesonide transport and the effects of combination with formoterol needs further study.

Ipratropium bromide incubation resulted in lower CF retentions with increasing concentrations in a dose-dependent manner. Ipratropium bromide competitively inhibits acetylcholine binding to the muscarinic receptor resulting in decreased guanosine 3',5'-cyclic monophosphate (cGMP) levels which lowers bronchoconstriction. There are no reports in the literature on its mechanism of action with respect to MRP1, but it can be speculated that cGMP might play a role in this process since cGMP is a substrate of other members of the MRP family, MRP4 and MRP5. [23]

Effects of NAC were similar to those observed with ipratropium bromide, i.e. MRP1 mediated transport was stimulated with increasing concentrations of NAC. This may be due to the fact that NAC elevates glutathione levels. Glutathione and many glutathione conjugates are substrates for MRP1, and therefore, intracellular glutathione levels are of major importance for MRP1 function in antioxidant defense. It is known that transport of anionic species such as CF can be stimulated with glutathione. [15, 24] It was observed that the compounds sulfinpyrazone and indomethacin stimulate glutathione transport by MRP2 at low concentrations, whereas relatively high concentrations inhibit GSH transport into the medium. [25] A possible explanation was given that both compounds are transported cooperatively. Our results with NAC and ipratropium bromide might be explained by a similar co-transport of these substances with CF. It can be speculated that certain (at present undefined) toxic inhaled substances can be transported by MRP1 more effectively with increasing glutathione levels as well. For example, the tobacco-derived NNAL-*O*-glucuronide was identified as an MRP1 substrate that requires glutathione for transport. [26]

Treatment of COPD is a matter of intensive investigation. There is no cure for COPD, and so far available treatment only improves symptoms and quality of life, and reduces the number of exacerbations. Since the positive clinical effects are clear, these treatments should be used in clinical practice. Our *in vitro* studies show that other, potentially positive or negative long-term effects of these drugs may be present as well, which clearly requires further

studies and specifically the role of MRP1 in this process.

The pulmonary drugs that we tested are, apart from NAC, clinically administered by inhalation. These drugs act directly on epithelial cells or pass the epithelial layer and act on e.g. smooth muscle cells or inflammatory cells. Clinically achievable concentrations at the level of airway epithelium are mainly unknown. The intracellular levels that can be reached with treatment highly depend on e.g. biochemical features of the substance, action of drug metabolism proteins (phase II conjugation enzymes such as cytochrome P450 isoforms), drug efflux pumps (phase III systems), as well as the route of administration. We have chosen the ranges of the applied drug concentrations based on published data [20, 21, 27-30] and theoretical models, i.e.ranges that will be clinically effective. MRP1 is located in bronchial epithelium, the first cells that drugs have to encounter to reach the underlying tissue to act on e.g. smooth muscle cells, fibroblasts and inflammatory cells. Thus, local high cellular concentrations of pulmonary drugs are very likely to affect MRP1 activity in bronchial epithelium which was indeed confirmed *in vitro* in the present study.

In conclusion, our studies show that drugs that are clinically used in COPD affect MRP1 function, a transporter that protects against oxidative stress and toxic compounds. Budesonide inhibits this MRP1 mediated transport and addition of formoterol on its turn reduces this inhibitory effect. Our data suggests that the combination of budesonide and formoterol or relatively high concentrations of ipratropium bromide and NAC may be beneficial for long-term treatment of COPD with regard to the protective properties of MRP1.

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